Thiolated hydroxypropyl-β-cyclodextrin as mucoadhesive excipient for oral delivery of budesonide in liquid paediatric formulation

Valentino Laquintana[a], Mulazim Hussain Asim[b,c], Angela Lopedota[a], Annalisa Cutrignelli[a], Antonio Lopalco[a], Massimo Franco[a], Andreas Bernkop-Schnürch[b,*], Nunzio Denora[a,d,*]

[a] Department of Pharmacy – Pharmaceutical Sciences, University of Bari "Aldo Moro", 70125 Bari, Italy
[b] Center for Chemistry and Biomedicine, Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innrain 80/82, 6020 Innsbruck, Austria
[c] Department of Pharmaceutics, Faculty of Pharmacy, University of Sargodha, 40100 Sargodha, Pakistan
[d] Institute for Chemical and Physical Processes (IPCF)-CNR SS Bari, Via Orabona 4, 70126 Bari, Italy

ARTICLE INFO

ABSTRACT

Budesonide (BUD) is a low water soluble ($S_0 = 5.028 \cdot 10^{-5}$ M) corticosteroid used as preferred therapy for the treatment of Eosinophilic Esophagitis (EoE), a chronic allergic-immune condition with an increased incidence in the paediatric population. Currently there are no commercial medicines indicated for EoE, and, therefore, in the hospital pharmacy the BUD is extemporaneously formulated as viscous oral suspension at the initial dose of 1–2 mg per day for children, highlighting the need of a mucoadhesive drug delivery system (MDDS) that adheres to the site of action and prolongs the therapeutic activity of the administered drug. Considering the ability of hydroxypropyl-β-cyclodextrin (HP-β-CD, 100 mM) to increase the water solubility of BUD ($S_{HP-\beta-CD} = 4.3 \cdot 10^{-3}$ M; $K_{1:1} = 861.11$ M^{-1}), a mucoadhesive thiolated HP-β-CD (HP-β-CD-SH) was synthesized and characterized by mass spectroscopy, ^{1}H- and ^{13}C NMR techniques. Phase-solubility studies demonstrated the capability of HP-β-CD-SH (100 mM) to form a reversible water soluble inclusion complex with BUD ($S_{HP-\beta-CD-SH} = 10.9 \cdot 10^{-3}$ M; $K_{1:1} = 2013.52$ M^{-1}), which increases its permanence on the target site as proved by mucoadhesive studies on porcine oesophagus mucosa. HP-β-CD-SH might be a useful MDDS for the pharmacist to prepare BUD oral liquid formulations at the recommended doses, with pH values below 5, which guarantee the chemical stability of the new mucoadhesive excipient.

1. Introduction

Eosinophilic Esophagitis (EoE) is a multifactorial inflammatory pathology of the oesophagus mediated by Th-2 interleukins with a high incidence in the paediatric population with a genetic predisposition. The oesophageal inflammation involves the alteration of the mucosal barrier with an anomalous reaction of the immune system to environmental allergens, which finally leads to oesophageal remodelling and fibrosis. It causes oesophageal dysfunction with chronic dysphagia, gastroesophageal reflux, chest pain, and food impaction (Attwood, 2019). Currently, therapies include topical steroids treatment, the use of proton pump inhibitors and dietary managements (Vinit et al., 2019). However, there are no commercial medicines indicated for EoE, and, therefore, pharmaceutical compounding is the alternative choice for this therapeutic need. In fact, even if topical steroids, such as budesonide (BUD) or fluticasone propionate (FP), are considered the first

choice recommended drug treatment for EoE in children and adults, they are administered through the so-called swallowed topical-acting corticosteroids (STCs) instead of being inhaled (Dellon et al., 2019), and, their prolonged use is responsible of local and systemic side effects such as oral and oesophageal candidiasis and adrenal insufficiency, respectively. Due to the nature of EoE in children and adults, and considering that dysphagia is accentuated in the paediatric population under 6–7 years of age, it is necessary that the extemporaneous dispensed medicines must be liquid formulations based on the use of a mucoadhesive vehicle that adheres to the site of action and prolongs the therapeutic activity of the administered STCs. BUD is a glucocorticoid used in inhalation formulations for the treatment of asthma, allergic rhinitis and chronic obstructive pulmonary disease (COPD) (Gupta et al., 2015). BUD is classified as a class II drug in the FDA's Biopharmaceutical Classification System (BCS, Amidon) with a good permeability (Log P = 3.2) and a poor aqueous solubility (Bhatt et al.,

