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Enhanced oral delivery of antimony from meglumine antimoniate/β-cyclodextrin nanoassemblies

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Abstract

The composition comprising the highly water-soluble drug meglumine antimoniate (MA) and β-cyclodextrin (β-CD) was shown previously to enhance the absorption of Sb by oral route and render MA orally active in a murine model of cutaneous leishmaniasis. This unexpected behaviour was attributed, in part, to the fact that the heating of equimolar mixture of MA and β-CD (first step of preparation of MA/β-CD composition) induced the depolymerization of MA from high-molecular weight Sb complexes into 1:1 Sb–meglumine complex, resulting in an enhanced oral bioavailability of Sb. In the present work, we demonstrate that the heated MA + β-CD mixture still produced significantly lower serum Sb levels when compared to the MA/β-CD composition, indicating that the freeze-drying process (second step of preparation of MA/β-CD composition) is required for achieving a high absorption of Sb by oral route. To get insight into the physicochemical alterations induced by the freeze-drying step, the MA/β-CD composition was further characterized by circular dichroism, ^{1}H NMR and ESI(−)-MS and photon correlation spectroscopy. The freeze-drying process was found to promote the formation of supramolecular nanoassemblies with a mean hydrodynamic diameter of 190 nm, comprising 1:2:1, 2:2:1 and 2:2:2 NMG–Sb–β-CD complexes. Another important observation was the ability of the MA/β-CD composition to act as a sustained release system of the antimonial drug MA, suggesting that this property may result in the change of the drug absorption site in the gastrointestinal tract. A model is proposed for the mechanisms involved in the enhanced absorption of Sb from the MA/β-CD composition.

Keywords: Cyclodextrin; Oral; Antimony; Meglumine antimoniate; ESI-MS; Freeze-drying

1. Introduction

The pentavalent organoantimonial drug, meglumine antimoniate (MA), is currently a drug of choice for the treatment of leishmaniasis (Berman, 1997). Recently, pentavalent antimonials were also found to exert activity against cancer, hepatitis C and AIDS (Yan et al., 2005). This compound is considered inactive when given enterally and is subject to rapid renal clearance after parenteral administration requiring a multiple dosing regimen.

It was reported previously that the association of MA with β-cyclodextrin (β-CD) enhances the absorption of Sb by oral route and renders MA orally active in a murine model of cutaneous leishmaniasis (Demicheli et al., 2004). According to the FDA Biopharmaceutical Classification System, MA belongs to Class III, i.e. drugs with high water-solubility (>300 mg/mL) and with low membrane permeability. In the case of Class III drugs, however, hydrophilic cyclodextrins, such as β-CD, are

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not expected to improve oral drug absorption (Loftsson et al., 2004a). Indeed, the MA/β-CD composition differs markedly from conventional inclusion complexes between hydrophilic cyclodextrins and poorly water-soluble drugs.

Progress was recently achieved towards the understanding of the mode of action of the MA/β-CD composition (Martins et al., 2006). The unexpected behaviour of the MA/β-CD composition was attributed, in part, to the physicochemical properties of MA in aqueous solution. MA consists of a mixture of oligomeric structures with the general formula $(NMG–Sb)_n$, $(NMG–Sb)_n$–NMG and $(Sb–NMG)_n$–Sb, where NMG represents *N*-methyl-D-glucamine (Demicheli et al., 2003). It was found that the first step of preparation of the MA/β-CD composition, which consists in heating of an equimolar mixture of MA and β-CD at 55 °C for 48 h, induces the dissociation of MA from high-molecular weight Sb complexes into 1:1 Sb–NMG complex. Furthermore, the observation that MA, after heating, was more effectively absorbed by the oral route led to propose that the dissociation of MA may contribute the enhanced absorption of Sb promoted by the MA/β-CD composition (Martins et al., 2006). However, the serum Sb levels achieved after heated MA were still significantly lower than those achieved after MA/β-CD composition, indicating that additional factors related to specific interactions of MA with β-CD should be involved in the mode of action of MA/β-CD. The characterization of the heated MA + β-CD mixture, using circular dichroism (CD) and electrospray ionization mass spectrometry (ESI-MS), indicated the formation of a ternary NMG–Sb–β-CD complex which may also contribute to the enhanced oral absorption (Martins et al., 2006).

