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Complexation with β-cyclodextrin confers oral activity on the flavonoid dioclein

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ABSTRACT

Dioclein is a flavonoid reported to have many beneficial effects on the cardiovascular system such as vasorelaxant, hypotensive, antioxidant and antiarrythmogenic activities. However, use as pharmaceuticals is limited due to the lack of oral activity and low water solubility. In this work, intending to improve its oral activity, we performed a 1:1 inclusion complex (IC) between dioclein and β-cyclodextrin (β-CD). The IC was characterized by nuclear magnetic resonance and infrared spectroscopy and its vasodilator and hypotensive effects were evaluated in mice. The inclusion of dioclein in β-CD increased the water solubility 44% compared to free dioclein. The IC (2.5 mg kg^{-1}) produced a higher and long lasting change in systolic blood pressure (SBP) after intraperitoneal administration compared to free dioclein. When given orally, free dioclein (10 mg kg^{-1}) showed no hypotensive effect while the IC induced a pronounced decrease in SBP. The *in vitro* vasodilator effect of dioclein was unchanged by its inclusion in β-CD showing that the IC does not change the interaction between dioclein and its cellular targets. In conclusion, our results show that the new complex prepared by inclusion of dioclein in β-CD improves the hypotensive effect of the flavonoid by increasing its bioavailability and enables dioclein to be effective after oral administration. The mechanism underling the increase in bioavailability is probably a consequence of a protective effect of β-CD against *in vivo* biodegradation by enzymes and possibly increased water solubility.

1. Introduction

Flavonoids consist of a large group of natural polyphenolic compounds mostly consumed in the daily diet (Ross and Kasum, 2002; Yao et al., 2004). The consumption of flavonoids in the human diet is well known to prevent cardiovascular diseases (Muldon and Kritchevsky, 1996; Riemersma et al., 2001), which could be explained by their pharmacological properties as vasodilators, anti-inflammatory, antioxidants and inhibitors of platelet aggregation. Despite the health benefits produced by polyphenolic compounds the therapeutic outcome is still dependent on improvement of the pharmacokinetic profile of these compounds after oral administration. The mechanisms of gastrointestinal absorption of polyphenols are complex. Flavonoids are poorly absorbed in their natural form in the intestine. It is believed that flavonoids are extensively degraded by intestinal microorganisms or enzymes and different metabolites can be produced. These metabolites, if absorbed, are subjected to the hepatic enzymatic system and new metabolites can be formed varying in bioactivity (Stahl et al., 2002). Moreover, many flavonoids have poor water solubility, what limits their pharmaceutical use.

Cyclodextrins are natural macrocyclic oligosaccharides that include six, seven or eight units of glucopyranose, well known for having torus-shaped structures with rigid lipophilic cavities. They are able to enclose highly hydrophobic molecules inside their hydrophobic cavity, constituting a true molecular encapsulation (Duchêne et al., 1999; Polyakov et al., 2004). Cyclodextrins have widely proved their usefulness as tools to generate aqueous drug solutions without the use of organic co-solvents, surfactants, or lipids, as formulation adjuncts (Duchêne et al., 1999; Polyakov et al., 2004). Moreover, formation of inclusion complex (IC) increases the guest's *in vivo* stability against hydrolysis, oxidation, decomposition and dehydration, consequently increasing bioavailability (Szejtli, 1998; Uekama et al., 1998; Hirayama and Uekama, 1999; Cortés et al., 2001).

Dioclein (Fig. 1) is a flavonoid present in the roots of *Dioclea grandiflora* Mart. Ex Benth (Bhattacharyya et al., 1995; Lemos

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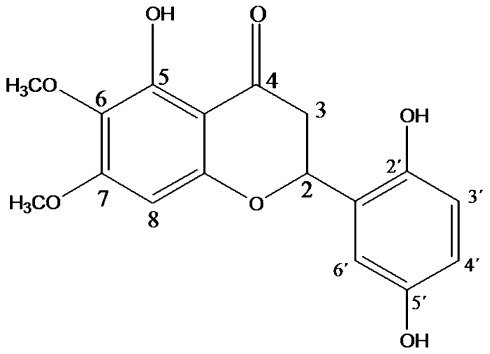

Fig. 1. Structure of dioclein (5,2′,5′-trihydroxy-6-7-dimethoxy-flavanone).

et al., 2006). Our group has reported several beneficial effects of dioclein in the cardiovascular system including vasodilation (Lemos et al., 1999; Cortes et al., 2001; Almeida et al., 2002) and antioxidant and antiarrythmogenic effects (Vianna et al., 2006). As a consequence, dioclein induces hypotension when administered intravenously in rats (Cortes et al., 2001). One problem with the use of dioclein as a therapeutic agent, in spite of the wide spectrum of pharmacological properties, is its lack of oral therapeutic efficacy and low water solubility. To circumvent the problem of solubility and possible intestinal degradation, we prepared and characterized a 1:1 IC between dioclein and β-cyclodextrin (β-CD) and measured its hypotensive effect after intraperitoneal (i.p.) or oral (gavage) administration.