---

2014). In the hospital practice, due to its limited water solubility, the BUD is formulated as viscous gel or mucoadhesive oral suspension at the initial dose of 1–2 mg per day for children and up to 8 mg per day for adults. Instead, FP powder is habitually directly dispersed in the oral cavity by nebulizer. Mucoadhesive BUD oral formulations appear to be more effective than FP aerosol therapy attributable to its increased contact time with the oesophagus mucosa (Oliva et al., 2017; Dellon et al., 2012; Lucendo et al., 2019). Therefore, an effective formulation, based on a mucoadhesive drug delivery system (MDDS) that increases the time of contact between the drug and the oesophageal mucosa, reducing the ingested dose and the systemic side effects, could be a valid alternative for the treatment of EoE in the paediatric population. In comparison to other regions of the gastrointestinal tract, the concentration of mucins on esophageal mucosa is certainly much lower (Hopwood et al., 1986; Burjonrappa, 2007; Meyer et al., 2009). However, mucoadhesion does not depend on the thickness of the mucus gel layer. Moreover, keratins are a major constituent of the esophageal epithelium that can likely also form disulfides with thiolated CDs (Grace, 1985). Recently, a new generation of biocompatible polymers that possess free and exposed thiol groups on the surface of the polymeric backbone have been shown to be effective in establishing an intimate contact with biological surfaces coated with mucous gel through chemical-physical mechanisms known as mucoadhesion (Bernkop-Schnürch, 2005). These thiolated polymers, also known as thiomers, are able to form disulphide bonds with cysteine-rich glycoproteins of mucus and have been successfully used to prepare suitable MDDS (Perez-Vilar and Hill, 1999; Bernkop-Schnürch and Steininger, 2000) including thiolated glycogen and glycol-chitosan mucoadhesive polymers (Denora et al., 2016; Perrone et al., 2017; Perrone et al., 2017). Recently, also oligosaccharides such as thiolated cyclodextrin have been developed as a new platform of mucoadhesive excipients suitable for the preparation of oral drug formulations (Moghadam et al., 2018; Asim et al., 2018; Ijaz et al., 2015). Considering all the above mentioned and the evidence that BUD/HP-β-CD inclusion complex showed a reduction of the neutrophil-induced inflammation in a COPD mouse model (dos Santos et al., 1859), the aim of this study was to synthesize a mucoadhesive thiolated HP-β-CD with the capability to form a reversible water soluble inclusion complex with BUD, increasing its permanence on the oesophagus mucosa. This inclusion complex might be useful to the pharmacist for the extemporaneous preparation of oral liquid paediatric formulations at the recommended doses, ensuring an increased water solubility of BUD and extending its residence time on the oesophageal mucosa, leading to an increased bioavailability of the drug in the site of action.

2. Materials and methods

2.1. Materials

Hydroxypropyl-β-cyclodextrin, (Cavasol W7, HP-β-CD with MW = 1540 and a molar substitution IR = 7) were obtained from Farmalabor, Canosa di Puglia, Italy, 5,5′-Dithio-bis(2-nitrobenzoic acid) (Ellman's reagent), 7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt (Alamar Blue, Resazurin sodium salt), Lithium bromine (anhydrous, free-flowing), 16,17-Butylidenebis(oxy)-11,21-dihydroxypregna-1,4-diene-3,20-dione (Budesonide, BUD), Thiourea, Triphenylphosphine, L-Cysteine hydrochloride, N-Bromosuccinimide (NBS), N,N-Dimethylformamide (anhydrous) were purchased from Sigma–Aldrich, Italy. The dialysis membrane (CE, MWCO 100–500 Da, Spectra/Por) were purchased from Spectrum Laboratories Inc., Italy. Reagent grade chemicals and solvents were used without further purification.

2.2. Methods

2.2.1. Synthesis of thiolated HP-β-CD

The thiolation of HP-β-CD was realized in two steps through a method based on the bromination of hydroxyl residues, followed by nucleophilic substitution with thiourea. The synthesis was conducted according to a method previously described but with slight modifications, as described below (Ijaz et al., 2015; Laffleur et al., 2016). First, was prepared the HP-β-CD bromine (HP-β-CD-Br) and in the second step, the brominated derivate was converted in thiolated HP-β-CD (HP-β-CD-SH). Reactions were performed under an argon atmosphere and the HP-β-CD, LiBr anhydrous, NBS, and Ph_3P were dried before experiments. For the first step, 0.5 g of HP-β-CD was dissolved in 5 mL of anhydrous DMF and the solution obtained was stirred at 80 °C for 1 h. After this time, LiBr (23 mmol, 2.0 g) was added and the reaction mixture was kept at 80 °C until complete dissolution of salt. Subsequently, the temperature was lowered to 70 °C, to avoid the degradation of the cyclodextrin and the mixture was agitated overnight. Thus, the reaction mixture was cooled in ice bath before added dropwise 1 mL of a solution of NBS (0.28 mmol, 50 mg) in anhydrous DMF and 1 mL of Ph_3P (0.38 mmol, 100 mg) in anhydrous DMF, under stirring. After 15 min, the reaction mixture was heated to 70 °C for 3 h and then, the temperature was lowered at room temperature and HP-β-CD-Br was precipitated using 100 mL of acetone.

The samples of cyclodextrin purified by precipitation were recovered using a Thermo Scientific SL 16 Centrifuge. Deionized water was used for the dialysis purification procedures, which was previously degassed by blowing helium.