As the second step of preparation of the MA/β-CD composition consists of freeze-drying of the heated MA + β-CD mixture, the freeze-drying step may promote additional interactions. However, it should be investigated to which extent each step (heating and freeze-drying) contributes to the enhanced Sb absorption by oral route and how the induced interactions mediate such an effect.

In the present work, the interactions between MA and β-CD induced by heating and freeze-drying were investigated using CD, ^{1}H NMR, ESI-MS and photon correlation spectroscopy (PCS) and their impact on Sb absorption by oral route was evaluated. Importantly, the freeze-drying process was found to generate supramolecular nanoassemblies and to contribute the most significantly to the enhanced Sb absorption by oral route. Upon dilution, MA/β-CD composition was found to act as a sustained release system for MA.

2. Materials and methods

2.1. Materials

N-methyl-D-glucamine (NMG) and $SbCl_5$ were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA), β-CD from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of at least reagent grade. Double distilled, deionized water was used throughout the experiments.

2.2. Preparation of MA and of MA/β-CD composition

MA was synthesized as previously described (Demicheli et al., 2003), from an equimolar mixture of NMG and freshly precipitated, hydrated Sb pentoxide, which was obtained from $SbCl_5$ hydrolyzed in water. The resulting product contained 29% of Sb by weight, as determined by inductively coupled plasma optical emission spectrometry. The MA/β-CD composition was prepared, as previously described (Demicheli et al., 2004) by mixing β-CD and MA in water at a 1:1 β-CD/Sb molar ratio, heating the mixture for 48 h at 55 °C under stirring and finally freeze-drying the resulting solution.

2.3. Absorption of Sb in mice by oral route from MA and MA/β-CD mixtures

Swiss mice (female, weighing 25 ± 3 g) were obtained from Cebio (Centro de Bioterismo do Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais). Free access was allowed to standard diet and tap water was supplied *ad libitum*.

Animals received by gavage the following preparations at 100 mg Sb/kg of body weight: meglumine antimoniate freshly prepared in water at 0.05 mol/L Sb and 25 °C (MA); equimolar mixture of MA and β-CD freshly prepared in water at 0.05 mol/L Sb and 25 °C (MA+β-CD); equimolar mixture of MA and β-CD in water at 0.05 mol/L Sb and 25 °C previously incubated for 48 h at 55 °C (MA + β-CDh); freeze-dried MA + β-CDh preparation reconstituted in water at 0.05 mol/L Sb and 25 °C (MA/β-CD). Four mice from each group were sacrificed 3 h after administration. Blood samples were obtained and the serum was recovered and frozen.

In a previous study (Demicheli et al., 2004), the serum pharmacokinetics of Sb after MA and MA/β-CD were determined in Swiss mice, indicating that both compounds exhibited similar elimination phases but different absorption levels of Sb. Therefore, in the present experiment aimed at comparing different AM compositions, a significant difference between serum Sb levels at 3 h could be interpreted as a difference in Sb absorption level.

Experimental protocols were performed in accordance with the guidelines for the humane use of laboratory animals and received approval from the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (protocol no. 004/03).

The serum was assayed for Sb by atomic absorption spectrometry with a graphite furnace (ETAAS) without digestion of the sample, as previously described (Demicheli et al., 2004), using Zirconium (Zr) and Rhodium (Rh) as permanent modifiers.

2.4. Physicochemical characterization of MA/β-CD mixtures

Circular dichroism (CD) spectra were recorded on a Jobin Yvon-Spex Mark CD6 dichrograph. The solutions containing

antimonial compound at 0.01 mol/L Sb were transferred to a 0.1 cm quartz cuvette and the CD spectra were immediately recorded. The CD signal is given as $\Delta\varepsilon$, which is the differential molar dichroic absorption coefficient ($\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ in $L\,cm^{-1}\,mol^{-1}$) and is expressed in terms of the molar concentration of Sb.

ESI-Q-ToF mass spectrometry analyses were carried out using a Q-ToF Micro™ (Micromass, UK) equipped with an electrospray ionization source operated in the negative or in the positive ion mode. Capillary voltage was 3–3.5 kV and sample cone voltages were 30–60 V. Mass spectrometer calibrations were made by using sodium iodide with caesium iodide in the *m/z* 100–2000 range. In the ESI-MS spectra, Sb–NMG complex ions were easily characterized as containing Sb by the distinctive isotope pattern of Sb (ratio of ^{121}Sb:^{123}Sb, 57:43). The number of Sb atom in each complex was also determined from the specific isotope patterns: doublet for one Sb atom, triplet for two Sb atoms and quadruplet for three Sb atoms. On the basis of the isotope pattern of Sb, monovalent ions showed peaks with a difference in atomic mass unit of 2, whereas divalent ions showed peaks with a difference of 1. The compounds were prepared in water at 0.01 mol/L Sb and introduced using a syringe pump with a flow rate of 5–10 L/min. Data were analysed by MassLynx® 4.0 software. Each species is indicated in the following with the *m/z* value of the first peak of its isotopic cluster.

