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Microwave synthesis and *in vitro* stability of diclofenac-β-cyclodextrin conjugate for colon delivery

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ABSTRACT

The aim of this work was to synthesize an ester prodrug of diclofenac and β-cyclodextrin suitable for colonic delivery. The synthesis of an ester linkage between diclofenac and β-cyclodextrin was conducted by the nucleophilic substitution of mono-6-tosyl-β-cyclodextrin under microwaves irradiation. After purification, the conjugate was characterized by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry; infrared (IR) spectroscopy; proton nuclear magnetic resonance (^{1}H NMR) spectroscopy; and two-dimensional rotating frame nuclear overhauser effect (ROESY) spectroscopy. The purity was qualified by high pressure liquid chromatography (HPLC). To assess its potential for colonic delivery, the conjugate was evaluated for stability in simulated gastric and small intestinal fluids, and in fecal material from humans processed within a slurry under anaerobic conditions. The conjugate was successfully synthesized with a yield of 20% following purification. The mass spectra showed the parent peak *m*/*z* 1434 corresponding to [conjugate+Na] adduct. IR and NMR results confirmed that the carboxyl group of diclofenac is covalently bound to one of the hydroxyl groups of cyclodextrin by an ester linkage. Moreover, ROESY data indicated that the formation of the conjugate is not accompanied by the inclusion of diclofenac within the cyclodextrin. The conjugate was otherwise stable in simulated gastric and small intestinal conditions, but was also readily hydrolyzed liberating diclofenac in less than 2 h within the human fecal slurry. This confirmed the potential for this new prodrug as a carrier for colonic delivery.

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1. Introduction

Oral colon-specific drug delivery has progressively gained importance for the localized delivery of therapeutic agents in the treatment of disorders such as ulcerative colitis and Crohn's disease. Additionally, colon-specific delivery provides an opportunity for the systemic delivery of drugs utilized in the treatment of diseases sensitive to circadian rhythms, including asthma, angina and arthritis (McConnell, Liu, & Basit, 2009).

Though a number of approaches have been proposed for the colonic delivery of therapeutic agents (Yang, Chu, & Fix, 2002), the use of gastrointestinal microbiota as a prompt offers a viable and attractive method of effecting drug release in the colon. This approach exploits the abrupt increase in bacterial population and associated enzymatic activity moving from the small intestine to the colon (McConnell, Fadda, & Basit, 2008). For example, azo-bonded prodrugs of mesalazine – including sulfasalazine, balsalazide and olsalazine – are used clinically for the treatment of ulcerative colitis. These prodrugs pass intact through the upper gastrointestinal tract, but once in the colon, the azo-bond is cleaved by the resident microbiota, releasing the active drug molecule from the carrier (Dhaneshwar & Vadnerkar, 2011).

The oligosaccharide β-cyclodextrin can function as a carrier for colonic delivery.

β-Cyclodextrin is a cyclic oligosaccharide derived from starch consisting of seven glucose units linked by α-1,4 glycosidic bonds in a doughnut-shaped form. As a consequence of its chemical structure, β-cyclodextrin can form inclusion complexes with smaller molecules that fit into their cavities. β-Cyclodextrin is not absorbed through biological membranes in the gastrointestinal tract, but it is fermented into small saccharides by the microbiota of the colon – in particular, *Bacteroides* species (Chourasia & Jain, 2003; Flourié et al., 1993; Park et al., 2000; Sinha & Kumria, 2001). Active molecules can

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be attached covalently to the primary or secondary hydroxyl groups of β-cyclodextrin producing prodrugs with the ability to remain intact in the upper gastrointestinal tract. Once such molecules reach the microbiota of the colon, they are subsequently cleaved to release the active drug (Uekama, 2004).

Moreover, fermentation of β-cyclodextrin leads to production of short-chain fatty acids (SCFA) that can contribute to the maintenance of the health and integrity of the colonic epithelium (Giardina & Inan, 1998). In this respect, therefore, the use of β-cyclodextrin as a carrier provides considerable health benefits and is additionally 'Generally Regarded As Safe' (GRAS) (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gándara, 2009).

