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Synthesis and physico-chemical characterization of a β-cyclodextrin conjugate for sustained release of Acyclovir

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ABSTRACT

We report the synthesis of an oligomeric prodrug of the antiviral agent Acyclovir (Acy) conjugated to β-cyclodextrin (β-CyD). The drug was selectively linked through a succinic spacer to one of the primary hydroxyl groups of β-CyD by ester linkage in a 1:1 molar ratio. The conjugate was purified by semipreparative reverse-phase chromatography and characterized by FAB mass spectrometry and NMR experiments. The release of Acy from the conjugate was evaluated both in acidic and in neutral conditions and in the presence of porcine liver esterase. In all cases we observed the release of both free Acy and Acy succinate (AcySucc) at differing rates as a function of the hydrolysis conditions. In the presence of esterase the release of free Acy was favoured over AcySucc, showing a release rate of 100% of Acy within 7 days.

1. Introduction

Cyclodextrins (CyDs) are cyclic oligosaccharides which have been used for many years as complexing agents for molecules carrying on different pharmacological activities. Due to complexation, the physico-chemical properties of included drugs are significantly modified, such as the increase of stability and water solubility, the enhancement of the dissolution rate and the bioavailability of the drug (Cannavà et al., 2013; Kurkov & Loftsson, 2013; Stella & He, 2008; Ventura et al., 2005; Venuti et al., 2014). Only physical forces are involved in the formation of the complexes, so under physiological conditions the drug is released quickly, the release-rate depending on the degree of the stability constant of the inclusion compounds. Therefore, it is impossible to realize sustained drug-release or drug targeting because the drug is released before it can reach a specific organ or tissue. In order to overcome these drawbacks and realize these objectives the drug could be covalently linked to free hydroxyls of CyD. In this case, the rate of drug release would depend on the type of bond chosen, on the presence of an eventual spacer and on the chemical and enzymatic environment that surrounds the conjugate. Different anti-inflammatory drugs were conjugated with natural α-, β- and modified β-CyD in order to obtain their selective release into the colon. These prodrugs, in fact, are able to resist chemical hydrolysis in the stomach and are hydrolyzed in rat caecal and faecal matter (Hyrayama, Minami, & Uekama, 1996; Kamada et al., 2002; Vadnerkar & Dhaneshwar, 2013; Ventura et al., 2003; Zou et al., 2005).

In this work, natural β-CyD was used to develop a sustained-release system for the antiviral drug Acyclovir (Acy). Acy is one of the most effective and selective agents presently in use against viruses of the Herpes group. It is active against herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2), the varicella zoster virus (VZV), the Epstein–Barr virus (EBV) and to a lesser extent against cytomegalovirus (CMV) (Wagstaff, Faulds, & Goa, 1994). Acy has low bioavailability (about 15–30%) when administered orally due to its poor gastro-intestinal absorption (Fletcher & Bean, 1985), so high doses must be administered, accompanied by notable adverse effects such us nausea, diarrhoea and headache. Topical therapy could be an alternative to oral administration, but this also has a low level of efficacy, due to the lack of penetration of

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a suitable amount of the drug to the target site (Qureshi, Jiang, Midha, & Skelly, 1998). In many instances intravenous administration in the form of a strong alkaline (pHs 10–11) solution of sodium salt is required in order to obtain a satisfactory response, but this can cause thrombophlebitis or perivascular inflammation (Shojaei, Berner, & Li, 1998.) Moreover, Acy is characterized by a short half-life (2.5–3.3 h) (Susantakumar, Gaur, & Sharma, 2011), so all therapeutic treatment requires repeated daily administrations (5–6 times), reducing patient compliance. To overcome these drawbacks, sustained release formulations have been developed, examples being polymeric nanoparticles (Fresta et al., 2001; Jwala et al., 2011) or ethosome formulations (Godin & Touitou, 2003; Zhou, Wei, Zhang, & Wu, 2010). Polymeric or macromolecular prodrugs can also be effectively used to increase the bioavailability of Acy and to obtain the desired sustained release (Giammona, Puglisi, Cavallaro, Spadaro, & Pitarresi, 1995; Hiramath et al., 2011; Sawdon & Peng, 2014). The conjugation of Acy with natural β-CyD could be an effective approach for producing a sustained release of the drug making it suitable for oral or other routes of administration such as intra-pulmonary or intra-vitreal, etc. Acy is widely used in the treatment of various ocular diseases such as herpes simplex keratitis and acute retinal necrosis; in the latter case intra-vitreal administration of the drug could be more efficacious than intravenous administration in the attempt to reach therapeutical concentrations in the intraocular region (Damico et al., 2012). However, due to the great invasiveness of the intra-vitreal injections, frequent administrations cannot be considered. A prolonged release of the drug could be of noteworthy therapeutical importance in this case. This work is a preliminary studies to synthetize Acy-β-CyD conjugate and evaluate its hydrolysis in media simulating physiological environments, in order to explore the potentiality of this prodrug for use through various routes of administration (oral, intraocular, inhalatory and so on). During experimentation Acy was selectively linked to one primary hydroxyl of β-CyD by ester linkage using succinic moiety as a spacer and was characterized through NMR studies. Two-dimensional NMR experiments were used to investigate the spatial arrangement of the conjugate. Water solubility, dissolution rate and the octanol/water partition coefficient of the conjugate were evaluated by comparison with free Acy. Chemical and enzymatic hydrolysis studies were performed to investigate the potentiality of the conjugates as sustained release systems for Acy. For enzymatic hydrolysis we used the isoenzymes of porcine liver esterase (PLE), a non-specific carboxylase in wide use for the last 30 years and highly active both as an esterase and amidase (Ge et al., 2013; Huang et al., 1996). A large number of papers report the use of the isoenzymes of PLE to study the hydrolysis of the prodrug, co-drug or macromolecular conjugate developed for various routes of administration, including parenteral (Lau, Heard, & White, 2013; Lee et al., 2011; Swartz, Zhang, Valeriote, Chen, & Shaw, 2013; Wang et al., 2012; Wu, Shaw, Dubaisi, Valeriote, & Li, 2014).

