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Mode of action of β-cyclodextrin as an absorption enhancer of the water-soluble drug meglumine antimoniate

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Abstract

It has been previously reported that β-cyclodextrin (β-CD) enhances the oral absorption of the pentavalent antimony (Sb) drug, meglumine antimoniate (MA). Contrary to the drugs commonly used in association with β-CD, MA is highly soluble in water (solubility >300 mg/mL) and, therefore, the mode of action of β-CD in this system requires clarification. ESI(−)-MS analysis of MA and of the MA/β-CD composition indicated the formation of a 1:1 association compound between 1:1 Sb–meglumine complex and β-CD. A stability constant on the order of 100 L mol^{-1} was determined for this association compound. When MA solution was heated for 48 h at 55 °C to mimic the conditions used to prepare MA/β-CD, MA was found to suffer dissociation, from high molecular weight Sb complexes into species of lower molecular weight. Strikingly, heated MA was found to be more extensively absorbed in mice by the oral route than MA freshly prepared at room temperature. *In vitro* skin permeation experiments using MA and MA/β-CD indicated a two-fold increase in the Sb flux for MA/β-CD. These findings support the hypothesis that the improved oral absorption of Sb arises from the increased permeation of MA across lipid bilayers, as a result of the enhanced availability of 1:1 Sb–meglumine complex.

Keywords: Cyclodextrin; Oral; Topical; Antimony; Meglumine antimoniate; ESI-MS

1. Introduction

Cyclodextrins are cyclic oligosaccharides composed of glucose units joined through α-1,4 glycosidic bonds, which are well known in recognition chemistry as molecular hosts capable of including, with a degree of selectivity, water-insoluble guest molecules via non-covalent interactions within their hydrophobic cavity (Szejtli, 1998). The ability of cyclodextrins to enhance oral or dermal absorption of drugs is a well-documented property, especially in the case of poorly water-soluble drugs (Rajewski and Stella, 1996; Szejtli, 1998; Hirayama and Uekama, 1999; Matsuda and Arima, 1999; Loftsson and Masson, 2001). This property has been attributed to the increase in thermodynamic activity of the drug in the vehicle and/or to the enhancement of the rate of drug dissolution.

The pentavalent organoantimonial drug, meglumine antimoniate (MA), has been used as the first-line drug for the treatment of leishmaniasis (Berman, 1997). Recently, pentavalent antimo-

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nials were also found to exert activity against cancer, hepatitis C and AIDS (Yan et al., 2005). We reported previously that the association of MA with β-cyclodextrin (β-CD) enhances the oral absorption of Sb in mice and renders MA orally active in a murine model of cutaneous leishmaniasis (Demicheli et al., 2004). However, since MA is a highly water-soluble compound and, most probably, does not interact with the internal hydrophobic cavity of β-CD (Demicheli et al., 2004), this association compound differs physicochemically from conventional inclusion compounds and its mechanism of action remains to be elucidated.

To obtain further insight into the mode of action of the MA/β-CD composition, MA and MA/β-CD were characterized by electrospray ionization mass spectrometry (ESI-MS), circular dichroism (CD) and vapor pressure osmometry. It is reported that the process used to prepare the MA/β-CD composition induces the dissociation of MA from high molecular weight Sb complexes into species of lower molecular weight. Moreover, MA in its dissociated state was found to be more effectively absorbed in mice by the oral route. The ability of MA/β-CD to enhance the permeability of Sb across lipid bilayers was also investigated using the *in vitro* skin permeation model.

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) and potassium hexahydroxoantimoniate ($KSb(OH)_6$) from Fluka Chemie GmbH (Switzerland). 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., 1999, 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. ESI mass spectrometric analyses of MA and MA/β-CD composition

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 100–2000 *m/z* range. On the basis of isotope ratios, most ionic species observed in MA appeared to have a charge of −1 or +1 such that the mass/charge ratio (*m/z*) is equal to the molecular weight. In the ESI-MS spectra, Sb–NMG complex ions were easily characterized as containing Sb by the distinctive isotope pattern of antimony (ratio of ^{121}Sb:^{123}Sb, 75:43). 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. The compounds were prepared in water, typically at 1 mg/mL, and introduced using a syringe pump with a flow rate of 5–10 μL/min. Collision-induced dissociation (CID) was performed to confirm the structure of the compounds, by using argon and collision energies in the range of 20–50 eV. 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.

2.4. Osmolarity measurements of aqueous MA solutions

A solution of MA was first prepared in water at 0.7 mol/L of Sb. At $t = 0$, this solution was diluted in water at 0.1 mol/L of Sb and the resulting solution was immediately divided into three groups of three samples. The first group was incubated at 25 °C, the second group was incubated at 37 °C and the third group was incubated at 55 °C. Osmolarity measurements were carried out at 25 °C at different time intervals using a Vapor Pressure Osmometer, Vapro5520 – Wescor®, Logan (Utah, USA).

2.5. Circular dichroism study of the interaction of MA with β-CD

UV absorption and CD spectra were recorded on a Jobin Yvon-Spex Mark CD6 dichrograph. In the study of the formation of MA complex, NMG and $KSb(OH)_6$ were mixed in water at an equimolar ratio and the resulting mixture was incubated at 55 °C. In the study of the formation of MA/β-CD association compound, MA was incubated for 90 min at 55 °C at a 10 mmol/L final Sb concentration, in the presence of β-CD at different concentrations (0, 10, 20, 30, 40 or 50 mmol/L). The solutions were then 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 NMG.