Diclofenac [2-(2,6-dicloranilino) phenylacetic acid] is a non-steroidal anti-inflammatory drug, and much interest surrounds the notion of targeting its release in the colon, given that such an approach would allow for circumvention of the adverse gastric effects notoriously associated with drugs of this class. It is also widely believed that the colonic delivery of diclofenac would provide a suitable tactic for the management of arthritis pain by chronotherapy (Lin & Kawashima, 2012). Furthermore, diclofenac is known to be well-absorbed in the colon (Gleiter et al., 1985), and has demonstrated an effective chemopreventive action in colon cancer (Jasmeet & Nath, 2010; Saini, Kaur, Sharma, & Sanyal, 2009).

In this present study, we have investigated a method to prepare diclofenac-β-cyclodextrin prodrug suitable for site-specific delivery in the colon. The preparation method involves modification of a β-cyclodextrin hydroxyl group by an electrophilic reagent followed by nucleophilic substitution under microwaves irradiation. The stability of the conjugate was determined in simulated stomach and small intestinal fluids, and in human fecal slurry to mimic the conditions of the colon, to determine the suitability of the conjugate to release diclofenac in the colon.

2. Materials and methods

2.1. Chemicals

β-Cyclodextrin hydrate, *p*-toluenesulfonyl chloride, DIAION HP-20 were obtained from Sigma. Sodium diclofenac was kindly provided by Labesfal Genéricos. Dimethylformamide (DMF) was purchased from Fisher Scientific. Other chemicals and solvents were of analytical reagent grade and deionized water was used.

2.2. Characterization

Thin layer chromatography (TLC) was conducted on aluminum plates percoated with silica gel F254 (Merck & Co.) and eluted with a mixture of 2-propanol/ethyl acetate/water/ammonia (6:1:3:1) or acetonitrile/water/ammonia (6:3:1).

For spot detection the plates were immersed in a mixture of *p*-anisaldehyde (2 mL)/ethanol (36 mL)/acetic acid (5–6 drops)/sulfuric acid (2 mL). The plate was heated to 150 °C for 5 min in order to visualize the spots.

The identification of the obtained compounds was confirmed by one or more of the following techniques: proton nuclear magnetic resonance (^{1}H NMR) spectroscopy; matrix-assisted laser desorption/ionization (MALDI) spectroscopy; infrared (IR) spectroscopy; and high performance liquid chromatography (HPLC).

^{1}H NMR spectra were acquired on a Varian Unity-500MHz spectrometer, using deuterated dimethyl sulfoxide (DMSO-d_6) as solvent and TMS was used as an internal reference.

MALDI-TOF mass spectra were obtained on an Autoflex III, Bruker using methanol and water as solvents and α-cyano-4-hydroxy-cinnamic acid (HCCA) as matrix.

IR spectra was obtained using Thermo Scientific Nicolet 6700 FT-IR spectrometer by scanning KBr discs of the samples.

An HPLC system (model HP1100 series, Hewlett Packard, Germany) equipped with an autosampler (Agilent 1100 series, Germany) was used. A reversed-phase X-Terra C-18 column, 5 μm, 4.6 mm × 250 mm (Waters, USA), with a pre-column, was employed. Mobile phase consisted of acetonitrile and 0.4% trifluoroacetic acid (TFA) in water with a flow rate 1.0 mL/min, an injection volume of 20 μL and detection at 254 nm at 30 °C.

2.3. Synthesis of diclofenac-β-cyclodextrin conjugate

Diclofenac-β-cyclodextrin was synthesized through a 2-step process involving tosylation of β-cyclodextrin (step 1) and nucleophylic substitution of tosylated β-cyclodextrin by sodium diclofenac under microwaves (step 2).