2. Materials and methods

2.1. Inclusion complex preparation

The IC was prepared by the freeze-drying method in the 1:1 molar ratio. In briefly, the aqueous solution containing dissolved β-CD was stirred with dioclein for 24 h to obtain the equilibrium between the species. After this the solution was frozen in liquid nitrogen and lyophilized for 48 h at Savant ModulyoD-Freeze Dryer, Thermo Electron Corp., Waltham, MA, USA (De Sousa et al., 2008). As control system for the physical–chemical characterization, a solid physical mixture of dioclein and β-CD in the same molar ratio of the IC was also prepared.

2.2. Determination of the solubility of dioclein

The solubility of dioclein was determined by ultraviolet visible (UV) spectroscopy, in HP 8453 spectrophotometer, in the absence and in the presence of β-CD (1:1 IC). Two solutions were obtained, one containing only dioclein (2.0 mg/mL) and another with dioclein (2.0 mg/mL) and β-CD (1:1 molar ratio), both dissolved in water. The solutions were kept in a thermostatic bath at 30 °C for 24 h to make sure that the equilibrium was reached. These solutions were centrifuged in an Eppendorf Centrifuge model 5415D (Westbury, NY, USA) at 13,000 rpm, during 10 min. The supernatant, a limpid solution, was used to determine dioclein solubility in both conditions, and 336 nm was used as reference wavelength. The dioclein standard solution was prepared in a concentration of 2.5 mg/mL in a solution of DMSO/water. Successive dilutions were taken from that and used to plot the calibration curve (Fig. 2). Each measurement was carried out in triplicate.

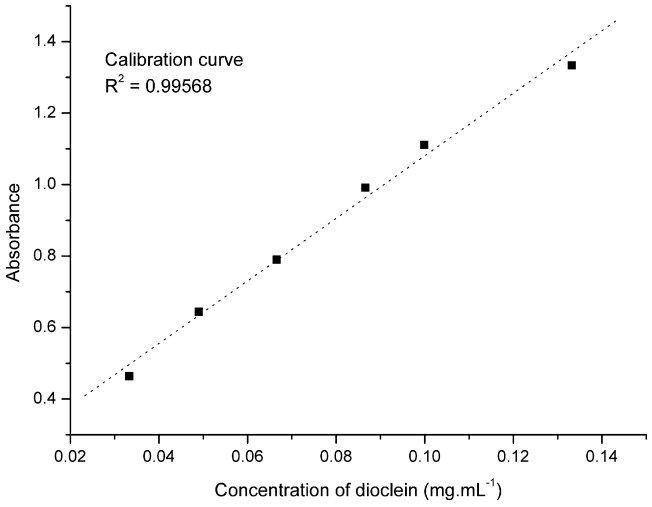

Fig. 2. Calibration curve for dioclein solubility.

2.3. FT-IR spectroscopy

In order to investigate the vibrational changes upon host:guest interaction between dioclein and β-CD, FT-IR spectroscopy was used. This spectroscopy technique is useful to identify which vibrational mode of dioclein and β-CD are being disturbed during the inclusion process, suggesting the interactions between these molecules in solid state (Denadai et al., 2006). All spectra were carried out in a Perkin Elmer spectrophotometer model (Spectrum GX Perkin Elmer, Boston, MA, USA) in KBr pellets. The spectra were obtained at the region of 4000–400 cm^{-1}, with 4 cm^{-1} of resolution and co-additions of 32 scans at room temperature (Denadai et al., 2006). In order to compare the interactions observed in the IC, the FT-IR spectrum of physical mixture, in a 1:1 molar ratio, was also carried out.

2.4. NMR spectroscopy

NMR spectra were recorded on Bruker DRX400-*AVANCE* spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 400 MHz at 27 °C equipped with ϕ5 mm inverse probe with z-gradient coil and DMSO-d_6 as solvent. The chemical shifts are reported in ppm using TMS (0 ppm) as internal standard. One-dimensional ^{1}H NMR spectra were acquired under standard conditions. Two-dimensional inverse hydrogen-detected heteronuclear shift correlation spectra were obtained by HSQC pulse sequence [^1J(C, H)] and HMBC pulse sequence [^nJ (C, H), n = 2, 3, and 4], ^{1}H homonuclear 2D-NOESY experiment was used to confirm the assignments of all hydrogens of the dioclein molecule and dioclein/β-CD complex (NOESY mixing time = 600 ms) (Bax and Davis, 1984).