Assuming that the main compound formed between β-CD and MA is a 1:1 association compound (named MA/β-CD), the molar concentration of MA/β-CD can be expressed as:

$$[\text{MA}/\beta\text{-CD}] = [\text{MA}]_{\text{tot}}(\Delta\varepsilon - \Delta\varepsilon^{\text{MA}})/(\Delta\varepsilon^{\text{MA}/\beta\text{-CD}} - \Delta\varepsilon^{\text{MA}})$$

where $[\text{MA}]_{\text{tot}} = [\text{MA}] + [\text{MA}/\beta\text{-CD}]$; [MA] is the molar concentration of free MA; and $\Delta\varepsilon^{\text{MA}}$ and $\Delta\varepsilon^{\text{MA}/\beta\text{-CD}}$ are the differential molar dichroic absorption coefficients of MA and of the MA/β-CD association compound, respectively.

The stability constant for the formation of MA/β-CD (K) was determined assuming the following equilibrium:

$$\text{MA} + \beta\text{-CD} \leftrightarrow \text{MA}/\beta\text{-CD}$$

and using the following equation:

$$1/[MA] = K\{([\beta\text{-CD}]_{tot}/[MA/\beta\text{-CD}]) - 1\}.$$

2.6. Oral absorption of Sb in mice from MA and MAβ-CD composition

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 libidum*.

Animals received, through oral inoculation with the aid of a needle gauge, the following preparations at 100 mg Sb/kg of body weight: MA in water, freshly prepared at 25 °C and 0.05 mol/L of Sb from acetone-precipitated MA (as described in Section 2.2.); MA in water, freshly prepared at 25 °C and 0.05 mol/L of Sb from freeze-dried MA after incubation for 48 h at 55 °C in water at 0.05 mol/L of Sb; MA in water, freshly prepared at 0.05 mol/L of Sb and 25 °C in the presence of 0.05 mol/L β-CD; MA/β-CD composition in water, freshly prepared at 0.05 mol/L of Sb from freeze-dried MA/β-CD obtained as described in Section 2.2. Four mice from each group were sacrificed 3 h after administration.

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 time of 3 h was chosen because it represents the phase of drug elimination, as evidenced by the serum pharmacokinetics of Sb previously determined for MA and MA/β-CD in Swiss mice (Demicheli et al., 2004). Blood samples were obtained and the serum was recovered and frozen.

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. Briefly, samples were diluted five times with 1% (v/v) nitric acid containing 0.02% cetyltrimethylammonium chloride (CTAC). Pyrolytic graphite coated tubes were pre-treated with 150 μL of noble metal solution (1000 mg/L) and then submitted to the temperature gradient program. This procedure was repeated five times in order to obtain a deposit of 500 μg Zr and 250 μg Rh. The diluted samples (20 μL) were then added into the graphite tube. All measurements were carried out using pyrolysis and atomization temperatures of 900 and 1900 °C, respectively, with a Hitachi (Mitorika, Ibaraki, Japan) Z-8200 atomic absorption spectrometer, equipped with a graphite furnace and an autosampler (SSC-300, Hitachi) and with polarized Zeeman effect background correction.

2.7. In vitro skin permeation of Sb from MA and MA/β-CD composition

Full thickness skin was excised from the abdominal surface of 6–8 weeks old hairless mice (HRS/J strain, originally obtained from Jackson Laboratories, Bar Harbor, ME) and immediately mounted in a Franz diffusion cell. Experiments were carried out, under non-occlusive conditions, in the absence or presence of the stratum corneum (SC). The SC was removed by stripping the skin with a cellophane tape. The available diffusion area of the diffusion cell was 1.76 cm^2. Receptor phase, maintained at 37 °C, was an isotonic phosphate buffer saline (PBS, pH 7.2) containing 0.01% of $HgCl_2$ as preservative. MA and MA/β-CD dissolved in water were applied onto the skin (100 μL containing 430 μg of Sb). The receptor fluid was removed at specific times (2, 4, 6, 8 h) and immediately replaced by fresh solution. After 8 h, the skin surface was washed three times with an aqueous solution containing 1% (w/v) of polyoxyethylene 20-oleyl ether and then dried with a cotton swab. The extraction of Sb from the skin fragments was performed by dilacerating the skin in 5 mL of PBS using an Ultra-Turrax.

Sb was assayed in the receptor phase and the skin extract by ETAAS, using a mixture of ruthenium (Ru) and rhodium (Rh) as permanent modifiers. Briefly, samples were diluted five times with 1% (v/v) nitric acid containing 0.02% CTAC. Pyrolytic graphite coated tubes were pre-treated, as previously described (Demicheli et al., 2004), with 150 μL of noble metal solution (1000 mg/L) and then submitted to the temperature gradient program. This procedure was repeated five times in order to obtain a deposit of 500 μg Ru and 250 μg Rh. The diluted samples (20 μL) were then added into the graphite tube. All measurements were performed, as described above, using pyrolysis and atomization temperatures of 900 and 1600 °C, respectively, which gave symmetric absorption peaks and Sb recuperation efficiency for Sb-contaminated samples close to 100%.

2.8. 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. Characterization of MA and MA/β-CD composition by ESI-MS

The chemical compositions of MA and of its association compound with β-CD (MA/β-CD) were investigated using electrospray ionization mass spectrometry in the negative mode (ESI(−)-MS). The MA/β-CD association compound could not be detected in the positive mode at the neutral pH of the MA/β-CD solution (data not shown), because of the zwitterionic character of this specie and, presumably, the very low pK_a of the antimonic acid group (Demicheli et al., 1999).