Step 1: Tosylated β-cyclodextrin was synthesized adapting the procedure described by McNaughton, Engman, Birmingham, Powis, and Cotgreave (2004). Briefly, to a solution of 5 g of β-cyclodextrin (4.4 mmol) in water (110 mL) 1.25 g of *p*-toluenesulfonyl chloride (6.55 mmol) was added and the resultant solution was stirred at room temperature for 2 h under inert atmosphere. Aqueous NaOH (2.5 M, 20 mL) was added and the solution stirred for 10 min before unreacted *p*-toluenesulfonyl chloride was filtered off. 5.8 g of ammonium chloride (108 mmol) was added to lower the pH to approximately pH 8. The solution was cooled overnight and the resultant white precipitate collected by filtration. The white powder was washed with acetone and water to remove the non-reacted cyclodextrin and then dried under vacuum. The product obtained is used directly in the next step.

Step 2: Sodium diclofenac (0.333 mmol) was dissolved in 3 mL of anhydrous DMF containing β-cyclodextrin tosylate (0.327 mmol) in an appropriate thick-walled glass vial. The reaction vessel was then sealed with a teflon cap and the reaction mixture magnetically stirred and heated at 140 °C for 40 min under focused microwave irradiation with an initial power setting of 75 W (CEM Discover S-class single mode microwave reactor). The product obtained was precipitated in acetone, filtered, washed several times with acetone and ethyl ether and dried. In order to obtain a purified sample 300 mg of the crude product was passed through DIAION HP-20 ion-exchange chromatography column eluting with water/methanol, and steadily increasing the methanol content. The conjugate was eluted with 80% methanol. Methanol in the eluate was removed under reduced pressure, the solution lyophilized in a freeze-dryer (Lyph-lock 6 apparatus, Labconco) for 72 h and the diclofenac-β-cyclodextrin was obtained with a yield of 20%.

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 7.52 (d, H-5′, H-3′ of Diclofenac), 7.22 (d, H-4″ of Diclofenac), 7.20 (t, H-4′ of Diclofenac), 7.05 (t, H-6″ of Diclofenac), 6.83 (t, H-5″ of Diclofenac), 6.25 (d, H-7″ of Diclofenac), 5.66–5.84 (m, OH-2 and OH-3 of β-cyclodextrin), 4.83 (d, H-1 of β-cyclodextrin), 4.21–4.55 (m, OH-6, H-6 of β-cyclodextrin), 3.50–3.90 (m, H-6, H-3 and H-5 of β-cyclodextrin), 3.25–3.43 (m, H-2, H-4 of β-cyclodextrin), MS (MALDI) *m*/*z* for $C_{56}H_{79}Cl_2NNaO_{36}$, found 1434.308 [conjugate+Na].

2.4. Stability of diclofenac-β-cyclodextrin in simulated gastric and small intestinal fluids.

Simulated gastric fluid (SGF) was prepared according to USP specifications. Sodium chloride (200 mg) was added to a 100 mL flask and dissolved in water followed by addition of 700 μL HCl (10 M) to adjust the pH to 1.2. Pepsin (320 mg) was then added to the medium. The simulated small intestinal fluid (SIF) was also prepared according to USP specifications through dissolution of 680 mg monobasic potassium phosphate in water. To this solution 7.7 mL of 0.2 M sodium hydroxide solution was added and the remaining water added to make the volume up to 100 mL.

After adjusting the pH to 6.8, 1 g pancreatin (from porcine pancreas, 3USP units activity/g) was added and shaken gently until dissolved.

Aqueous solutions of diclofenac-β-cyclodextrin conjugate 0.8 mg/mL were prepared in SGF and in SIF. To guarantee the complete dissolution of the conjugate in the medium 1% of DMF was used. These solutions were incubated at 37 °C and shaken at 100 rpm. At fixed intervals, 100 μL of fluid was withdrawn, mixed with 400 μL of methanol and centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was then removed and analyzed *via* HPLC to determine the concentration of conjugate. Each experiment was performed in triplicate.