2.5. Animals

All experimental protocols were performed in accordance with guidelines for the human use of laboratory animals at our Institute and were approved by local authorities (protocol # 025/07, Comitê de Ética em Experimentação Animal (CETEA), Federal University of Minas Gerais (UFMG)). We used male Swiss mice (14–18 weeks) and male Wistar rats (12–14 weeks). All animals were obtained from Cebio (Centro de Bioterismo do Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais). Free access was allowed to standard diet (Labina) and tap water was supplied

ad libitum. Both rats and mice were kept in cages containing at the most five animals, under controlled temperature (24 °C) and a light dark cycle 12/12 h, with lights on at 7:00 a.m and lights off at 7:00 p.m. Efforts were made to avoid any unnecessary distress to the animals.

2.6. Evaluation of the relaxant effect of free dioclein and dioclein/β-CD inclusion complex in resistance arteries

Branch II or III of mesenteric resistance arteries of male rats were cleaned of fat and connective tissue, and a segment 1.6–2.0 mm in length was removed. The segments were then mounted in physiological salt solution (PSS) of the following composition (in mmol/L): NaCl, 119; KCl, 4.7; KH_2PO_4, 0.4; $NaHCO_3$, 14.9; $MgSO_4$, 1.17; $CaCl_2$, 2.5; glucose, 5.5, as previously described (Cortes et al., 2001). Mechanical activity was recorded isometrically by a force transducer (Kistler-Morse, DSG BE4). After mounting, the vessel was stretched to a length that yields a circumference equivalent to 90% of that given by an internal pressure of 100 mmHg; this required a load of about 200 mg. After an equilibration period of 60 min, it was challenged twice with 3 μM phenylephrine to elicit reproducible contractile responses. The presence of functional endothelium was assessed by the ability of acetylcholine (ACh; 1 μM) to induce more than 60% relaxation of vessels pre-contracted with phenylephrine (3 μM), otherwise they were discarded.

The vasorelaxant activity of dioclein and complexed dioclein were measured in vessels pre-contracted with phenylephrine (3 μM). These compounds were added in increasing cumulative concentrations once the response to phenylephrine had stabilized. To evaluate if β-CD alone had relaxing properties, concentration–response curves were constructed in another fragment of the same vessel with this compound.

2.7. Evaluation of the hypotensive effect of free dioclein and dioclein/β-CD inclusion complex

For monitoring systolic blood pressure (SBP) we performed an adaptation of the current tail-cuff method (Krege et al., 1995; Whitesall et al., 2004). SBP was measured by the tail-cuff method using a XBP1000 Series Rat Tail Blood Pressure System (Kent Scientific, Torrington, CT). Conscious male Swiss mice were conditioned to restraint at the warming chamber controlled at 37 °C at most for 5 min. Thereafter an integrated sensor-cuff occluder was placed and used to take at last five different measurements of SBP through the pressure value necessary to stop the heart beat registered in tail. Waveforms could be visualized in a RTP-computerized blood pressure monitor. Measurements were taken every 15 or 30 min for periods varying from 2 to 5 h. In this way, it was possible to assemble curves representing the profile of blood pressure of each animal. This procedure was repeated at least five times for each animal on alternate days, consisting of a training period to minimize the stress caused by restraint and heat (Irvine et al., 1997; Gross and Luft, 2003). To obtain an accurate blood pressure reading, conscious mice must have remained still and undisturbed throughout the measurement period.

After measurement of the basal blood pressure, each animal received an i.p. or oral dose of vehicle, free dioclein, the IC or β-CD alone. After each drug administration, the blood pressure was monitored for a period varying from 2 to 5 h, in intervals of 15 or 30 min depending on the profile of changes in SBP induced by each drug administration.