2. Materials and methods

2.1. Materials

β-Cyclodextrin (β-CyD) was purchased from Cyclolab R & D Laboratory (Budapest, Hungary) and used after desiccation in a vacuum with phosphoric anhydride for 24 h at 90 °C. Acyclovir (Acy), anhydrous *N*,*N*-dimethylformamide (DMF), anhydrous pyridine (Py), succinic anhydride, triethylamine, thionyl chloride, porcine liver esterase (PLE) (EC 3.1.1.1., lyophilized powder, ≥15 units/mg solid) and phosphate buffer solutions (PBS) were Sigma–Aldrich (Milano, Italy) products. Lichroprep RP-18 (E. Merck, 40–63 μm) was used for reverse-phase flash chromatography with different eluent mixtures. All reactions were followed by thin-layer chromatography (TLC) using aluminium sheets precoated with silica gel 60 RP-18 F_{254} (Merck) and detection by UV light and/or with ethanolic 10% sulfuric acid. Solvents were dried by distillation according to standard procedure (Perrin, Armarego, & Perrin, 1988) and stored over 4 Å molecular sieves activated for at least 24 h at 400 °C. Double-distilled water was used throughout the study.

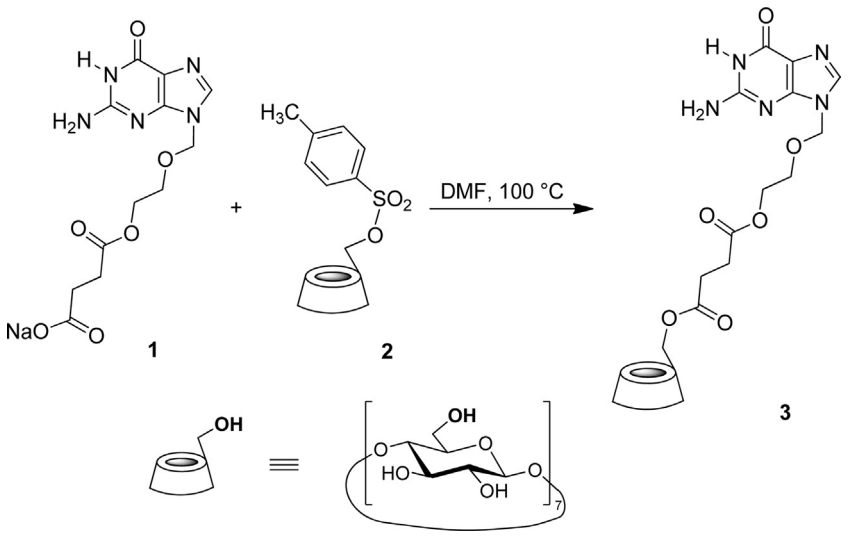
2.2. Synthesis of 6^A-O-{9-[[2-[(3-carboxypropionyl)oxy]ethoxy]methyl]guanine}-β-cyclodextrin conjugate (β-CyDAcySucc, **3**)

Sodium 4-{2-[(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl) methoxy]ethoxy}-4-oxobutanoate (AcySuccNa, **1**) (0.30 g, 0.88 mmol) (Colla, De Clercq, Busson, & Vanderhaeghe, 1983) was added to a solution of 6A-O-(p-toluenesulfonyl)-β-cyclodextrin (β-CyDOTs, **2**) (1.13 g, 0.88 mmol) (Djedaini-Pilard, Désalos & Perly, 1993) in DMF (20 mL), and the resulting mixture was stirred at 100 °C for 24 h. The solvent was then removed under reduced pressure, yielding a residue which was washed with water (50 mL) to remove the unreacted β-CyDOTs **2**. After filtration, the obtained filtrate containing β-CyDAcySucc **3** was concentrated under vacuum and the resulting crude product was purified by reverse phase chromatography on Lichroprep RP-18 (gradient eluent: from 0% to 35% of methanol in water). The fractions containing β-CyDAcySucc **3** were collected and the eluent was removed under reduced pressure. Finally, the β-CyDAcySucc **3** obtained was lyophilized to yield 0.57 g (0.39 mmol, yield 45%).

β-CyDAcySucc **3**; pale yellow crystals; m.p. 249–250 °C; Rf, 0.27 (propanol/H_2O/NH_4OH/AcOEt, 5:3:2:1, v/v/v/v); α = +133 (c = 1, H_2O); ^{1}H NMR (500 MHz, D_2O): 2.64 (m, 2H, COCH_2), 2.72 (m, 2H, COCH_2), 3.54 (t, 1H, β-CyD H-4A), 3.65–3.73 (m, 13H, β-CyD H-2 and H-4), 3.76 (m, 2H, OCH_2 CH_2), 3.83–3.98 (m, 25H, β-CyD H-6, H-5 and H-3), 4.10 (m, 1H, β-CyD H-6A), 4.18 (m, 2H, OCH_2CH_2), 4.79 (d, 1H, β-CyD H-6A), 5.09–5.10 (m, 7H, β-CyD H-1), 5.56 (s, 2H, N-CH_2), 7.89 (s, 1H, H-8); ^{13}C NMR (125 MHz, D_2O): δ 29.0, 29.5 (COCH_2), 60.1 (C-6), 64.0 (OCH_2CH_2), 64.6 (OCH_2CH_2), 66.7 (C-6A), 70.7 (C-5), 72.0 (C-3), 72.7 (N-CH_2), 73.3 (C-2), 81.7 (C-4), 102.1 (C-1), 116.1 (C-3′), 140.0 (C-5′), 152.3 (C-4′), 154.2 (C-2′), 159.0 (C-6′), 172.0 (C=O), 174.1 (C=O); FAB MS (glycerol/water), 1443 (M+1) m/z.