^1H NMR spectra were recorded on an 11.7 T (500 MHz) Varian Unity INOVA spectrometer operating with a 5 mm gradient indirect detection probe at 25 °C. The resolution obtained after shimming was assessed by the measurement of the linewidth at half-height of the water resonance. Identical resolutions were achieved for spectra obtained on identical samples after different times. The spectra in D_2O (128 transients) were obtained with a 4 s of relaxation delay including the water presaturation pulse of 2 s at 0.03 W, followed by a 90° pulse. Data were acquired on 16 K data points for a 6000 Hz spectral width. The Free Induction Decay (FID) was processed with an exponential multiplication corresponding to 0.3 Hz lines broadening prior to Fourier transform. Chemical shifts were referenced to internal signal of water (4.78 ppm at 25 °C).

The particle size distribution in the aqueous MA/β-CD solution was determined by photon correlation spectroscopy at 25 °C with a 90° scattering angle and using a channel correlator (ZEN3500, Malvern Instruments, UK) in conjunction with a laser of wavelength 532 nm. Samples were prepared in deionized water at the final β-CD concentration of 2.5 mmol/L and kept at 25 °C. The mean hydrodynamic diameter and polydispersity index were determined different times after the preparation of the solution.

2.5. Statistical analysis

Comparisons between serum Sb levels were performed by analysis of variance (one-way ANOVA, with Tukey's Multiple Comparison Post-test). A P value of <0.05 was considered statistically significant.

3. Results

3.1. Absorption of Sb by oral route from MA/β-CD mixtures

Fig. 1 shows the Sb concentrations in the serum of mice 3 h after administration by oral route of different compositions of MA: a solution of MA in water, freshly prepared at 0.05 mol/L of Sb and 25 °C (MA); an equimolar mixture of MA and β-CD at 0.05 mol/L of Sb in water, either freshly prepared at 25 °C (MA+β-CD) or heated for 48 h at 55 °C (MA + β-CDh); the conventional MA/β-CD composition freshly prepared in water at 0.05 mol/L of Sb at 25 °C.

When MA was given to mice as a physical mixture of MA and β-CD, instead of MA alone, no significant increase of Sb level was observed, even though a slight (but not significant) increase was observed after heating of the mixture for 48 h at 55 °C. Surprisingly, when the MA + β-CD mixture was freeze-dried a significant 2- to 3-fold increase of Sb concentration was observed, indicating that the freeze-drying process greatly improves the delivery of Sb by the oral route.

3.2. Physicochemical characteristics and kinetics of dissociation of MA/β-CD composition

Since freeze-drying of the heated MA + β-CD mixture markedly enhanced the serum Sb level after oral administration, the impact of this process on the physicochemical state of MA/β-CD was investigated. Fig. 2 shows the CD spectra obtained for MA, MA + β-CDh and MA/β-CD, immediately after their dilution in water at 0.01 mol/L Sb and 25 °C. The MA/β-CD composition exhibited immediate dissolution, as evidenced by the lack of turbidity. As reported previously (Martins et al., 2006), upon heating of MA in the presence β-CD, the CD spectrum of MA shows a decrease of its intensity and a red-shift, as a result of the formation of a ternary NMG–Sb–β-