Fig. 1A and B display the negative ESI spectra obtained for MA and MA/β-CD, respectively. Table 1 shows the molecular weight of the main species observed in the spectra as well as their proposed structure.

ESI(−)-MS data for MA shows Sb–*N*-methyl-*D*-glucamine (Sb–NMG) major complex ions at *m/z* 364, 516, 541, 675

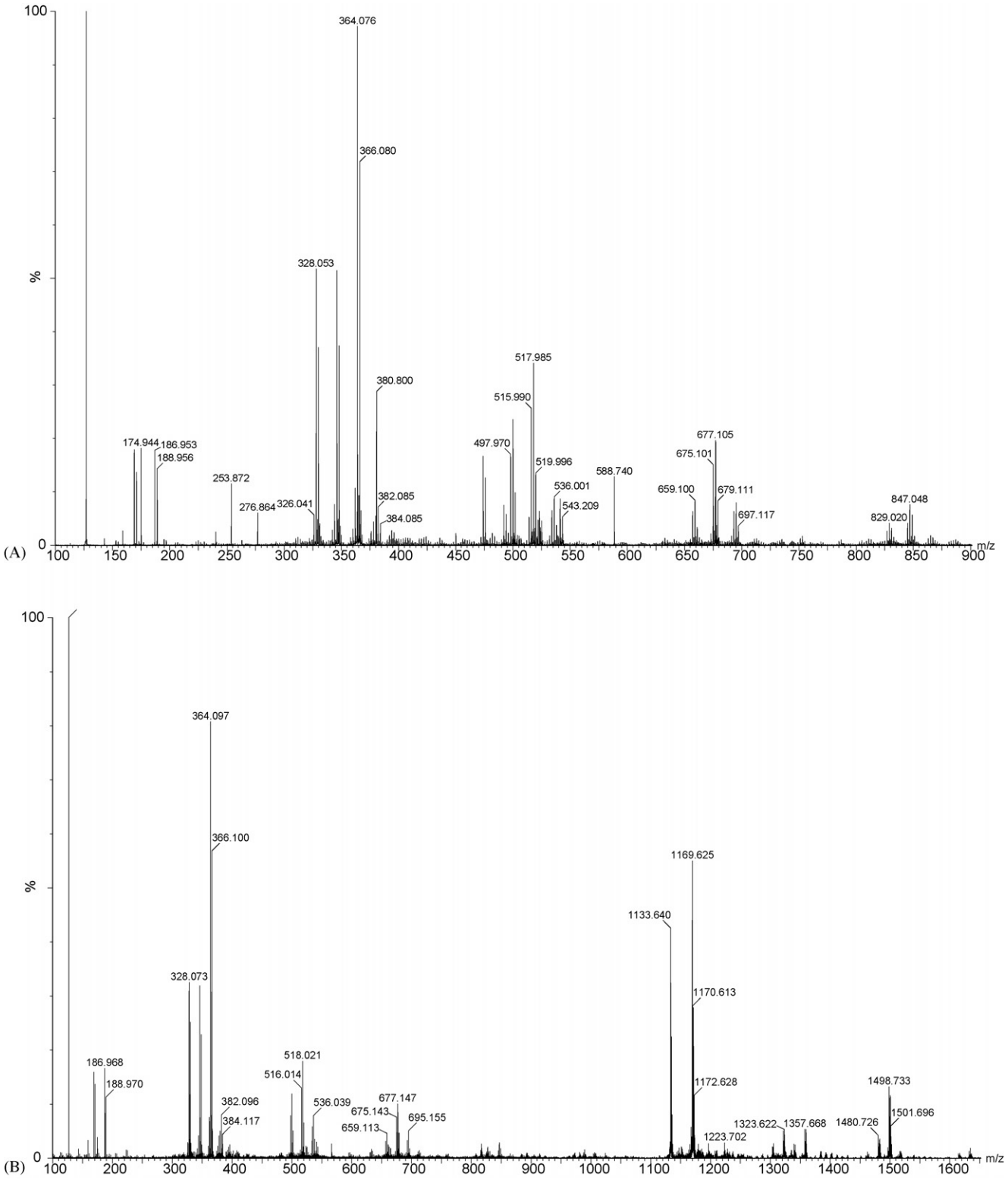

Fig. 1. ESI(−)-MS spectra obtained for MA (A) and MA/β-CD (B) in water.

and 845. This data confirms previous ESI(+)-MS observations (Roberts et al., 1998; Demicheli et al., 2003), indicating that MA consists of a mixture of polymeric structures with the general formula, $(NMG–Sb)_n$, $(NMG–Sb)_n$–NMG and $(Sb–NMG)_n$–Sb. Taking into account the value of the pK_a of NMG amine group on the order of 10 (Demicheli et al., 1999), the major 1:1 Sb–NMG complexes observed in the negative mode (*m*/*z* of 328, 346 and 364) are in equilibrium with their zwitterionic form, which should predominate at neutral pH. This notion is further supported by the observa-

Table 1
Ionic species identified in the ESI(−)-MS spectra obtained with meglumine antimoniate and its association compound with β-CD