2.3. Apparatus

Fast atom bombardment (FAB) mass spectra were recorded on a VG ZAB-25E spectrometer, in a positive mode using glycerol water. The chromatographic analyses were performed using a HPLC system Varian ProStar model 230 (Varian, Milan, Italy) using a reverse-phase column (C_{18}) Waters Symmetry (5 μm × 4.6 mm × 15 cm). The HPLC apparatus was equipped with an auto-sampler Varian model 410 and Galaxie software for data elaboration. The mobile phase consisted of a mixture 88/12 (v/v) of acetonitrile/ammonium acetate buffer 20 mM (pH 3.7). All analyses were carried out at room temperature, at a flow rate of 1.0 mL/min. Twenty microlitres of each sample were injected and the column effluent was monitored continuously at 254 nm. The amount of Acy was calculated by reporting the peak area of a sample on a standard calibration curve in the range between 0.4 and 10.0 μg/mL of Acy (r^2 = 0.9922). ^{1}H NMR spectra were recorded with a Varian VnmrJ instrument at 500 MHz in the aforementioned solvent. All samples were solubilized in D_2O; no internal standard was added to the samples in order to avoid its interaction with the β-CyD cavity. The residual sign of HOD at 4.83 ppm was used as reference (Ivanov, Salvatierra, & Jaime, 1996). ^{13}C NMR spectra were recorded at 125 MHz. Chemical shifts are given in ppm (δ).



Scheme 1. Synthesis of β-CyDAcySucc **3** conjugate.

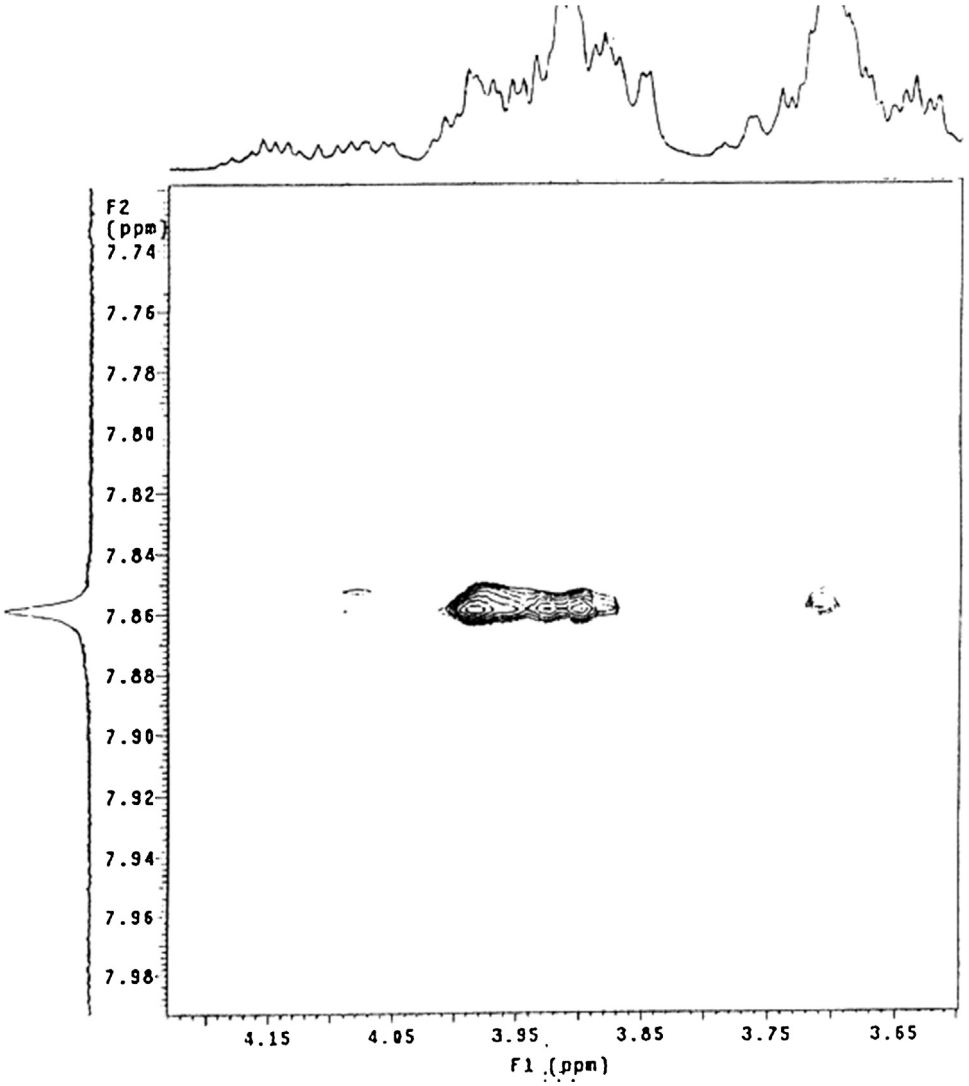


Fig. 1. T-Roesy experiment of β-CyDAcySucc **3** conjugate.

2.4. Solubility measurements and dissolution rate

A weighed amount of free Acy (5.0 mg) and a corresponding amount of conjugate were separately poured into two screw-capped vials and 1.0 mL of water was added. All suspensions were shaken at 25.0 ± 0.5 °C. When equilibrium was reached (after 3 h), the suspensions were filtered through 0.45 μm Sartorius Minisart®-SRP 15PTFE (Germany) filters and the solutions were analyzed by HPLC to determine Acy and conjugate concentrations. No degradation of the conjugate was observed during this procedure.