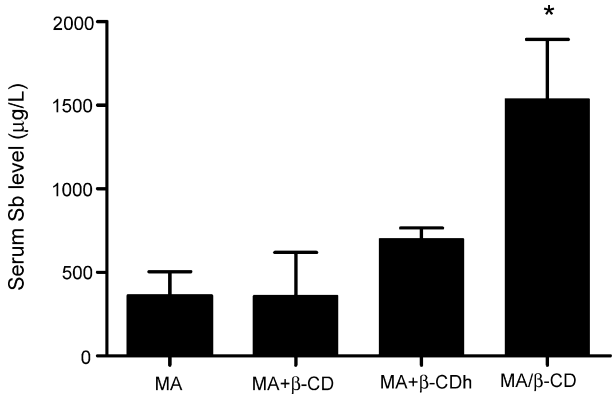

Fig. 1. Sb concentration in the serum of Swiss mice 3 h after oral administration of different compositions of MA at 100 mg Sb/kg. MA: meglumine antimoniate freshly prepared in water at 0.05 mol/L Sb and 25 °C; MA + β-CD: equimolar mixture of MA and β-CD freshly prepared in water at 0.05 mol/L Sb and 25 °C; MA + β-CDh: equimolar mixture of MA and β-CD in water at 0.05 mol/L Sb and 25 °C pre-incubated for 48 h at 55 °C; MA/β-CD: freeze-dried MA + β-CDh freshly reconstituted in water at 0.05 mol/L Sb and 25 °C. Data are given as means ± S.D. ($n = 4$). *$P < 0.01$ for comparisons between MA/β-CD and the other groups (one-way ANOVA followed by Tukey's Post-test).

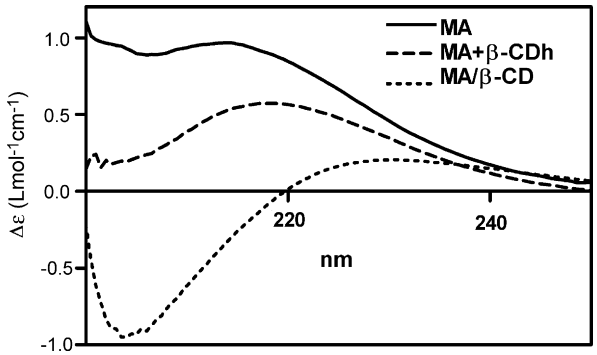

Fig. 2. Circular dichroism spectra of different compositions of MA in water at 0.01 mol/L Sb. MA: MA prepared in water at 0.05 mol/L Sb and then diluted to 0.01 mol/L Sb at 25 °C just before measurement; MA + β-CDh: equimolar mixture of MA and β-CD in water at 0.05 mol/L Sb first heated for 48 h at 55 °C and then diluted to 0.01 mol/L Sb at 25 °C just before measurement; MA/β-CD: freeze-dried MA + β-CDh reconstituted in water at 0.01 mol/L Sb and 25 °C just before measurement.

CD complex. Strikingly, following submission of the heated MA + β-CD mixture to freeze-drying, MA spectrum suffered marked changes, with the appearance of a negative Cotton effect centered at 205 nm, indicating the occurrence of additional interactions between MA and β-CD.

As shown in Fig. 3, when the CD spectrum of MA/β-CD composition was registered as a function of time, it was found to return slowly to a spectrum characteristic of MA, suggesting that the MA/β-CD composition released MA. Fig. 3 insert displays also the kinetics of change of the CD signal at 220 nm at 25 °C. The kinetic was found to be biphasic with a first rapid phase and a slow late phase. The changes in the physicochemical state of MA were also evidence by ^{1}H NMR. Fig. 4 shows the region of the ^{1}H NMR spectrum of MA/β-CD in D_2O, corresponding to the hydrogens of the methyl group of MA, different times after its preparation at 0.01 mol/L Sb. Since these spectra were obtained with identical resolutions, one can infer that the linewidths of the methyl resonances decreased with time. The thinner peaks may be attributed to longer T_2 relaxation times and to molecular entities of smaller size.

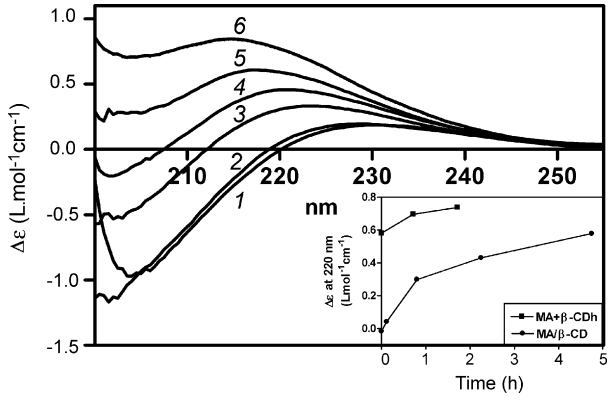

Fig. 3. Circular dichroism spectra registered different times after preparation of MA/β-CD preparations in water at 0.01 mol/L Sb at 25 °C (1:0 min; 2:7 min; 3:48 min; 4:2.25 h; 5:4.75 h; 6:3 days). The insert shows the kinetics of variation of the $\Delta\varepsilon$ at 220 nm, after the preparation of MA + β-CDh and MA/β-CD in water.