2.5. Stability of diclofenac-β-cyclodextrin in human fecal slurry

Fecal slurries were utilized to simulate the conditions of the colon. Fresh feces were collected from healthy adults and transferred into an anaerobic workstation at 37 °C and relative air humidity of 70% as soon as possible after defecation. The feces were diluted with phosphate buffer saline (British Pharmacopeia) pH 6.8 in order to obtain a 40% w/w slurry. The mixture was then homogenized and sieved through an open mesh fabric (Sefar NitexTM, pore size 350 μm) to remove any unhomogenised fibrous material. Afterwards, the sieved homogenized fecal slurry was diluted 50% (w/w) with basal medium containing peptone water, yeast extract, NaCl, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 6H_2O$, $NaHCO_3$, Haemin, L-cysteine HCl, bile salts, tween 80, vitamin K and resazurin (Basit & Lacey, 2001).

Solutions of diclofenac-β-cyclodextrin (2.4 mg/mL) were prepared in PBS 6.8 (British Pharmacopeia) with DMF. 300 μL of this solution was mixed with 900 μL of fecal slurry in the anaerobic workstation. The final concentrations of the conjugate and DMF were 0.6 mg/mL and 1.0%, respectively. A control experiment was also run in parallel in fecal slurry that was subjected to autoclaving at 130 °C for 20 min. This was conducted to inactivate the bacterial enzymes in the slurry.

Thereafter, these solutions were incubated and shaken at 100 rpm. At fixed intervals, 100 μL of fluid was withdrawn, mixed with 400 μL of methanol and centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was then removed and analyzed by HPLC. Each experiment was performed in triplicate.

3. Results and discussion

3.1. Synthesis of diclofenac-β-cyclodextrin conjugate

Initially, various approaches were investigated to synthesize the cyclodextrin conjugate. These included: nucleophylic substitution with conventional heating (Hirayama et al., 2000); activation of diclofenac using carbodiimides (Dev, Mhaske, Kadam, & Dhaneshwar, 2007; Udoa et al., 2010), and the formation of an acid chloride (Cassano et al., 2010; Ventura et al., 2003). All three approaches were unsuccessful in the synthesis of the conjugate. We came to the conclusion that these approaches did not work due to the particular structure of diclofenac since these strategies had success in other cyclodextrin conjugates. As support to this conclusion the work of Roy et al. (2001) and also the work of Tudja, Khan, Mestrovic, Horvat, and Golja (2001) showed that under heating conditions diclofenac can suffer dehydratation and an intramolecular cyclization. In other case where an activation of the carboxylic acid was attempted we suggest that nucleophylic attack of the hydroxyl group of cyclodextrin cannot compete with the more favorable intramolecular acylation by the nearby amino group.

In contrast to these initial approaches, we succeeded in synthesizing of the diclofenac-β-cyclodextrin conjugate by the nucleophylic substitution of a 6-tosyl group under microwave radiation. The major challenge of this first step was to achieve monotosylation of cyclodextrin specifically to the 6-position without considerable amounts of primary and secondary side multi-tosylated by-products (Brady, Lynam, O'Sullivan, Ahern, & Darcy, 2000; Khan, Forgo, Stine, & D'Souza, 1998). This selective sulfonylation generally occurs in dry pyridine (Tang & Ng, 2008), or in water at alkaline pH (Brady et al., 2000). Pyridine is a non-user-friendly solvent and forms a pyridinium complex with the cyclodextrin cavity, complicating the process of purification (Khan et al., 1998). Consequently, the method using deionized water as a solvent was followed in order to prepare the 6-*p*-toluenosulfonyl-β-cyclodextrin derivative (Brady et al., 2000). Following the procedure as described above we obtained gram amounts of the 6-*p*-toluenosulfonyl-β-cyclodextrin that was characterized by NMR spectroscopy and MALDI-TOF spectrometry. For the substitution reaction, this product was used without purification, despite the presence of small amounts of di- and tri-tosilated derivatives as verified by MALDI analysis.