The dissolution rates of free Acy (5.0 mg) and a corresponding amount of the conjugate were determined in a medium simulating gastro-intestinal juice (acid medium at pH 1.1 and PBS at pH 6.8) and at pH 7.4 at 37.0 ± 0.5 °C. Samples were suspended in the release medium (5.0 mL) in stoppered bottles and shaken at 100 strokes min^{-1} for 1 h. Aliquots of 500 μL were withdrawn at fixed time intervals, filtered and assayed by HPLC to determine the amount of dissolved Acy and β-CyDAcySucc present. The sample volumes were replaced with the same amounts of fresh medium. The obtained values were corrected for the dilution used during sampling. The experiments were carried out in triplicate and data expressed as mean ± standard deviation (S.D.).

2.5. Octanol/water partition coefficient

A certain amount of free Acy (1.0 mg) and a corresponding amount of conjugate were separately poured into two screw-capped vials, dissolved in 10.0 mL of water and an equal amount of octanol was added to each. Both the octanol and the water were pre-saturated. The mixtures were vigorously shaken at 25.0 ± 0.5 °C for 3 h, and then centrifuged, in order to separate the two phases. The aqueous phases were analyzed by HPLC to determine the drug or conjugate concentration. The concentration of the octanol phase was obtained by determining the difference between the total weighed amount of the drug and the amount present in the aqueous phase.

Table 1
Physico-chemical properties of Acy and its β-CyD conjugate.

	Acy	β-CyDAcySucc **3**
Molecular weight (Da)	225.20	1442.25
Solubility in water (mg/mL)[a]	1.8 ± 0.2	22.7 ± 1.5 (3.6 ± 0.1)[b]
log *P*	0.027 ± 0.011	−0.061 ± 0.023

[a] At 25.0 ± 0.5 °C.
[b] Amount of Acy contained.

2.6. Release of Acy from the β-CyDAcySucc conjugate

The chemical hydrolysis of β-CyDAcySucc was studied in an acid solution (HCl solution at pH 1.1) and in PBS at pHs 6.8 and 7.4 at 37.0 ± 0.5 °C. Solutions of β-CyDAcySucc were prepared by dissolving 5.0 mg in 10.0 mL of preheated solution, respectively. At timed intervals, 500 μL of each sample was collected and analyzed by HPLC for released species and the remaining

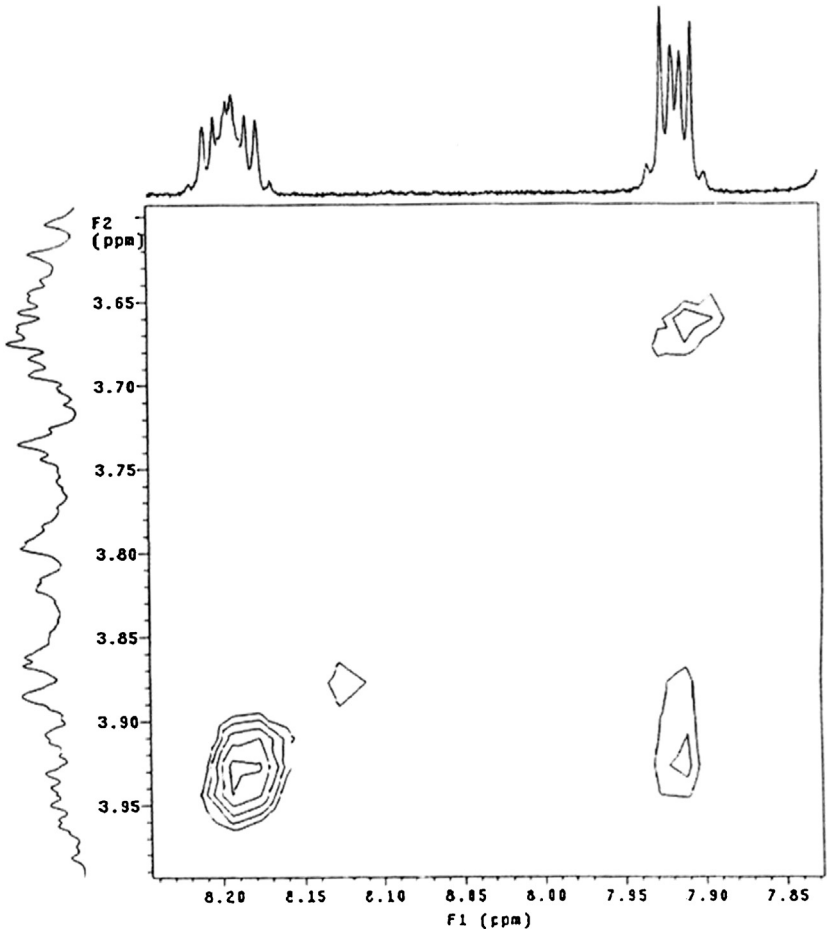


Fig. 2. T-Roesy experiment of β-CyDAcySucc **3** conjugate in the presence of AnSNa.