2.8. Chemicals and solutions

β-CD was obtained from Xiamen Mchem, Xiamen (China), phenylephrine, acetylcholine and dimethylsulphoxide (DMSO) from Sigma (USA). Dioclein was synthesized according to the technique described previously (Spearing et al., 1997). Because of the low solubility of dioclein in water, the *in vivo* experiments were performed using water/DMSO (oral route, 200 μL 5% DMSO) or saline/DMSO (i.p. route, 100 μL 5% DMSO) as vehicles for dioclein. However, for IC and β-CD a lower final concentration of DMSO (1%) in the vehicle was needed to promote the complete solubilization, since the water solubility of these compounds was better than dioclein. At the concentrations used, DMSO had no effect in SBP (data not shown). In the *in vitro* experiments, the final concentration of DMSO never exceeded 0.01% in the bath and was also without effect (data not shown). Calculations were done to assure the same amounts of dioclein, free or complexed with β-CD, during *in vitro* or *in vivo* experiments.

3. Results and discussion

3.1. Determination of the solubility of dioclein

It has been extensively reported in the literature, that β-CD molecules are able to increase the guest molecule solubility (Szejtli, 1998; Uekama et al., 1998; Loftsson and Duchene, 2007). In the present work, free dioclein had a solubility of 0.085 ± 0.005 mg/mL in water, while the solubility of dioclein in a 1:1 IC was increased by 44% (0.123 ± 0.018 mg/mL). This phenomenon could be associated to the interaction between host:guest molecules, during the supramolecular complex formation. This higher solubility of the supramolecular complex could contribute, at least in part, to a greater *in vivo* activity of dioclein (Stella et al., 1999).

3.2. FT-IR spectroscopy

The β-CD, dioclein, IC and physical mixture spectra, in KBr pellet, is depicted in Fig. 3. The analysis of the β-CD spectrum, carried out at 4000–400 cm^{-1}, demonstrates the presence of their most characteristic vibrational modes, such as: the ν_{OH} at 3400 cm^{-1}, ν_{CH} at 2930 cm^{-1}, δ_{OH} at 1400 cm^{-1} and also the ν_{COC} at 1030 cm^{-1} (Egyed, 1990).

The dioclein FT-IR bands presented in Fig. 3 between 650 and 400 cm^{-1}, indicate the crystalline profile of this molecule, and in addition, the main characteristic vibrational modes to this molecule can be assigned, as follows: ν_{OH} at 3470 and 3280 cm^{-1}, symmetric and asymmetric ν_{CH} at 2940 cm^{-1}, $\nu_{C=O}$ at 1650 and 1600 cm^{-1}, the region near 1400 cm^{-1} corresponding to the aromatic ring $\nu_{C=C}$, and others ν and δ corresponding to both aromatic rings between 1000 and 700 cm^{-1}.

FT-IR spectrum of the IC, also presented in Fig. 3, depicts a change in its profile when this is compared to the isolated molecules. The main vibrational modifications might be assigned to the: $\nu_{C=O}$ at 1600 cm^{-1}, the aromatic moieties near 1400 cm^{-1}, the ν_{COC} at 1290 cm^{-1}, and also to the ν and δ_{CH} at 1000–700 cm^{-1}, belong to the aromatic rings. The similarity of IC and physical mixture spectra might be associated to the KBr pellets preparation, in which the preparation process and the force applied to obtain these pellets could be responsible to promote the non-covalent interactions between the species, as well as in the IC. It could occur because the pellets preparation is similar to the dry mixing method used to prepare ICs in solid state; this method involves mixing the cyclodextrin with the guest molecule with no added water (Hedges, 1998). These