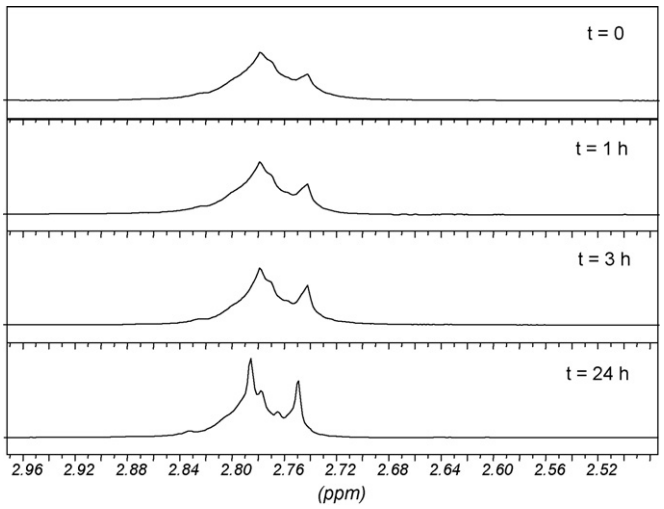

Fig. 4. Region of the ^{1}H NMR of MA/β-CD in D_2O corresponding to the hydrogens of the methyl group of MA, different times after reconstitution of the composition at 0.01 mol/L Sb.

Further information on the molecular changes in the MA/β-CD composition upon dissociation were obtained by comparing the ESI(−)-MS spectra of MA/β-CD, immediately and 24 h after its dissolution in water (Figs. 5 and 6). Strikingly, the relative abundance of the species with *m/z* 602.2, 758.0, 815.5, 913.4 and 1479.4 was found to decrease as a function of time, suggesting that some of these species may contribute to the enhanced absorption of Sb by oral route. Table 1 displays the proposed structural formula for some of these anionic species. The formation of 1:2:1, 2:2:1 and 2:2:2 NMG–Sb–β-CD complexes is proposed. When compared to the ternary 1:1:1 NMG–Sb–β-CD complex previously identified (Martins et al., 2006), these new species exhibit higher-molecular weight and ionization state, presumably as a result of multiple associations.

Fig. 7 shows the time-course of variation of the mean hydrodynamic diameter of particles, as determined by photon correlation spectroscopy, after reconstitution of MA/β-CD composition in deionized water at 2.5 mmol/L of β-CD. Initially, a mean hydrodynamic diameter of 190 nm with a polydispersity index of 0.37 was measured and two different particle populations could be identified: smaller particles with a mean diameter of 45 nm (33%, v/v) and larger ones with a mean diameter

Table 1
Sb–β-CD complexes identified in the ESI(−)-MS spectrum of the MA/β-CD composition

Sb–β-CD complexes	*m/z*
$[CD(Sb)_2(O)_4(K)_2–4H]^{2-}$	757
$[CD(NMG)(Sb)_2(OH)_4–8H]^{2-}$	816
$[CD(NMG)(Sb)_2(OH)_4–8H]^{2-} + H_2O$	825
$[CD(NMG)(Sb)_2(OH)_4–8H]^{2-} + 2H_2O$	834
$[CD(NMG)_2(Sb)_2(OH)_3–9H]^{2-}$	904
$[CD(NMG)_2(Sb)_2(OH)_3–9H]^{2-} + H_2O$	913
$[CD(NMG)_2(Sb)_2(OH)_3–9H]^{2-} + 2H_2O$	922
$[CD(NMG)(Sb)(OH)_2–4H]_2^{2-}$	1479
$[CD(NMG)(Sb)(OH)_2–4H]_2^{2-} + H_2O$	1488
$[CD(NMG)(Sb)(OH)_2–4H]_2^{2-} + 2H_2O$	1497