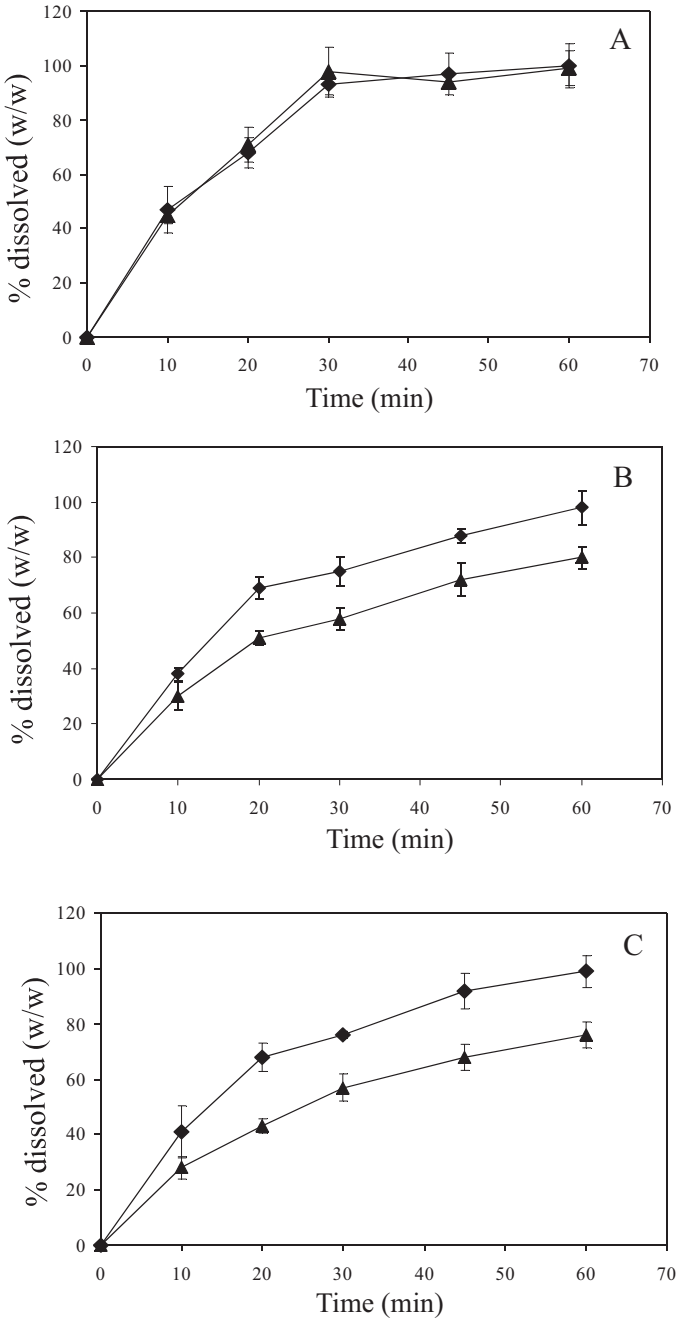


Fig. 3. Dissolution profiles of Acy (triangle) and β-CyDAcySucc **3** conjugate (diamonds) at pH 1.1 (A), at pH 6.8 (B) and at pH 7.4 (C), at 37.0 ± 0.5 °C.

β-CyDAcySucc. Enzyme-catalyzed hydrolysis of the conjugate was carried out in PBS (pHs 6.8 and 7.4 at 37.0 ± 0.5 °C), using PLE. β-CyDAcySucc (5.0 mg) was solubilized in 5.0 mL of PBS (pH 6.8 or 7.4) and then 5.0 mL of the enzyme solution was added (prepared by solubilization of the enzyme in PBS at pH 6.8 or 7.4; ionic strength = 0.15 mol dm^{-3}, at a final concentration of 1 unit/mL). The solutions were maintained at 37.0 ± 0.5 °C under stirring. At timed intervals, 500 μL of each solution was collected and treated with an equal amount of 1% $ZnSO_4$ water/methanol (70/30, v/v) solution for protein denaturation. The obtained suspensions were filtered with 0.22 μm Sartorius Minisart®-SRP 15PTFE filters (Germany) and the filtrate was analyzed by HPLC to determine the amount of species present in solution.

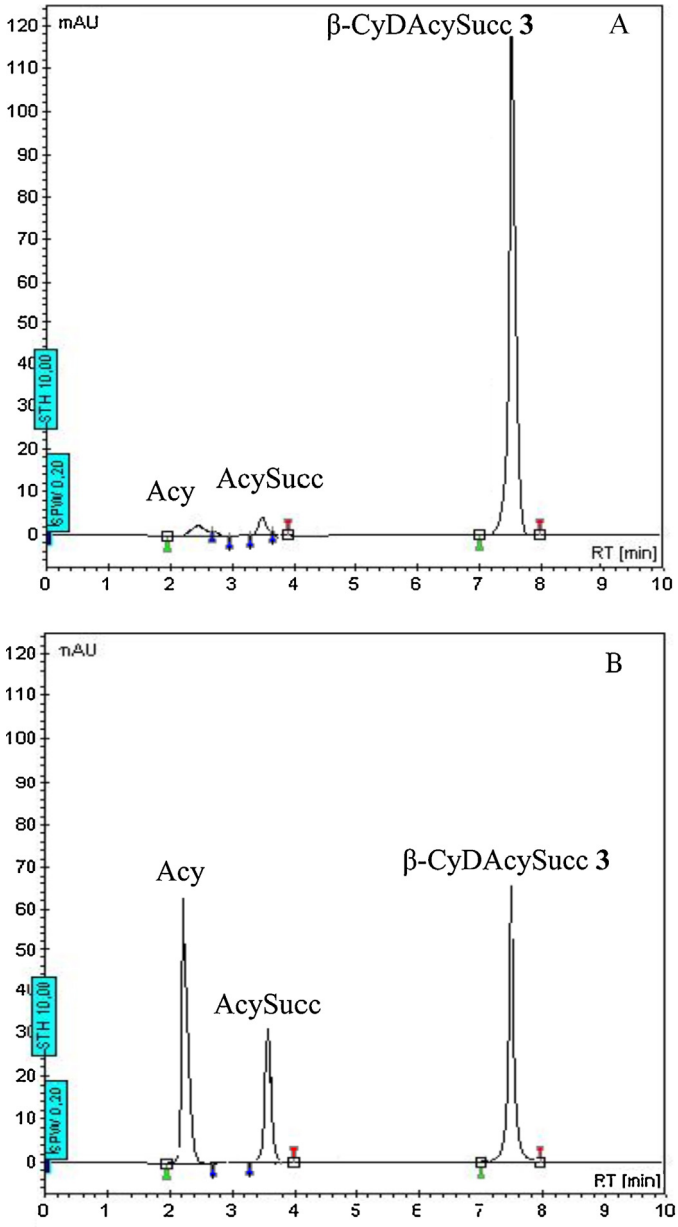


Fig. 4. HPLC chromatogram of β-CyDAcySucc **3** at pH 1.1 and at 37.0 ± 0.5 °C after 15 min (A) and 48 h (B) of hydrolysis.