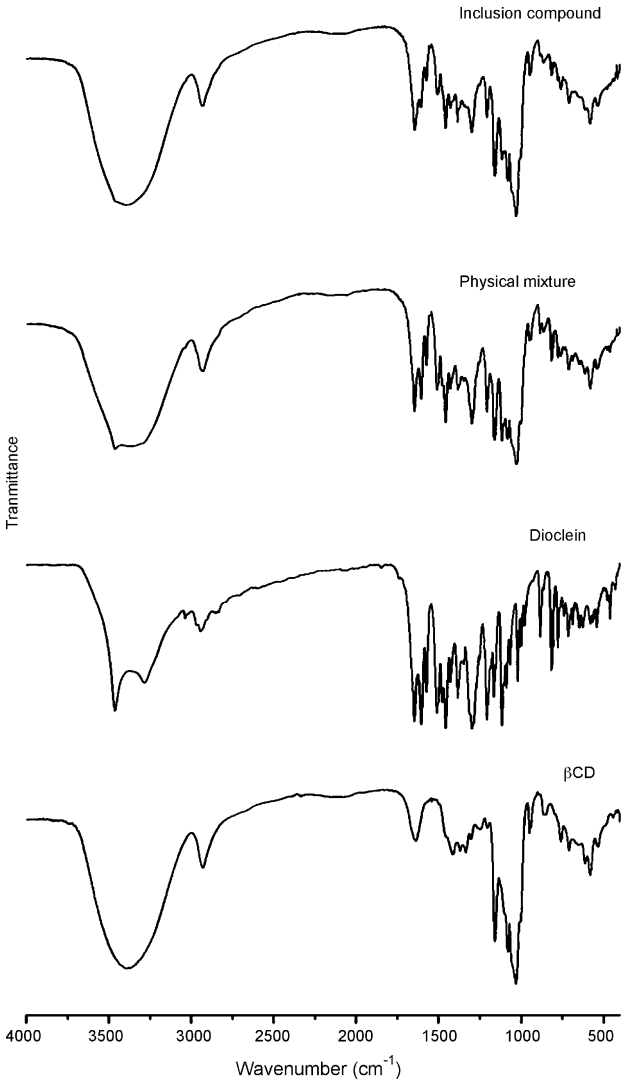

Fig. 3. FT-IR spectra of (a) complexed dioclein, (b) dioclein:β-CD physical mixture, (c) dioclein and (d) β-CD.

results suggest an interaction between β-CD and dioclein, mainly by the aromatic rings. To confirm this hypothesis, the 1D and 2D NMR experiments were carried out.

3.3. NMR spectroscopy

Modern NMR technique based on gradient-pulsed field was used in this study in order to make the assignment and the determination of the dioclein and its IC structures (Derome, 1987; Claridge, 1999). ^{1}H NMR resonance assignments of the dioclein molecule were carried out by 2D shift-correlated NMR techniques. The results of this study were used to confirm dioclein assignments previously reported (Bhattacharyya et al., 1995; Lemos et al., 2002).

In order to evaluate the host:guest interaction, ^{1}H NMR spectrum of dioclein/β-CD complex, in DMSO-d_6 solution, was obtained (Fig. 4). The chemical shifts (^{1}H) and relaxation times (T_1) of pure dioclein and in its β-CD complex are summarized in Table 1.

Comparing the ^{1}H NMR spectrum of dioclein (Fig. 4a), and ^{1}H NMR spectrum of dioclein/β-CD complex in DMSO-d_6 solution (Fig. 4b) changes in NMR signals can be assigned. The most important difference observed was the broadening of the signal attributed to 5-OH group and its little shift from δ_H 11.95 to δ_H 11.75 ($\Delta\delta$ 0.20). This difference might be attributed to the interaction between dioclein and β-CD molecule.

Spin-lattice relaxation times (T_1) values can provide very important information about hydrogen mobility of this supramolecular system, once the correlation times of guest molecules assembled in a supramolecular form are modified due to the mobility restriction upon complexation. According to the results presented in Table 1, it can be observed that the T_1 values for the dioclein/β-CD complex are different to the T_1 values obtained to pure dioclein solution. The results suggest a change in the environment of the hydrogens 5-OH (δ_H 11.95), 7-OCH_3 (δ_H 3.88), H8 (δ_H 6.32) of the ring A and the hydrogens 2′-OH (δ_H 9.10), H4′ (δ_H 6.63), 5′-OH (δ_H 8.85), H6′ (δ_H 6.89) of the aromatic moiety of the guest molecule as consequence of its interaction with β-CD molecule (Gil and Geraldes, 1987; Schneider et al., 1998).

Nuclear Overhauser Effect (NOE) measurements are one of the most important tools to confirm the guest inclusion in the β-CD cavity, and this NMR experiment is very useful to provide information about supramolecular topology. The NOEs detected in the 2D-NOESY experiments cross peak correlations between the hydrogens 2′-OH (δ_H 9.10), 5′-OH (δ_H 8.85) of dioclein molecule and hydrogens H-3 (δ_H 3.89) and H-5 (δ_H 3.69–3.80) of β-CD, could

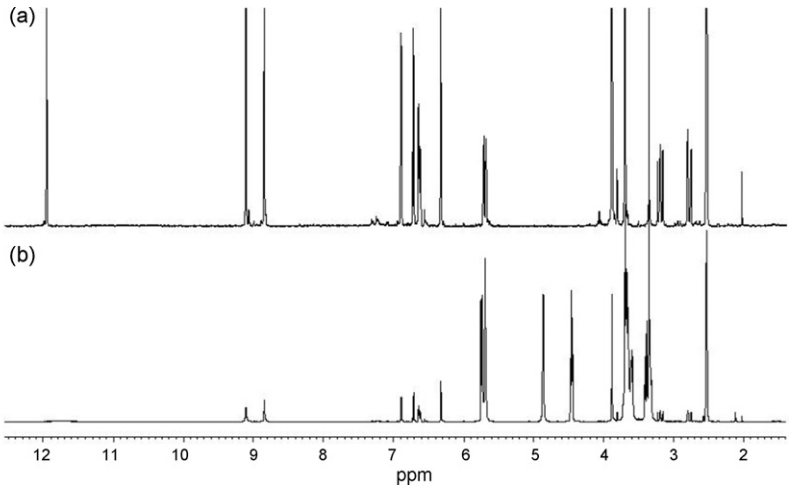

Fig. 4. ^{1}H NMR of (a) dioclein and (b) complexed dioclein (DMSO-d_6, 400 MHz).