Ionic species	*m/z*
$[Sb(O)_2(OH)_2]^-$	187
$[(NMG)Sb(O)–4H]^-$	328
$[(NMG)Sb(O)(OH)–3H]^-$	346
$[(NMG)Sb(O)(OH)_2–2H]^-$	364
$[(NMG)(Sb)_2(O)_2(OH)_2–5H]^-$	498
$[(NMG)(Sb)_2(O)_2(OH)_3–4H]^-$	516
$[(NMG)_2Sb(O)–4H]^-$	523
$[(NMG)_2Sb(OH)_2–4H]^-$	541
$[(NMG)_2(Sb)_2(O)(OH)–8H]^-$	657
$[(NMG)_2(Sb)_2(OH)_3–8H]^-$	675
$[(NMG)_2(Sb)_3(O)_5–6H]^-$	827
$[(NMG)_2(Sb)_3(O)_4(OH)_2–6H]^-$	845
$[β\text{-CD–H}]^-$	1134
m/z 364 + β-CD	1499
m/z 346 + β-CD	1481
m/z 328 + β-CD	1463

tion that MA behaves as a weak electrolyte (Demicheli et al., 1999).

The ESI(−)-MS spectrum obtained for MA/β-CD association compound (Fig. 1B) shows peaks related to the presence of free MA (at *m/z* 328, 346, 364, 498, 516, 657, 675, 693, 827, 845) and free β-CD (at *m/z* 1134, 1170). Some other peaks, corresponding to new species containing one atom of Sb, could also be observed at *m/z* 1499, 1481 and 1463. The molecular structure proposed for the *m/z* 1499 species was further confirmed by a CID experiment. As shown in Fig. 2, the fragmentation of the *m/z* 1499 species led to the appearance of a major complex ion at *m/z* 364. The appearance of this species is in agreement with the formation of a compound in which one 1:1 Sb–NMG complex is associated to one β-CD molecule (364 + 1135 = 1499).

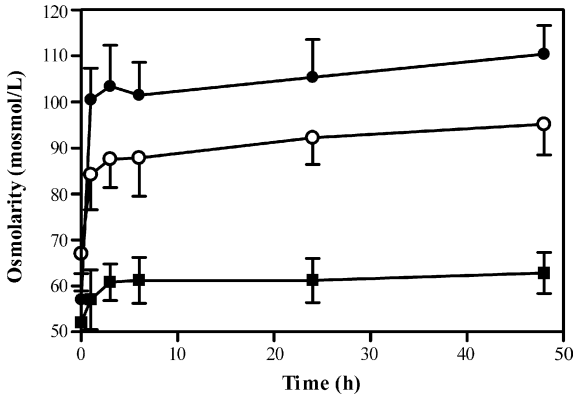

Fig. 3. Time-course of osmolarity changes of freshly prepared MA solution at 0.1 mol/L of Sb at different temperatures: 25 °C (■), 37 °C (◯) and 55 °C (●). Data are given as means ± S.D. ($n = 3$).

3.2. Study of the dissociation of MA at different temperatures

The state of dissociation of MA in water was evaluated from vapor pressure osmometry measurements. Fig. 3 depicts the time course of osmolarity changes of MA solutions in water (at 0.1 mol/L of Sb) incubated at different temperatures, immediately after their dilution from a concentrated MA solution (at 0.7 mol/L of Sb). The initial osmolarity was equal to about