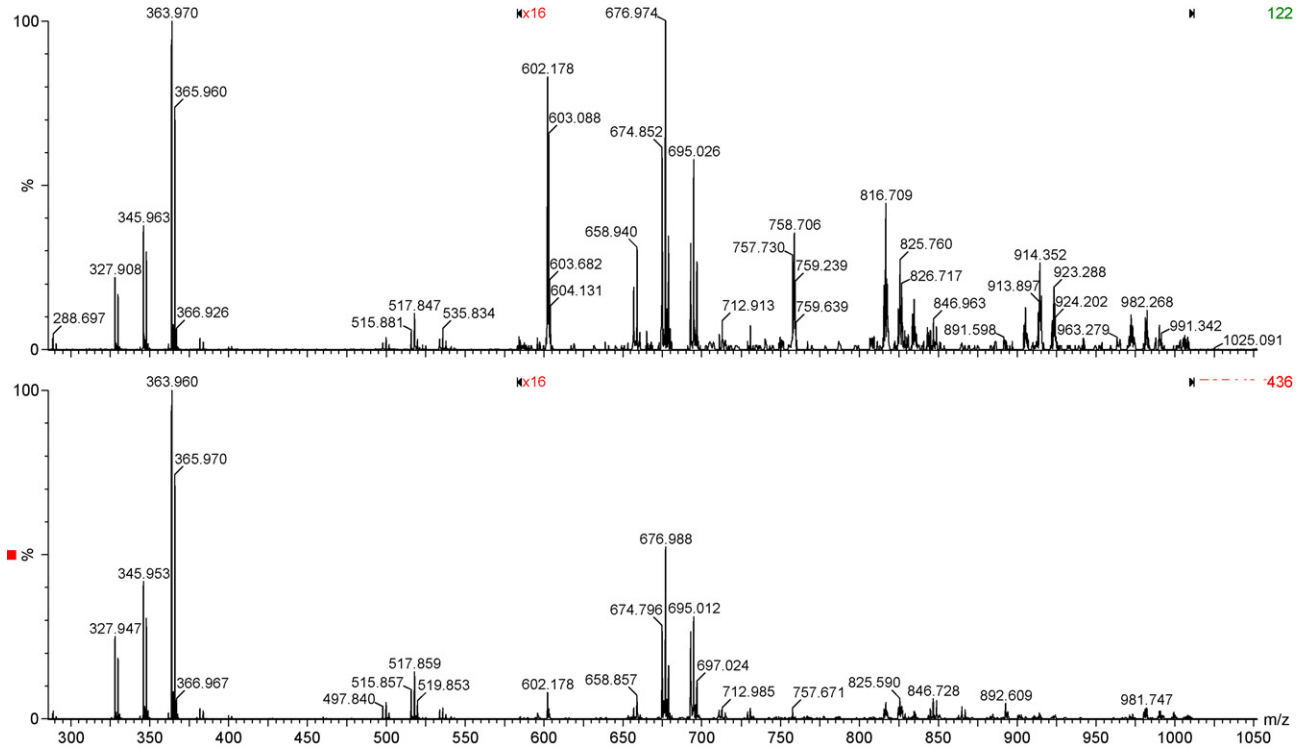

Fig. 5. ESI(−)-MS of MA/β-CD in the *m/z* range of 280–1050, immediately (top) and 24 h (bottom) after its preparation in water at 0.01 mol/L Sb at 25 °C.

of 176 nm (67%, v/v). The mean hydrodynamic diameter was found to increase as a function of time. From 0 to 3 h, the increase in particle size was accompanied by a decrease of the particle count (data not shown). At 24 h, a mean diameter of 971 nm was registered with a polydispersity index of 0.75. When β-CD alone was studied in the same condition of concentration, lower particle counts were observed, mean hydrodynamic diameters higher than 1000 nm with polydispersity indexes of 1 were determined.