2.7. Statistical analysis

All data were examined by the analysis of variance (ANOVA) or Student *t*-test, using Minitab statistical software (Origin Lab vers. 7.0, MA, USA). A *p* value of less than 0.05 was accepted as statistically significant. Results are the average of three experiments ± S.D.

3. Results and discussion

The synthesis of β-CyDAcySucc 3 (Scheme 1) was carried out by heating one equivalent of AcySuccNa **1** with β-CyDOTs **2** in anhydrous DMF at 100 °C for 24 h. After workup, the conjugate β-CyDAcySucc **3** was purified from the unreacted product by means of reverse-phase Lichroprep RP-18 flash-chromatography.

We adopted the notation system with the glucopyranosidic rings identified as A, B, C, D, E, F and G going counter-clockwise and viewed from the side of the primary hydroxyl. A is the functionalized ring.

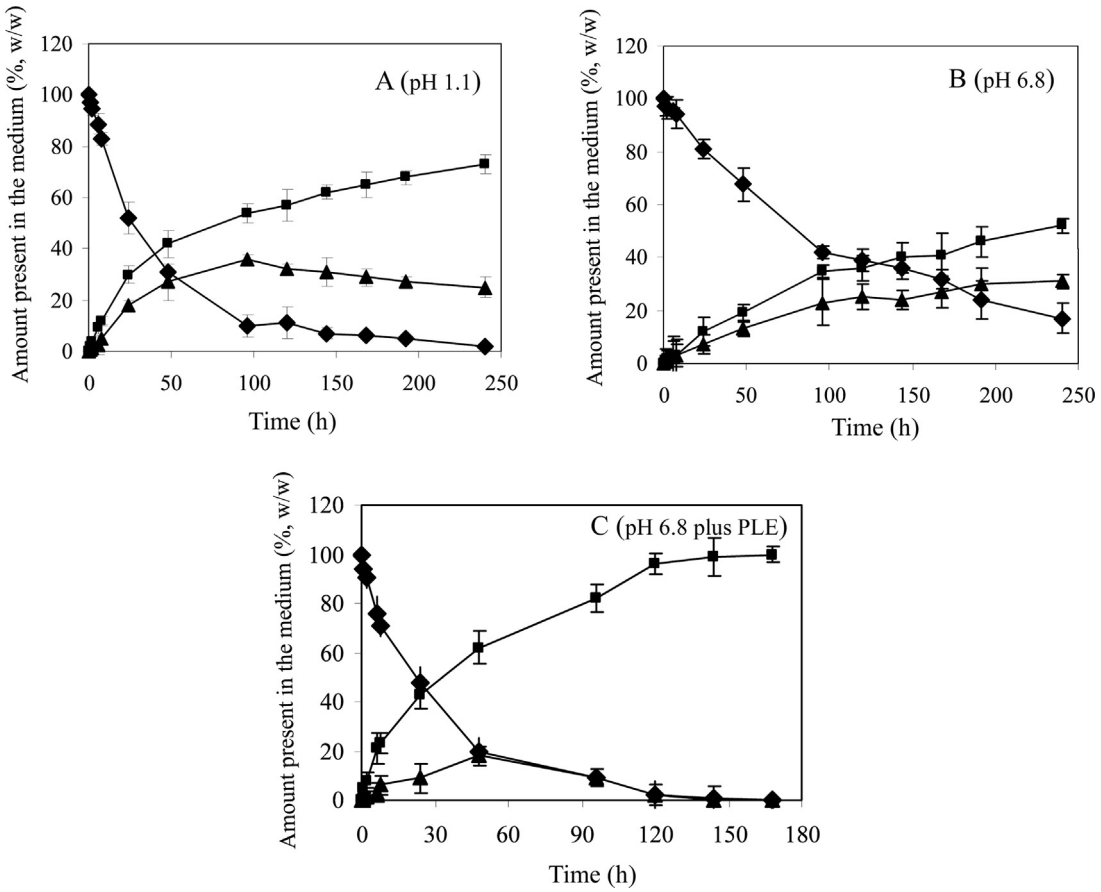


Fig. 5. Time course for the hydrolysis of β-CyDAcySucc **3** (diamonds) and subsequent appearance of AcySucc (triangle) and Acy (square) at 37.0 ± 0.5 °C, in simulated gastric (A) and intestinal mediums in the absence (B) or in the presence of PLE (C).

The chemical structure of β-CyDAcySucc **3** was characterized by ^{1}H, ^{13}C NMR and FAB mass spectrometry, as reported in the experimental section. The ^{1}H NMR spectrum shows the lack of the characteristic signals of the tosyl group and especially the downfield shift of two H6A and H6′A protons of the modified glucose unit, due to the formation of an ester bond between the C6 hydroxyl group and the succinoyl moiety of Acy. The difference in the chemical shift of one H6A proton (δ = 4.79 ppm) with respect to the other (δ = 4.10 ppm) is particularly evident. The H5A proton of the modified glucose unit was also shifted downfield. A shift to higher fields was observed in the ^{1}H NMR for the H4A proton of the functionalized glucose moiety, and for the methylene protons of the succinic spacer of the condensation product β-CyDAcySucc **3**, from 2.34–2.42 ppm (for AcySucc) to 2.64–2.72 ppm. Moreover, analogous shifts were observed for the corresponding carbon atoms in the ^{13}C NMR spectrum. Other diagnostic signal shifts included those of the carbonyl group of the succinoyl spacer, which resonate at 172.0 and 174.1 ppm. These resonances further confirm the presence of the bond between the carboxyl group and the cyclodextrin C-6 oxygen atom. When DMSO-d_6 was used as solvent, the ^{1}H NMR spectrum of β-CyDAcySucc **3** showed the two amine protons as a singlet at 6.53 ppm.