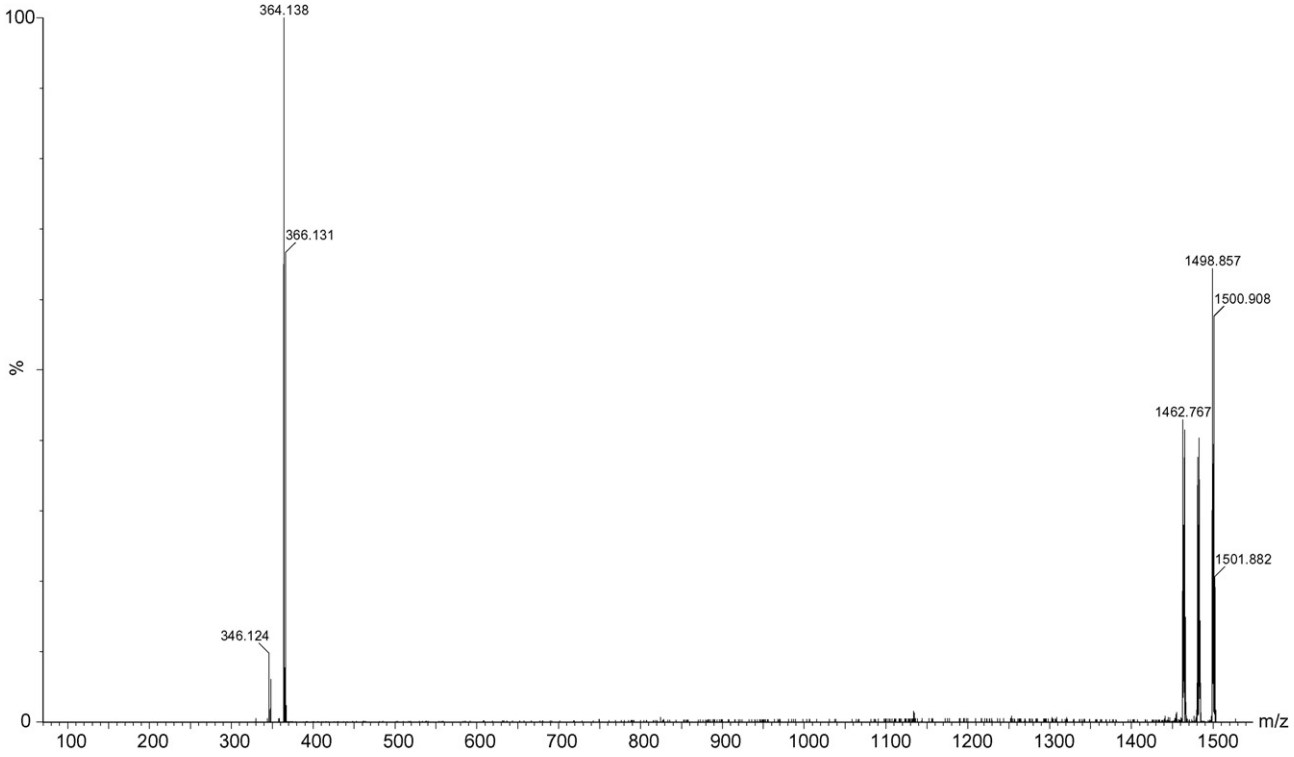

Fig. 2. CID experiment performed for *m/z* 1499 species identified in the ESI(−)-MS spectrum of MA/β-CD.

0.055 osmol/L, indicating an average of two Sb atoms per particle in solution. At the three temperatures studied, an increase in osmolarity was observed during the first 3 h of incubation. These changes can be attributed to the dissociation of MA from high molecular weight Sb complexes into species of lower molecular weight. Increases on the order of 10%, 60% e 85% were observed at 25, 37 and 55 °C, respectively. The final value of osmolarity of 0.11 ± 0.01 osmol/L achieved at 55 °C corresponds to an average of one Sb atom per particle in solution, suggesting the predominance of 1:1 Sb–NMG complex.

When the MA solution, after 48 h of incubation at 55 °C, was freeze-dried and then reconstituted in water at its original concentration, an osmolarity of 0.10 ± 0.01 osmol/L was obtained, indicating that the freeze-drying process preserved the higher abundance of low molecular weight species in MA.

ESI(−)-MS and ESI(+)-MS experiments were also performed in order to evaluate the molecular changes in MA induced by the heating. After heating of the MA solution (0.1 mol/L of Sb) for 3 h at 55 °C, significant changes in the relative abundance of the different peaks were observed in the positive mode, but not in the negative mode (data not shown). The main changes in the positive mode were a two-fold increase of the relative abundance of NMG peak ($m/z = 196$) and a two-fold decrease of the relative abundance of the peak at m/z 818, previously attributed to a 2:3 Sb–NMG complex (Roberts et al., 1998). This data further supports the notion that the heating of the MA solution promoted the dissociation of MA into species of lower molecular weight. Nevertheless, one should keep in mind that the mass spectrum does not necessarily reflects the composition of the starting material, since detection efficiency may decrease with mass and some aggregation may occur as an artifact of the electrospray process.

3.3. Determination of the stability constant for MA/β-CD association compound

We have shown previously that incubation of potassium antimoniate with NMG at 55 °C leads to the formation of MA (Demicheli et al., 2003). Antimoniate in water exhibits an UV absorption below 250 nm but did not show any detectable CD band. We report here that the reaction of potassium antimoniate with NMG is accompanied by the appearance of a CD band with a maximum intensity at 215 nm, evidencing an induced chirality as a consequence of the binding of NMG to Sb. Fig. 4 shows the kinetics of appearance of the CD signal at 215 nm, following the reaction of Sb with NMG at 55 °C.

We took advantage of this characteristic CD signal to investigate the interaction of MA with β-CD. Fig. 5 displays the changes induced in the CD spectrum of MA in the presence of β-CD at varying concentration, after incubation for 90 min at 55 °C. A red shift and a decrease of CD signal intensity were observed until a plateau was reached.

Assuming that MA consists mainly of a 1:1 Sb–NMG complex at 55 °C and that β-CD forms essentially a MA/β-CD association compound according to the following equation:

$$\text{MA} + \beta\text{-CD} \leftrightarrow \text{MA}/\beta\text{-CD}$$

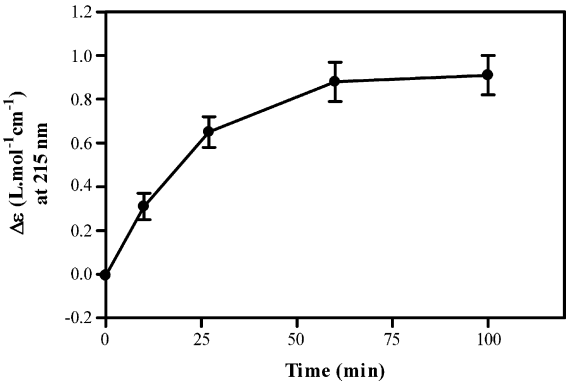

Fig. 4. Kinetics of appearance of circular dichroism signal at 215 nm, following reaction of $KSb(OH)_6$ with NMG at 55 °C. [$KSb(OH)_6$] = [NMG] = 10 mmol/L. Data are shown as means ± S.D. ($n = 3$).

A stability constant (K) for MA/β-CD could be estimated. Its value was found to be equal to 104 ± 24 L mol^{-1}.