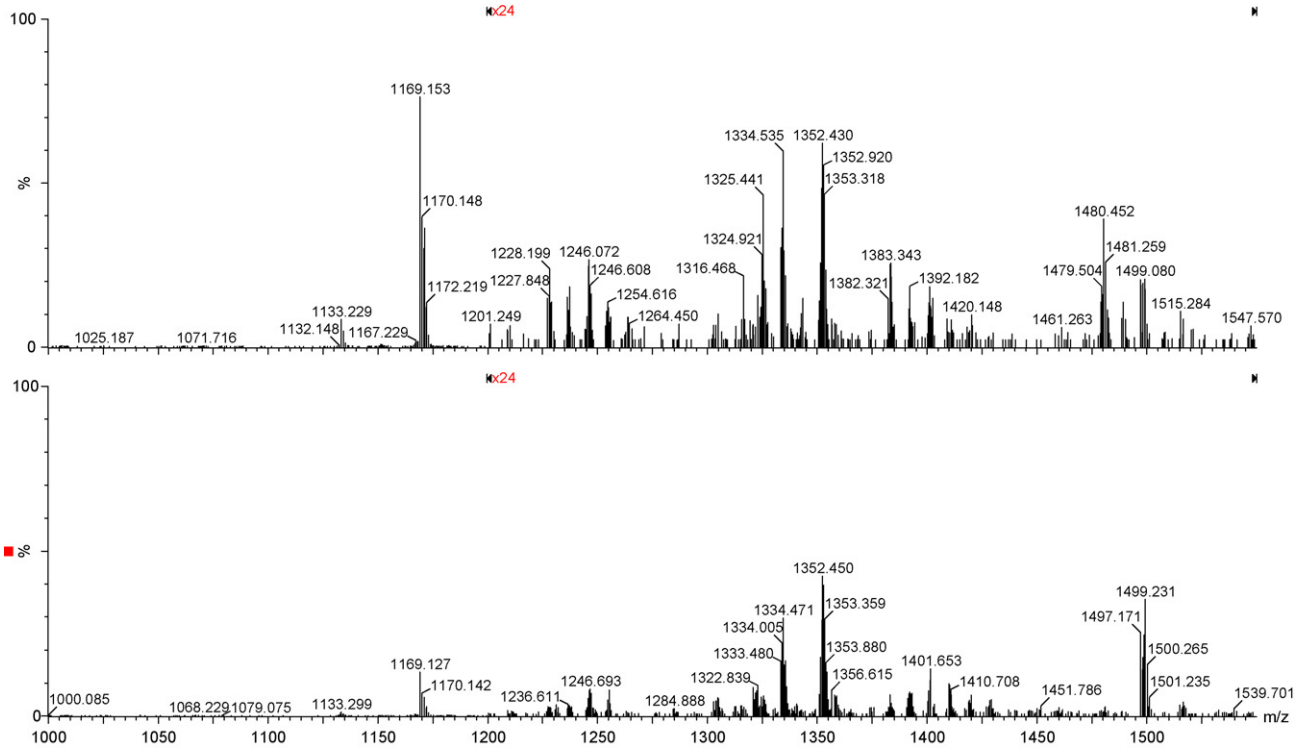

Fig. 6. ESI(−)-MS of MA/β-CD in the *m/z* range of 1000–1550, immediately (top) and 24 h (bottom) after its preparation in water at 0.01 mol/L Sb at 25 °C.

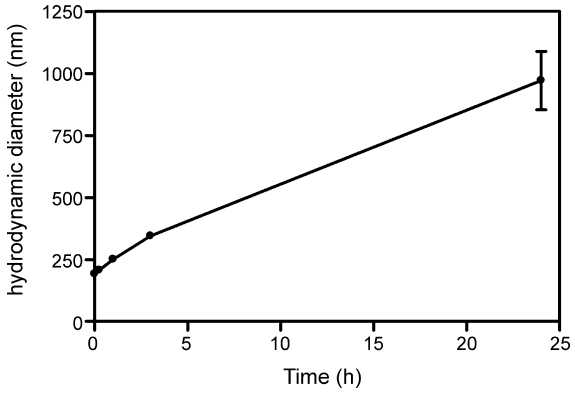

Fig. 7. Time-course of variation of the mean hydrodynamic diameter of particles after reconstitution of MA/β-CD composition in deionized water at 2.5 mmol/L of β-CD, as determined by photon correlation spectroscopy. Data are given as means ± S.D. ($n = 3$).

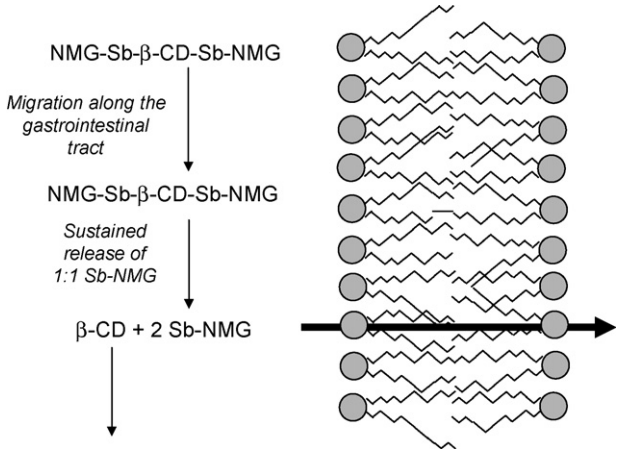

Fig. 8. Model proposed for the mechanisms involved in the enhanced absorption of Sb from the MA/β-CD composition given orally. The MA/β-CD nanoassemblies comprising high-molecular weight MA/β-CD complexes, such as NMG–Sb–β-CD–Sb–NMG species, migrate along the gastrointestinal tract. The MA/β-CD nanoassemblies then slowly release MA in the form of 1:1 Sb–NMG complex which permeates by simple diffusion across the intestinal epithelium. β-CD continues migrating up to the colon where it is degraded.