Microwave irradiation has long been viewed as an interesting alternative to the use of classical heating systems, and particularly in the field of organic synthesis, largely due to frequent increases in reaction rates, improved yields and selectivities associated with this technique (Richel, Laurent, Wathelet, Wathelet, & Paquot, 2011).

Nucleophylic substitution of tosyl group by the sodium diclofenac was achieved in DMF under microwave irradiation. The diclofenac-β-cyclodextrin conjugate was obtained after a systematic study of temperature and microwave conditions.

An HPLC study of different samples prepared under different reaction temperatures showed that the use of 140 °C produced the best yield. As temperature increases, it is also possible to observe a decrease in diclofenac-β-cyclodextrin yields. This is likely due to the degradation of diclofenac at high temperatures (Galmier et al., 2005; Tudja et al., 2001). The use of microwave radiation additionally led to the conjugate formation, with a short reaction time and less solvent comparatively to conventional processes required for the preparation of cyclodextrin conjugates (Hirayama et al., 2000). After chromatographic purification, about 20% yield of product was achieved, and the microwave approach to the synthesis of this conjugate proved to be consistently reproducible.

The chemical structure of the conjugate was confirmed and characterized by IR, ^{1}H NMR, ROESY spectroscopy and MALDI-TOF spectrometry, and the purity confirmed by HPLC.

3.2. Characterization of diclofenac-β-cyclodextrin

Mass spectrum (MALDI-TOF) of the product shows only a signal *m*/*z* of 1434.308 corresponding to the [M+Na] adduct. No other relevant mass signal was observed as shown in Fig. 1. The formation of the conjugate was proved by comparison of the ^{1}H NMR spectrum of the free β-cyclodextrin (Fig. 2II-A); and of the sodium diclofenac (Fig. 2II-B); with the spectrum of the diclofenac-β-cyclodextrin conjugate (Fig. 2II-C). The ester bond occurs at the hydroxyl of the 6-position of cyclodextrin once the protons of cyclodextrin secondary hydroxyl groups do not demonstrate a significant shift when compared to the dramatic upfield shift observed in the protons of primary hydroxyl (change from 4.60 ppm to 4.21–4.55 ppm). Moreover, the 6-hydroxyl substitution is also confirmed by the reduction in the integration of the peak due to the hydroxyl protons in the 6-position. The H-6 protons linked to diclofenac resonate downfield relative to the unsubstituted H-6 protons, and present a distinct multiplicity.

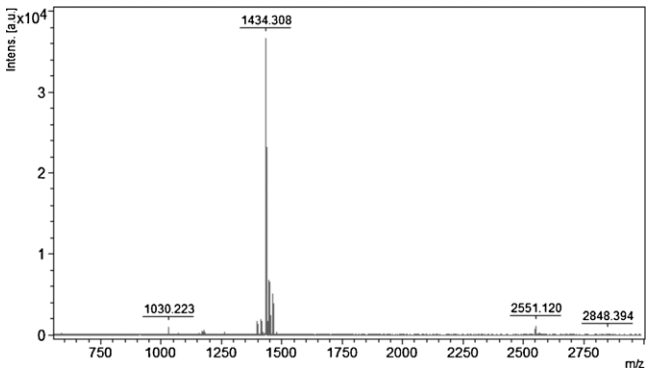

Fig. 1. MALDI mass spectrum of diclofenac-β-cyclodextrin conjugate.