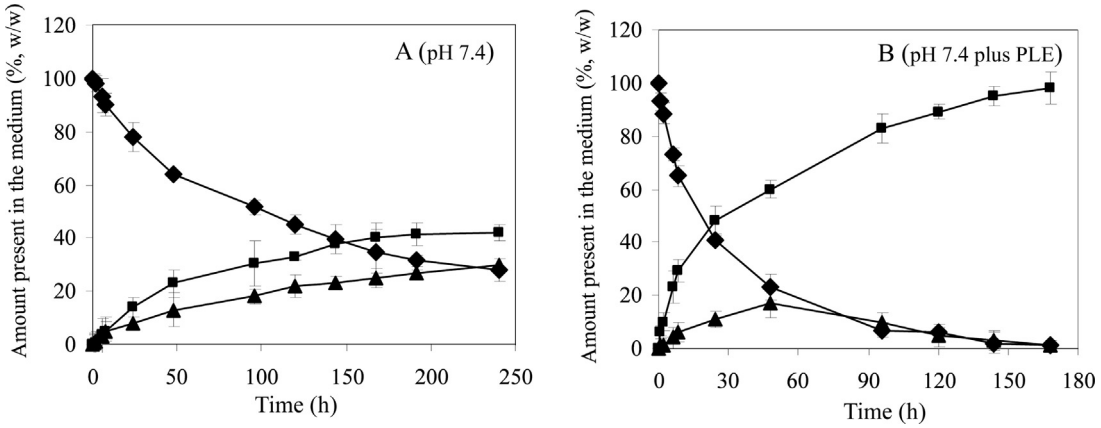


Fig. 6. Time course for the hydrolysis of β-CyDAcySucc **3** (diamonds) and subsequent appearance of AcySucc (triangle) and Acy (square) at 37.0 ± 0.5 °C, at pH 7.4 in the absence (B) and in the presence of PLE.

T-Roesy experiments were performed in order to investigate the possible conformation of the conjugate in water. In particular, the spectrum showed the NOE correlation peak of the aromatic proton of the Acy moiety with H3, H5 and H6 protons of β-CyD (Fig. 1). These data suggest an interaction of the heterocyclic ring with the cavity of the macrocycle, in aqueous solution. However, the intensity of the cross peak is not high, suggesting that the ring of the guanosinic residue is not deeply included.

In order to obtain further information on the conformation of β-CyDAcySucc **3** in water, T-Roesy spectra were performed after the addition of sodium anthraquinone-2-sulfonate (AnSNa) in equimolecular ratio, as competitive guest (Fig. 2). This compound was selected since its inclusion in β-CyD in water was extensively studied by NMR (Djedaini & Perly, 1990; Lin, Creminon, Perly, & Djedaini-Pilard, 1998; Puglisi et al., 2009), where the association constant determined with β-CyD was 850 M^{-1}. AnSNa was also selected on the basis of its aromatic character, which induces a large shift of NMR proton signals upon inclusion.

All non-diagonal peaks were indicative of spatial proximities among protons. Dipolar contacts were observed between H3 and H5 protons of the β-CyDAcySucc **3** cavity and AnSNa aromatic protons, indicating the formation of an inclusion complex, while the signal of the β-CyDAcySucc **3** aromatic proton disappeared. This suggested that the included AnSNa replaces the AcySucc ring, breaking its weak interaction with the cavity.

In Table 1, we report the physico-chemical properties of β-CyDAcySucc **3** by comparison with free Acy. An approximately twofold increase of Acy solubility in water is observed when it is conjugated with β-CyD. This is due to the chemical binding of Acy to a hydrophilic matrix, such as β-CyD, and probably to the partial inclusion (as evidenced from our NMR studies) of the guanosine nucleus of Acy into the macrocycle cavity to which it is linked or to another CyD. The more hydrophylic characteristic of β-CyDAcySucc **3** with respect to free Acy was also evidenced by the decrease in the partition coefficient value, which becomes negative for the conjugate (Table 1).

In Fig. 3A and C, we reported the dissolution profiles of β-CyDAcySucc **3** and free Acy in the medium simulating gastric (pH 1.1) (panel A) and intestinal juices (pH 6.8) (panel B) and at pH 7.4 (panel C). No particular influence was exerted by the β-CyD on this property when measured at acidic pH, and a similar, rapid dissolution rate was observed for the conjugate and the free Acy, with quantitative dissolution occurring within 30 min. At the considered pH value Acy is ionized, a condition which plays a more important role in the solubility of the drug and on its dissolution rate as compared to when it binds to β-CyD. Different results were obtained at higher pH values. In fact, both in mediums simulating intestinal juices (pH 6.8) and at the pH of 7.4 an increase in the dissolution profiles for the conjugate was observed as compared to free Acy. At these pH values the free drug was almost totally unionized so its solubility increased, due to the fact that its binding with β-CyD influenced dissolution in a positive manner.

In order to evaluate the ability of the conjugate to act as a sustained release system for the oral administration of Acy, we carried out hydrolysis studies through the incubation of β-CyDAcySucc **3** with pH values simulating gastric (pH 1.1) and intestinal mediums (pH 6.8). In this latter condition hydrolysis was performed in the absence or in the presence of PLE. The chromatograms obtained after 15 min and 48 h of hydrolysis of the β-CyDAcySucc **3** conjugate at pH 1.1 were reported in Fig. 4A and B.