Table 1
^{1}H NMR spectral data for free dioclein and the inclusion compound (400 MHz, DMSO-d_6).

Hydrogen	Dioclein		Dioclein/β-cyclodextrin		
	δ (ppm)	T_1 (s)	δ (ppm)	T_1 (s)	ΔT_1 (s)
H2	5.69	1.502	–[a]	–[a]	–
$H3_{(a\ and\ b)}$	2.77 and 3.19	0.381 and 0.366	2.77 and 3.19	0.360 and 0.420	0.021 and −0.054
5-OH	11.95	1.892	11.75	1.740	0.152
6-OCH_3	3.69	0.956	–[a]	–[a]	–
7-OCH_3	3.88	0.658	3.88	0.930	−0.272
H8	6.32	1.369	6.32	1.140	0.229
2′-OH	9.10	1.612	9.10	1.400	0.212
H3′	6.72	1.153	6.72	1.150	0.003
H4′	6.63	1.639	6.63	1.530	0.109
5′-OH	8.85	1.596	8.85	1.470	0.126
H6′	6.89	1.485	6.89	1.350	0.135

[a] Signals covered by β-cyclodextrin hydrogen signals.

only arise if a dioclein/β-CD complex has been formed (Fig. 5). This data suggests that dioclein aromatic rings are included into the torus β-CD cavity, in accordance to the FT-IR results, confirming the complexation. In addition, the hypothesis of a 1:1 complex stoichiometry might arise, since a specific site of the guest molecule presented dipolar correlation, in 2D-NOESY contour map, with the β-CD molecule.

3.4. Vasorelaxant effect of free dioclein and dioclein/β-CD inclusion complex on isolated resistance mesenteric arteries

Vascular tone of small arteries and arterioles underlies the maintenance of peripheral resistance in the circulation and is a major contributor to the control of blood pressure (White et al., 1996). We showed in a previous work that dioclein has a potent vasodilator effect in resistance arteries, mainly due to activation of potassium channels (Cortes et al., 2001). We speculated if the inclusion of dioclein in β-CD could change the vasodilator property of this flavonoid. In rat mesenteric arteries pre-contracted with 3 μM phenylephrine, dioclein produced a concentration-dependent vasorelaxation, with an IC_{50} value of 2.21 ± 1.12 μM (Fig. 6). Complexed dioclein was also able to produce a concentration-dependent vasorelaxation which was not different from dioclein (IC_{50} = 1.37 ± 0.38 μM). Both substances were able to induce 100% of relaxation in mesenteric arteries. β-CD alone did not produce a significant vasorelaxant effect compared to dioclein or complexed dioclein. These results indicate that the inclusion of dioclein in β-CD does not change its *in vitro* vasorelaxant properties.

3.5. Changes in SBP caused by free dioclein and dioclein/β-CD inclusion complex

Fig. 7 shows the decrease in SBP induced by free dioclein and complexed dioclein when given i.p. in mice. β-CD alone was