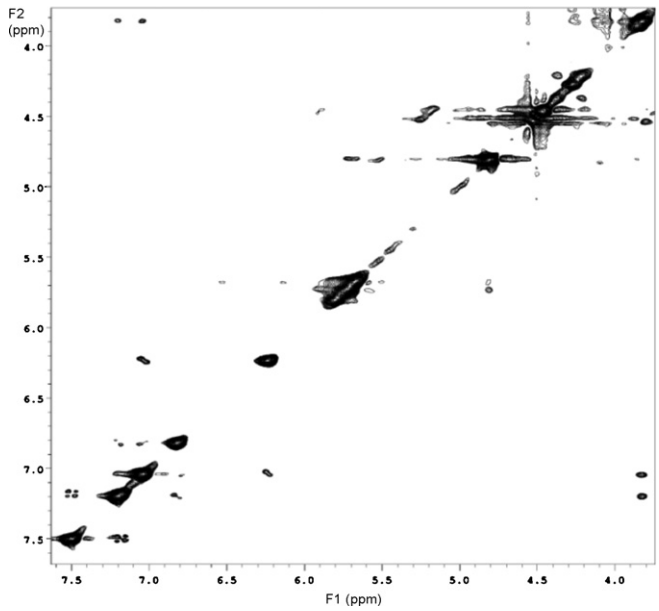

Fig. 3. ^{1}H NMR ROESY spectra of the diclofenac-β-conjugate.

ROESY experiments shown in Fig. 3 indicate that the only intermolecular correlations observed were between the H-4″ and H-6″ protons of the phenyl acetate ring and the H-6 protons of the β-cyclodextrin, corroborating the hypothesis of diclofenac non-penetration into the hydrophobic core of cyclodextrin.

IR spectroscopy also aided the identification of the diclofenac-β-cyclodextrin conjugate (see Fig. 4) (Hamdi, Abderrahim, & Meganem, 2010; Jing, Yanping, Baoguo, & Chengtao, 2011; Özkan, Atay, Dïkmen, Işimer, & Aboul-Enein, 2000; Wang, Han, Feng, & Pang, 2006). The spectrum of the conjugate (Fig. 4C) shows a pattern distinct from the spectrum of β-cyclodextrin (Fig. 4B) and of the diclofenac (Fig. 4A). The broad band at 3300–3500 cm^{-1} due to O—H stretching is narrower and more closely resembles the corresponding β-cyclodextrin. Equally, the region at 1000–1200 cm^{-1} due to C—O—C stretching is similar in spectrum (B), but with a new prominent band located at 1028 cm^{-1}. Near the H—O—H bending band of β-cyclodextrin at 1650 cm^{-1}, a new band appears at 1729 cm^{-1} due to the created ester bond.

3.3. Stability studies of diclofenac-β-cyclodextrin in simulated gastric and small intestinal fluids and in human fecal slurry

Diclofenac-β-cyclodextrin must pass intact through the stomach and the small intestine, and reach the colon where it will act upon enzymes of the colonic microbiota to release the active drug. Thus, in order to determine the stability of the conjugate in the stomach and small intestine, simulated gastric and small intestinal fluids were used. Results from these *in vitro* hydrolysis studies, as shown in Fig. 5, revealed that the conjugate is stable in these simulated fluids.