At both points in time, we were able to observe the presence of both free Acy and AcySucc. In 2000, Mehvara, Dannb, and Hogansonb (2000) demonstrated that the pro-drug dextran-methylprednisolone succinate hydrolyzes by utilizing two

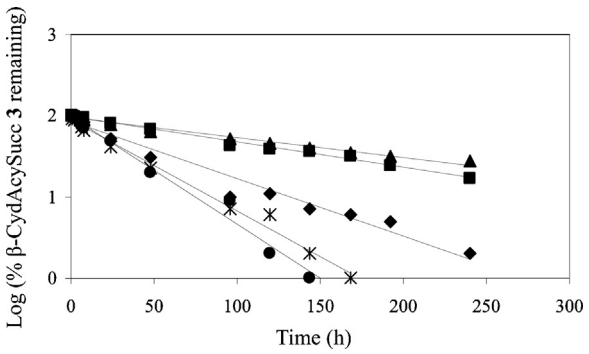


Fig. 7. First order kinetics plots for the disappearance of β-CyDAcySucc **3** at 37.0 ± 0.5 °C at different pH values. pH 1.1 (diamonds), pH 6.8 (square), pH 6.8 plus PLE (star), pH 7.4 (triangle), pH 7.4 plus PLE (circle).

mechanisms—along similar lines, our β-CyDAcySucc **3** conjugate either released Acy directly by cleavage of the ester bond adjacent to the drug or indirectly through AcySucc, by cleavage of the ester bond adjacent to β-CyD. A similar trend was observed at a pH of 6.8, but both the free form of Acy and AcySucc appeared in a more prolonged time frame. A different trend was observed in the presence of the enzyme; a more rapid hydrolysis was observed, with a quantitative release of the drug occurring within 7 days. The hydrolysis curves of the conjugate under different experimental conditions besides the subsequent appearance of free Acy and AcySucc are shown in Fig. 5A–C. About 4% (w/w) within 2 h at pH 1.1, and about 3% (w/w) and 23% (w/w) within 6 h at pH 6.8 in the absence or in the presence of PLE, respectively, of the free drug was released. At a more prolonged time we observed a release of about 50% (w/w) within 10 days of the experiments and a quantitative release within 7 days in the absence or in the presence of the enzymes, respectively. These results demonstrated that β-CyDAcySucc **3** cannot be used as a sustained release system for the oral administration of Acy, because the amount of Acy released from the conjugate during the period of gastro-intestinal residence is very limited. However, a long degradation time of the conjugate could be useful for alternative administration routes. With this in mind, we also performed hydrolysis studies in PBS at a pH of 7.4 in the absence and presence of PLE. A trend similar to that observed at pH 6.8 was observed. About 40% (w/w) of Acy was released within 10 days from the conjugate in the absence of the enzyme, the release was quantitative within 7 days in the presence of PLE (Fig. 6A and B).

Both at pHs 6.8 and 7.4 no trace of AcySucc was detected in the chromatograms within the first 50 min of the experiment. Within this period only free Acy was released from the conjugate. In addition, the amount of AcySucc present in the media (both pHs 6.8 and 7.4) during the whole experiment was very low (Fig. 6A and B) with respect to the amount observed in the absence of PLE. Based on these results, it is conceivable that there is a great influence of the enzyme on the release from the conjugate of free Acy rather than AcySucc. It is probable that the presence of macrocycle in β-CyDAcySucc **3** reduced the contact of the enzyme with the target site between β-Cyd and succinic spacer through steric hindrance, thus facilitating the cleavage of the ester bond between succinic spacer and Acy.

The pseudo-first order plots for the disappearance of the conjugate under different experimental conditions are shown in Fig. 7 and the observed rate constants (K_{obs}) for the overall degradation rate calculated by linear regression analysis of the slope of these lines based upon the equation $ln[\beta\text{-}CyDAcySucc]_t/[\beta\text{-}CyDAcySucc]_0 = -K_{obs}t$ (where $[\beta\text{-}CyDAcySucc]_t$ and $[\beta\text{-}CyDAcySucc]_0$ are the concentrations of the conjugate at time t and initial time, respectively) are reported in Table 2. The influence of the enzyme on the hydrolysis rate of the conjugate

Table 2
Observed rate constants (K_{obs}) for the overall degradation rate of the conjugate under different experimental conditions.

Experimental conditions	K_{obs} (h^{-1})
pH 1.1	0.0163 ± 0.0012
pH 6.8	0.0071 ± 0.0015
pH 7.4	0.0055 ± 0.0019
pH 6.8 plus PLE	0.0306 ± 0.0026
pH 7.4 plus PLE	0.0258 ± 0.0024

was evident at a value of about four times greater than chemical hydrolysis occurring at the corresponding pH values.

4. Conclusions

The conjugation of Acy with β-CyD produces a slight increase in its water solubility and a sustained release of the drug. About 75%, 50% and 40% (w/w) of the drug was released within 10 days at pHs 1.1, 6.8 and 7.4, respectively, but in the presence of porcine liver esterase a quantitative release of Acy within 7 days was observed at both pHs close to neutrality. Due to the prolonged release time observed in media simulating gastric and intestinal juice (about 4% (w/w) within 2 h at pH 1.1 and about 23% (w/w) within 6 h at pH 6.8 in the presence of PLE), this conjugate cannot be used for sustained release in the oral administration of Acy. However, the results obtained at the 7.4 pH value suggest that the β-CyDAcySucc **3** conjugate could have therapeutical relevance as a prolonged release system for Acy through alternative routes of administration, such as intra-vitreal or intra-pulmonary which would solve the problems connected with the short half-life of this drug. Other in vitro/in vivo studies are in progress to evaluate these observations.

Conflicts of interest

The authors report no conflicts of interest.

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