3.4. Oral bioavailability of Sb from dissociated MA

The observation that MA suffers dissociation into 1:1 Sb–NMG complex in the conditions of temperature and concentration used in the process of preparation of the MA/β-CD composition led us to investigate the contribution of MA dissociation to the improved oral absorption of MA/β-CD. Four different preparations of MA were obtained and evaluated for their ability to promote the oral absorption of Sb in mice: solutions of MA in water, freshly prepared at 25 °C and 0.05 mol/L of Sb, in the absence (MA) or in the presence of 0.05 mol/L β-CD (MA + β-CD); a solution of MA in water, freshly prepared at 25 °C and 0.05 mol/L of Sb, from freeze-dried MA previously heated for 48 h at 55 °C and 0.05 mol/L of Sb (MAh); the conventional MA/β-CD association compound freshly prepared in water at 0.05 mol/L of Sb.

Fig. 6 displays the level of Sb achieved in the serum of mice, 3 h after administration of each preparation at 100 mg Sb/kg of body weight. A significantly greater Sb level was observed after dissociated MA (MAh), when compared to non-dissociated

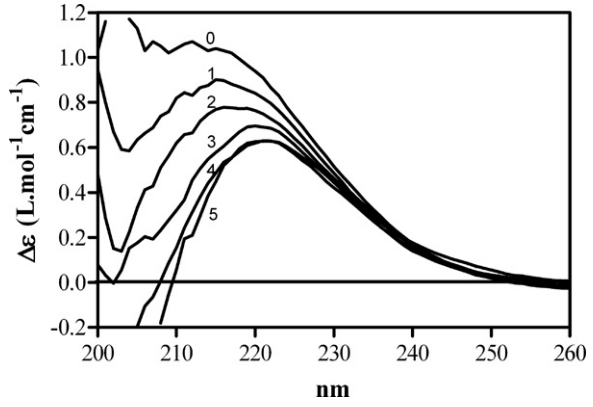

Fig. 5. Circular dichroism spectra of solutions of MA in the presence of increasing concentration of β-CD (0, 10, 20, 30, 40, 50 mmol/L for spectra 0, 1, 2, 3, 4 and 5, respectively), after incubation for 90 min at 55 °C. [Sb] = 10 mmol/L.

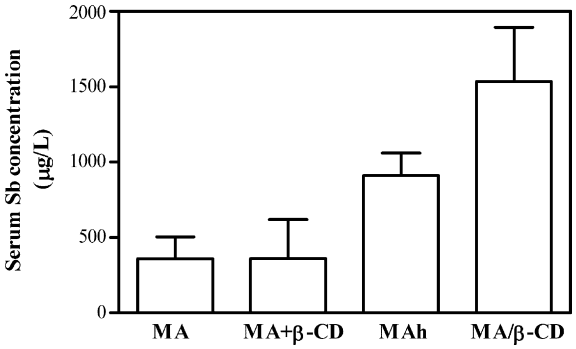

Fig. 6. Serum Sb concentrations in Swiss mice, 3 h after oral administration of MA under different chemical forms at 100 mg Sb/kg. MA: MA in water, freshly prepared at 25 °C and 0.05 mol/L of Sb from acetone-precipitated MA; MAh: MA in water, freshly prepared at 25 °C and 0.05 mol/L of Sb from freeze-dried MA after incubation for 48 h at 55 °C in water at 0.05 mol/L of Sb; MA + β-CD: MA in water, freshly prepared at 25 °C and 0.05 mol/L of Sb in the presence of 0.05 mol/L β-CD; MA/β-CD: MA/β-CD in water, freshly prepared at 0.05 mol/L of Sb from freeze-dried MA/β-CD association compound. Data are given as means ± S.D. ($n = 4$). Statistically significant differences were found between MAh and MA ($P < 0.05$) or MA + β-CD ($P < 0.05$), between MA/β-CD and all other groups, using One-Way ANOVA followed by Tukey's Post-test.

MA (MA), indicating that the dissociation of MA resulted in an improved oral absorption of Sb. Nevertheless, MAh was significantly less effective than MA/β-CD composition, indicating a specific contribution of β-CD to the enhanced oral absorption of MA. The fact that β-CD, in the MA + β-CD physical mixture, failed to improve the oral absorption of MA strongly suggests that specific interactions between MA and β-CD are required for achieving an enhanced oral absorption of Sb.

3.5. In vitro skin permeation of Sb from MA and MA/β-CD composition

The ability of MA/β-CD composition to enhance the permeability of Sb across lipid bilayers was investigated using the *in vitro* skin permeation model. Fig. 7A shows the *in vitro* skin permeation profiles of Sb across hairless mouse skin, when Sb was given as MA or MA/β-CD. A linear relationship was obtained when the total amount of Sb in the receptor phase was plotted against time. The amounts of permeated Sb after 8 h, across intact skin, were 7% and 14% of the applied dose for MA and MA/β-CD, respectively. Therefore, the association of MA to β-CD enhanced two times the flux of Sb across the skin. Strikingly, the skin retention of Sb was also increased significantly from 20.3 ± 1.5 μg (MA) to 27.0 ± 2 μg (MA/β-CD).

Stripped skin was employed to investigate the importance of the SC as a diffusion barrier for MA and MA/β-CD and also to simulate the loss of SC barrier as frequently observed in cutaneous leishmaniasis when lesions progress to ulcers. Fig. 7B shows the permeation profiles of Sb across stripped skin. As consequence of SC removal, the flux of Sb was strongly enhanced for both compounds. Furthermore, Sb flux was six- to eightfold greater for MA/β-CD than for MA. This data is consistent with the idea that the SC is the main diffusion barrier for both compound and that β-CD easily permeates across stripped skins (Loftsson and Masson, 2001).