4. Discussion

Cyclodextrins have been applied to improving the oral delivery of poorly water-soluble drugs through formation of inclusion complexes which increase the apparent drug solubility and/or the rate of drug dissolution (Rajewski and Stella, 1996; Szejtli, 1998; Hirayama and Uekama, 1999). However, in the case of the highly water-soluble drug MA, the formation of an inclusion complex with β-CD was not observed (Demicheli et al., 2004) and the role of β-CD as an absorption enhancer remained to be elucidated.

The present paper underlines the importance of the freeze-drying process in the preparation of MA/β-CD for the enhancement of Sb absorption by oral route. It has been shown that the heated MA + β-CD mixture still produced a significantly lower serum Sb level when compared to the final MA/β-CD composition, indicating that the freeze-drying is required for achieving a high absorption of Sb by oral route.

Importantly, the freeze-drying was found to promote additional interactions between MA and β-CD, which resulted in the formation of supramolecular nanoassemblies. The high-molecular weight 1:2:1, 2:2:1 and 2:2:2 NMG–Sb–β-CD complexes identified by ESI-MS most likely represent the main building blocks for these nanoassemblies. At least two types of interaction are expected to stabilize these nanostructures: (i) labile coordination linkages between Sb and the numerous hydroxyls of β-CD, leading to multiple Sb binding and to cross-linked β-CDs; (ii) hydrogen bonds between β-CD molecules as well as between NMG and β-CD.

β-CD was previously shown to self-associate in aqueous solution. A detailed study of the structures and size of β-CD aggregates in water indicated the occurrence of polymorphism depending on the β-CD concentration (Bonini et al., 2006). Polydisperse nearly spherical objects with diameters of about 100 nm were identified at concentrations lower that 3 mmol/L, whereas micrometer planar aggregates were predominantly observed at higher concentrations. Therefore, one can infer that the β-CD contributes, in both the free and complexed states, to the formation of nanoparticles in the MA/β-CD composition. It is likely that the small sized particle population with a mean diameter of 45 nm, which was detected neither in MA/β-CD after 24 h nor in β-CD solution, represents the main population of MA/β-CD nanoassemblies.

There is a growing body of evidence that supports the important contribution of non-inclusion based aspects for the solubilization of poorly water-soluble drugs by cyclodextrins, as a result of surfactant-like effects and molecular aggregation (Loftsson et al., 2004b). It is noteworthy that the present work presents the first example of an association between a water-soluble drug and a hydrophilic cyclodextrin, exclusively via non-inclusion interactions, which results in enhanced drug absorption.

Another important observation was the ability of MA/β-CD nanoassemblies to act as a sustained release system of the antimonial drug MA, following dilution in water. The dissociation of the nanoassemblies as a function of time was supported by NMR, CD and ESI-MS data and the decrease of particle count in PCS. The recovery of free MA was also evidenced by CD and NMR data. As a consequence of the slow drug release property of these nanoassemblies, one can expect a change of the drug absorption site in the gastrointestinal tract, when compared to MA alone or the heated MA + β-CD mixture.

Fig. 8 displays a model for the mechanisms involved in the enhanced absorption of Sb from the MA/β-CD composition. Accordingly, nanoassemblies comprising high-molecular weight MA/β-CD complexes, such as the NMG–Sb–β-CD-Sb–NMG (or 2:2:1 NMG–Sb–β-CD) species, would slowly dissociate into the 1:1 Sb–NMG complex, upon dilution along the gastrointestinal tract. The low-molecular weight 1:1 Sb–NMG complex would then permeate readily by simple diffusion through the intestinal epithelium (Martins et al., 2006). On the other hand, the released β-CD, because of its low membrane permeability, would continue migrating along the intestine up to the colon where it would be degraded enzymatically (Hirayama

and Uekama, 1999). MA/β-CD nanoassemblies would therefore improve the oral bioavailability of Sb by changing the site of drug absorption and making the 1:1 Sb–NMG complex readily available.

Acknowledgments

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