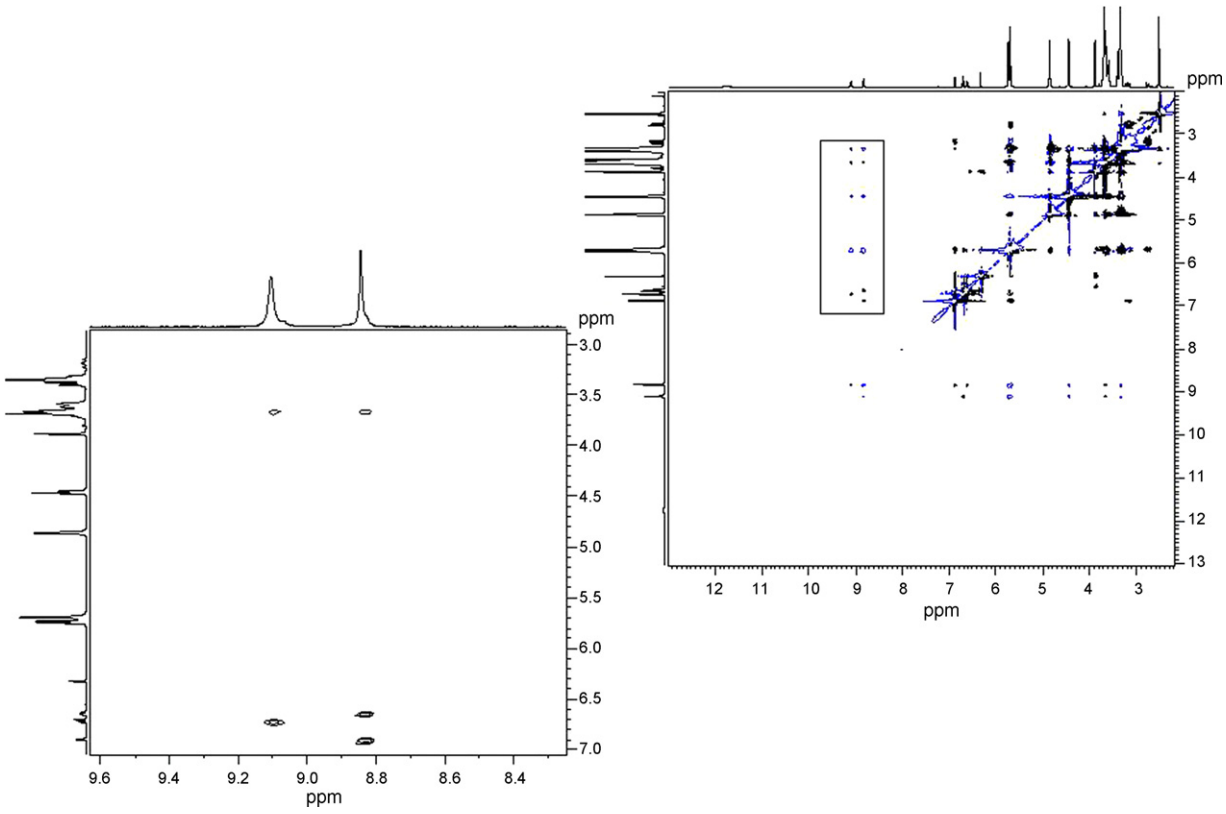

Fig. 5. A two-dimensional NOESY spectrum of the complexed dioclein (400 MHz, DMSO-d_6).

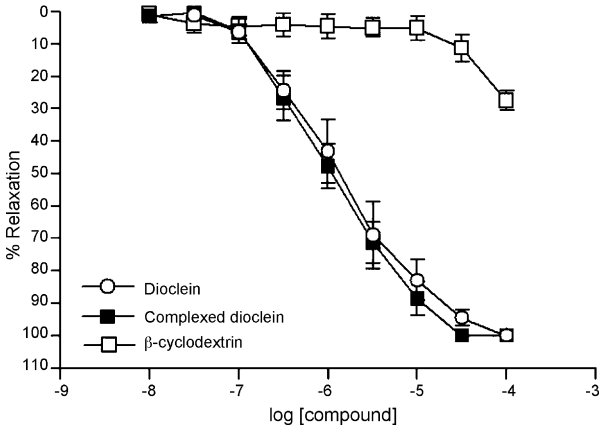

Fig. 6. Vasodilator effect of dioclein and complexed dioclein and β-CD in resistance mesenteric arteries pre-contracted with phenylephrine. The results are means ± S.E.M. of at least five experiments.

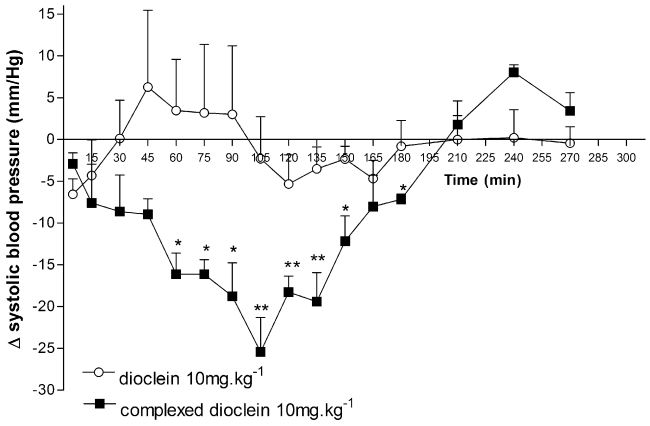

Fig. 8. Hypotensor effect induced by dioclein and complexed dioclein when administrated orally in mice. The values are means ± S.E.M. of at least eight animals (*$P < 0.05$; **$P < 0.01$) (Student's *t*-test).

not able to produce any modification in the SBP when given i.p. (data not shown). However, both dioclein and complexed dioclein induced hypotensive responses (Fig. 7). A profile of the hypotensive response of our compounds was obtained during 3 h monitoring SBP. Interestingly complexed dioclein induced a higher change in SBP when compared to dioclein alone (Fig. 7). The duration of the response was also increased when complexed dioclein was administered i.p. compared to dioclein alone (Fig. 7).