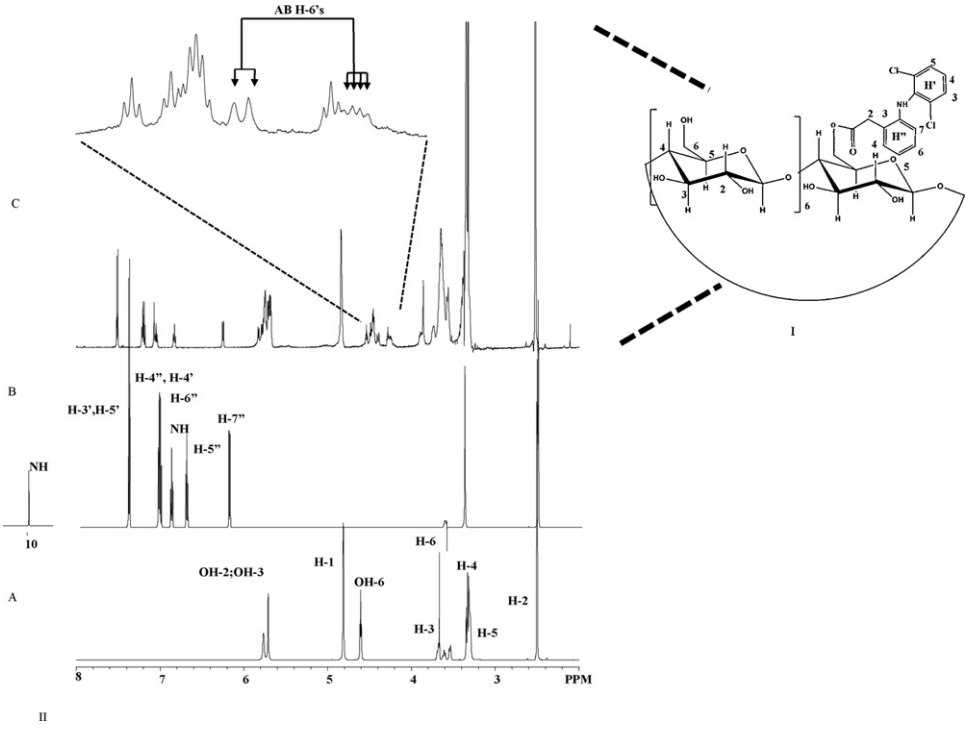

Fig. 2. I-Structure of the diclofenac-β-cyclodextrin; II-A-^{1}H NMR spectrum of hydrated β-cyclodextrin hydrated. II-B-^{1}H NMR spectrum of sodium diclofenac. III-C-^{1}H NMR spectrum of diclofenac-β-cyclodextrin conjugate.