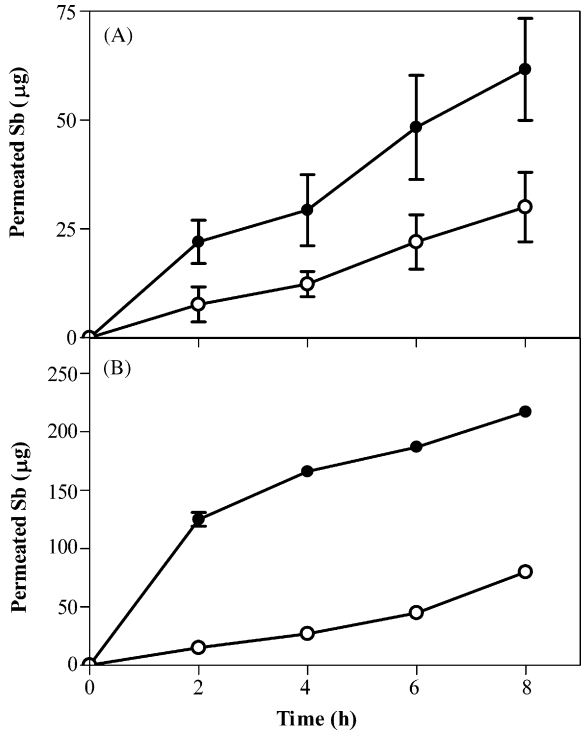

Fig. 7. Kinetic profiles of skin permeation of Sb from MA (open symbols) and MA/β-CD (closed symbols) across intact (A) or stripped (B) skins of hairless mice. Compounds were applied as 430 μg of Sb in 100 μL of water. Data are shown as means ± S.D. ($n = 3$). The error bars smaller than the symbols were not shown.

4. Discussion

Cyclodextrins have been applied to optimizing the oral and dermal delivery of drugs (Rajewski and Stella, 1996; Szejtli, 1998; Hirayama and Uekama, 1999; Matsuda and Arima, 1999; Loftsson and Masson, 2001). This relevant property, however, has been reported mainly for poorly water-soluble drugs. It has been attributed to the increase in the drug thermodynamic activity and/or in the rate of drug dissolution, as a result of the formation of an inclusion compound.

In the present study, new insights into the mode of action of β-CD as an absorption enhancer of the water-soluble drug MA were achieved.

In a first step, MA and its association compound with β-CD were characterized physicochemically by ESI-MS and CD. Structural studies of the β-CD association compounds should take into account the truncated cone-shaped structure of β-CD that exhibits a hydrophilic outer surface and a hydrophobic central cavity. The occurrence of interactions between β-CD and MA was previously suggested through the changes induced in the spin lattice relaxation times of protons in both compounds (Demicheli et al., 2004). However, the fact that no change in ^{1}H NMR resonance of the protons of both MA and β-CD was observed upon formation of the association compound and that β-CD hydrophobic cavity did not loose the included water molecules led us to propose that MA/β-CD is not a conventional drug/cyclodextrin inclusion compound but, rather, an association compound in which MA interacts with the hydrophilic

outer surface of the β-CD molecule, presumably at the largest rim's torus, where OH-3 (hydroxyls at the 3 position) are located (Demicheli et al., 2004).

In the present study, the MA/β-CD composition was further characterized physicochemically. It was found to contain a new chemical entity, in which one 1:1 Sb–NMG complex is associated to one β-CD molecule. Although other antimonial/β-CD compounds resulting from multiple associations may also be formed, those were not detected by ESI-MS and are not expected to be predominant at the 1:1 Sb/β-CD molar ratio used in our work. The low stability constant determined for the 1:1 MA/β-CD association compound further supports the hypothesis that it is a non-covalent complex and that weak interactions, most probably hydrogen bonds, are taking place between MA and β-CD. The high stability constant determined previously for MA ($K \sim 8600\,\mathrm{L\,mol^{-1}}$) (Ferreira et al., 2006) is also in agreement with the notion that the 1:1 Sb–NMG complex does not suffer significant dissociation upon formation of MA/β-CD association compound.

ESI(+)-MS analyses of MA have suggested the existence of a mixture of polymeric structures with the general formula, $(\mathrm{NMG–Sb})_n$–NMG, $(\mathrm{Sb–NMG})_n$–Sb and $(\mathrm{NMG–Sb})_n$ (Roberts et al., 1998; Demicheli et al., 2003). The existence of high molecular weight polymers in concentrated aqueous MA solutions is also supported by particle size analysis using photon correlation spectroscopy that reveals the presence of nanoparticles with a mean hydrodynamic diameter in the range of 1.5–3 nm (Frézard et al., unpublished results). It was also reported that, upon dilution of concentrated aqueous MA solution, a slow increase in the osmolarity of MA solution occurs, evidencing a depolymerization and/or dissociation of Sb–NMG complexes in MA (Roberts et al., 1998).

This observation led us to investigate possible changes in MA's polymerization state under the conditions used to prepare the association compound as well as the influence of the state of polymerization of MA on its oral bioavailability. The heating of the MA solution at 55 °C was found to promote the dissociation of MA, presumably into 1:1 Sb–NMG complex, and the predominance of these low molecular weight species persisted after freeze-drying. Moreover, our data obtained in mice indicate that the dissociation of MA resulted in an improved oral absorption of the drug. These findings suggest that the heating of the MA solution before administration may be an effective means to improve the oral bioavailability of Sb and, thus, this process may encounter applications for the oral treatment of leishmaniasis (Demicheli and Frézard, 2005), cancer, hepatitis C or AIDS (Yan et al., 2005). This data is of great importance, especially if we consider that conventional antimonial chemotherapy consists of daily intramuscular injections for at least 30 days and is often accompanied by local pain (Berman, 1997). Thus, an effective oral formulation would result in fewer side effects and in improved patient compliance, reducing treatment failures.