We have previously shown that the hypotensive effect of dioclein was likely to be a consequence of its vasodilator effect on resistance vessels (Cortes et al., 2001). The unchanged potency of the vasodilator effect of dioclein when complexed with β-CD, as described above (Fig. 6) suggests that the interaction of dioclein with its cellular targets is not modified in the complexed compound and that changes in kinetic parameters are probably the main factor involved.

Flavonoids are, in general, extensively conjugated after first pass through the liver and this could contribute to low bioavailability reported for these polyphenolic compounds (Ross and Kasum, 2002; Scalbert and Williamson, 2000). i.p. administration is in part subject to first pass metabolism (Aghazadeh-Habashi et al., 2002) and a considerable amount of dioclein could be inactivated by this system, decreasing its *in vivo* activities after administration by this route. One possible explanation for the increased activity after i.p. administration could be the protective effect of β-CD against *in vivo*

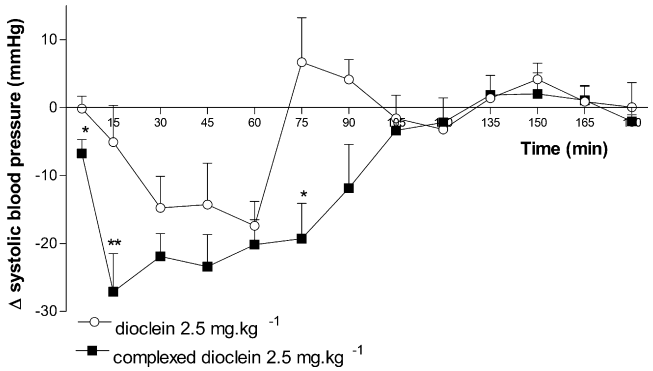

Fig. 7. Hypotensor effect induced by dioclein and the complexed dioclein when administrated intraperitoneally in mice. The values are means ± S.E.M. of at least eight animals (*$P < 0.05$; **$P < 0.01$) (Student's *t*-test).

biodegradation by enzymes, mainly hepatic, that could increase the delivery of dioclein to the systemic circulation after passing through the liver.

When administered orally (Fig. 8), dioclein did not produce any significant effect in SBP even in doses up to 10 mg kg^{-1} (fourfold the dose used in i.p. studies). Interestingly, complexed dioclein (10 mg kg^{-1}) produced a potent and long-lasting hypotensive effect after oral administration (Fig. 8). As expected the onset of the hypotensive effect of complexed dioclein after oral administration is observed later (Figs. 7 and 8) and the maximal effect takes longer to be achieved (Figs. 7 and 8) compared to i.p. administration. This is probably due to the longer time necessary to the compound to reach the systemic blood circulation from intestinal absorption after dioclein:β-CD IC dissociation. The duration of the hypotensive effect (Figs. 7 and 8) is also extended after oral absorption of complexed dioclein almost certainly due to the slow absorption of the compound into the blood stream from the gastrointestinal tract or to a higher bioavailability obtained when dioclein is complexed with β-CD.

The results reported here show that dioclein has poor oral absorption, as reported for many other flavonoids (Ross and Kasum, 2002; Stahl et al., 2002; Yao et al., 2004; Sildeberg et al., 2005). Many studies attribute this poor gastrointestinal absorption of polyphenolic compounds to reduced dissolution rate (Arcari et al., 1992; Liu et al., 2006) or degradation by enzymes (from bacteria and host) in the gastrointestinal tract and liver (Scalbert and Williamson, 2000; Stahl et al., 2002). In most of cases, complexation of many hydrophobic substances using β-CD can improve oral bioavailability by increasing both the dissolution rate (Arcari et al., 1992; Tommasini et al., 2004; Loftsson and Duchene, 2007) and/or protecting against biodegradation (Salmaso et al., 2007). The resultant complex obtained from the inclusion of dioclein in β-CD has greater pharmacological effectiveness compared to dioclein alone probably due to changes in solubilization properties or/and biodegradation that enable complexed dioclein to be active by oral route.

Acknowledgements

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