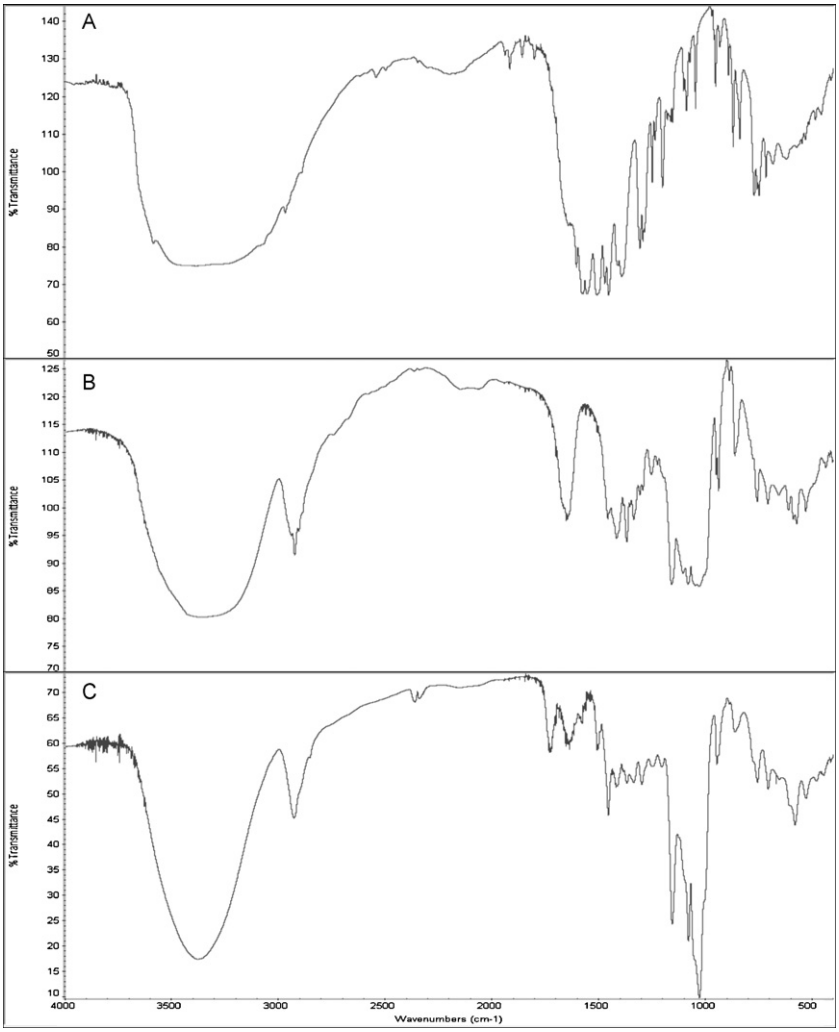

Fig. 4. IR spectra of diclofenac (A), β-cyclodextrin (B) and diclofenac-β-diclofenac conjugate (C).

The lack of a Pharmacopeia simulated colonic fluid for *in vitro* studies warrants the use of other methodology to mimic the environment of the human colon, in this case, the use of fresh human feces within a suitable medium has been identified as a suitable alternative (Sousa et al., 2008). The conjugate was added to the slurry and the results demonstrated that the conjugate was readily hydrolyzed in the human fecal slurry, and within 2 h the conjugate is completely degraded, as evidenced by the rapid liberation of free diclofenac (Fig. 6). By contrast, the conjugate was stable in the autoclaved fecal slurry (control). These results confirm that cleavage of the conjugate is due to bacterial enzymatic activity in the slurry. Moreover, release of the drug from the prodrug depends not only of the hydrolysis of the ester linkage but also of the integrity of the cyclodextrin, since release only takes place where cyclodextrin are fermented into small saccharides by the colonic microbiota (Flourié et al., 1993).

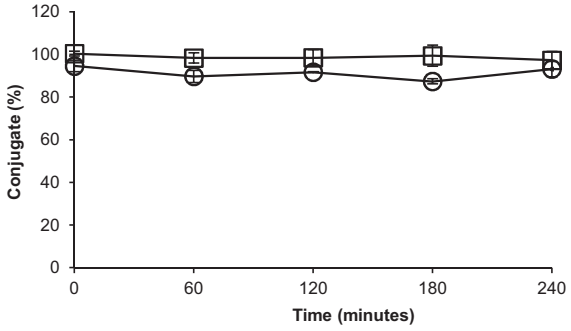

Fig. 5. Mean levels of diclofenac-β-cyclodextrin conjugate in simulated gastric (□) and small intestinal (○) fluids. Each point represents mean ± S.D. ($n = 3$).

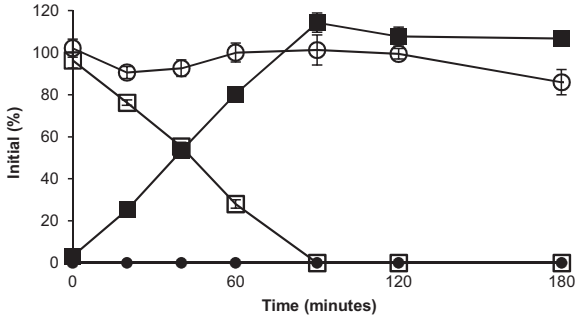

Fig. 6. Mean levels of diclofenac-β-cyclodextrin (□) and diclofenac (■) in human fecal slurry (test). Mean levels of diclofenac-β-cyclodextrin (○) and diclofenac (●) in autoclaved fecal slurry (control). Each point represents mean ± S.D. ($n = 3$).

4. Conclusion

In this work, synthesis of diclofenac-β-cyclodextrin conjugate was made possible with the use of microwave irradiation as a principal energy source. After synthesis, this prodrug was fully characterized and the purity verified by HPLC. Stability studies revealed that the conjugate is able to target the colon, given that it is completely stable in simulated gastric and in simulated small intestinal fluids but is degraded in the fecal slurry. This stability behavior is due to specific hydrolysis of β-cyclodextrin by colonic microbiota which was probed using the fecal slurry system. These preliminary results are promising, and suggest that the prodrug is capable of facilitating release of the active, diclofenac, in the colon.

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