These results also suggest that the ability of the MA/β-CD association compound to improve the oral absorption of Sb arises, at least in part, from the dissociated state of MA, which resulted in a higher concentration of low molecular weight permeable Sb species. The fact that the MA/β-CD association compound was more effective than the heated MA and the MA + β-CD physical mixture also suggests that factors related to the specific interaction of MA with β-CD are involved in the mode of action of MA/β-CD. It may be that MA/β-CD maintains a higher proportion of the 1:1 Sb–NMG complex when compared to the heated MA or promotes an ionization state of the 1:1 Sb–NMG complex more favorable to its permeation across the gastrointestinal barrier. Alternatively, since cyclodextrins are poorly digested in the small intestine but are almost completely degraded in the colon, the association of MA to β-CD may change the drug absorption site (colon). However, such a property has been described only for drugs covalently bound to cyclodextrins and is not expected in the case of drug/cyclodextrin complex of very low stability constant (Hirayama and Uekama, 1999).

The mechanism of permeation of pentavalent antimonials across biological membranes is still poorly understood. Whereas aquaglyceroporins and multidrug-resistance associated proteins (MRP) were found to mediate the passive and active transport, respectively, of Sb(III) across biological membranes (Borst and Ouellette, 1995; Vernhet et al., 1999; Liu et al., 2004), these transporters do not seem to recognize Sb(V) (Brochu et al., 2003; Yan et al., 2005; Dzamitika et al., 2006). Since no transporter has been identified so far for Sb(V) compounds, it is likely that pentavalent antimonials cross biological membranes either by endocytosis or by simple diffusion through the lipid bilayer.

In order to evaluate the permeation of Sb(V) across the lipid bilayer and test the hypothesis that MA/β-CD enhances the availability of low molecular weight permeable Sb species, the permeation of Sb was studied across lipid bilayers, from MA and its association compound with β-CD, using the *in vitro* skin permeation model. A two-fold increase in the Sb permeation flux across the intact skin was observed for MA/β-CD association compound. Moreover, the partition of Sb into the skin was also enhanced significantly with the association compound. Since the experiment was performed under non-occlusive conditions, water evaporation should take place and the proportion of β-CD-associated MA is expected to increase rapidly. Assuming that β-CD does not cross the intact skin and does not affect the skin barrier (Matsuda and Arima, 1999; Loftsson and Masson, 2001), the present data strongly supports the model that the permeability of Sb was enhanced as a consequence of the modified state of polymerization of MA. Indeed, if improved permeability did not take place, one would expect on the contrary, a decreased Sb flux because of the reduced availability of MA as a result of its association with β-CD (Matsuda and Arima, 1999; Loftsson and Masson, 2001). Presumably, the predominance of 1:1 Sb–NMG complex in the MA/β-CD composition and the low stability constant of MA/β-CD resulted in a higher concentration of the permeable species. Although the permeable specie(s) in MA is not yet known, it is expected that the species, that are, at the same time, electrically uncharged and of low molecular weight, should exhibit the highest permeability coefficient across lipid bilayers. Taking into account the Sb species identified by ESI-MS, one can propose that the neutral [O=Sb–NMG] species, with a molecular weight of 329, may be the permeable species.

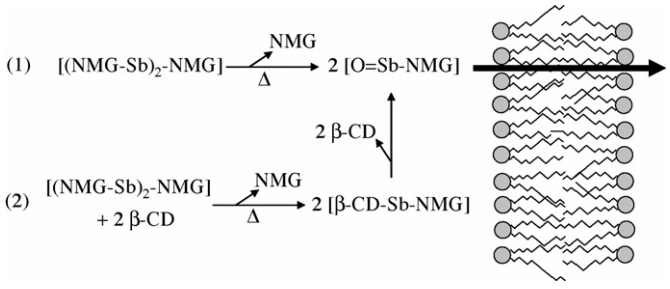

Fig. 8. Proposed model for the effect of heating of MA, in the absence (1) or presence (2) of β-CD, and its impact on the permeation of Sb(V) across biological membranes.

Considering the efficacy of intralesional MA in the treatment of cutaneous leishmaniasis (Alkhawajah et al., 1997), our data also suggest that the ability of MA/β-CD composition to control the permeation of Sb across the skin may be useful in the development of topical formulation for the treatment of cutaneous leishmaniasis.

Fig. 8 illustrates the model proposed for the effect of heating of MA, in the absence or presence of β-CD, as well as its impact on the permeation of Sb(V) across biological membranes.

In conclusion, the physicochemical characterization of MA/β-CD composition indicated the formation of an association compound, in which one 1:1 Sb–NMG complex binds to one β-CD molecule through weak hydrophilic interactions. Our data strongly suggests that the dissociated state of MA, induced by the heating and the binding of MA to β-CD, resulted in the enhanced oral absorption of Sb in mice. Finally, it is proposed that the improved oral absorption of Sb may arise from the increase in the permeability coefficient of the antimonial drug across lipid bilayers.

This work also suggests that the ability of cyclodextrins to enhance the oral absorption of drugs can be extended to some water-soluble drugs that self-associate in aqueous solution and can bind to the hydrophilic surface of the oligosaccharide.

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