The precipitate was separated by centrifugation (13,200 rpm, 15 min at 25 °C) after transferring the suspension in falcon 15 mL centrifuge tubes. The precipitate was washed three times with acetone and dried under nitrogen flow. The product obtained was re-dissolved in water and dialyzed exhaustively against water for 2 days, employing a cellulose membrane tube and then, it was freeze dried at −80 °C and 0.01 mbar (ALPHA 1–4 LSC model, CHRIST freeze-drier, Osterode am Harz, Germany). Afterwards, in the second step 0.3 g of HP-β-CD-Br was dissolved in 5 mL of a solution of 0.5% w/v thiourea in DMF and the solution was stirred at 70 °C for 20 h. Then, the reaction mixture was cooled in ice bath and a solution of 3 M NaOH (2 mL) was added dropwise. After 15 min, the reaction mixture was deactivated at room temperature with 1 M H_2SO_4 until pH 7.0 and, HP-β-CD-SH was precipitated using 200 mL of acetone. The precipitate was recovered by centrifugation and then it was purified by dialysis and freeze dried as described above. The so-prepared thiomer was stored at 4 °C until further use.

2.2.2. Characterizations of thiolated HP-β CD

The structure of brominated cyclodextrin HP-β-CD-Br and the thiomer HP-β-CD-SH were fully characterized by FT-IR, UV, ^1H NMR, ^{13}C NMR, DEPT, HSQC and MS Q-TOF.

FT-IR spectra were recorded on a PerkinElmer 1600 FT-IR spectrometer, using KBr pellets (2% of sample). ^1H NMR and ^{13}C NMR spectra, 13C Distortionless Enhancement by Polarization Transfer (DEPT) experiment, 1H, 13C Heteronuclear Single Quantum Correlation (HSQC) experiment and two-dimensional (2D-) Nuclear Overhauser Effect Spectroscopy (NOESY) were carried out on Agilent VNMRS 500 MHz spectrometer, using DMSO-d_6 as solvent. Chemical shifts were referenced by using the residual solvent signal, as internal reference (DMSO-d_6: 2.48 ppm for 1H and 40.10 ppm for 13C). Mass spectrometry was performed using Agilent 6530 accurate mass Q-TOF. Mass spectra were achieved in positive (ESI +) and negative (ESI-) electrospray ionization mode.

HP-β-CD-Br: Yield 66% w/w (330 mg), 65% mol/mol. FT-IR (KBr): 3504, 1594, 1030 cm^{-1}. ESI$^+$ MS: calculated for $C_{55}H_{93}O_{36}Br_3$, $[M + 2Na]^{2+} = 1612$ (m/z = 806). Found: m/z 806.3630; ^1H NMR (DMSO-d_6): 5.8–6.1 (m, 1H, OCHOH), 4.3–5.2 (m, 2H, CHOH), 4.0–3.2 (2H, CH$_2$OH), 1.00 (s, 3H CH$_3$), ppm.

HP-β-CD-SH: Yield 47% w/w (141 mg), 51% mol/mol. FT-IR (KBr): 3380, 1590, 1177, 635 cm^{-1}. ESI$^+$MS: calculated for $C_{55}H_{96}O_{36}S_3$, $[M + 2Na]^{2+} = 1634$ (m/z = 817). Found: m/z 817.2825; ^1H NMR

X= OH, or Br

R= OH, or SH

Reagents and conditions:

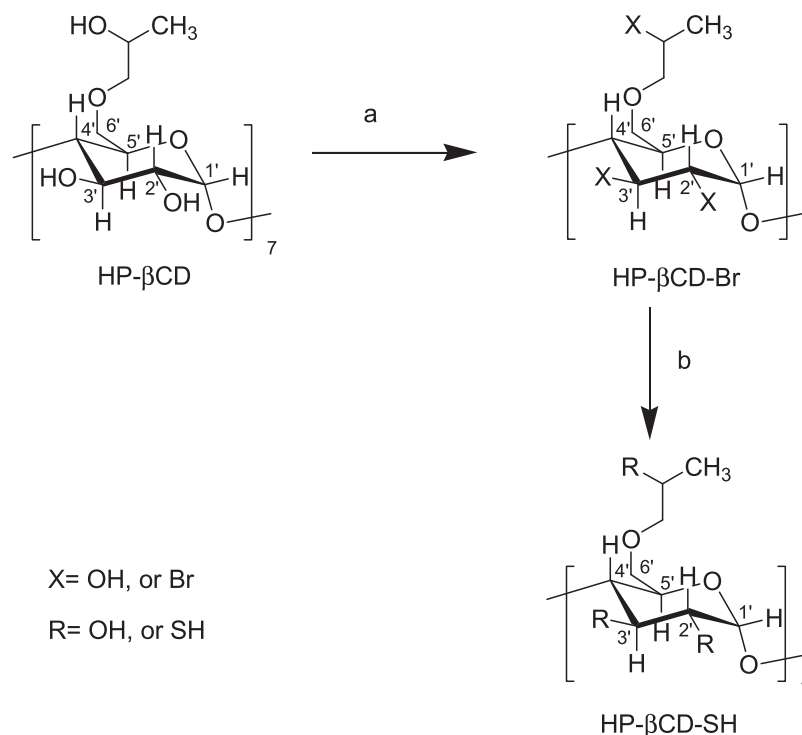a) LiBr, NBS and Ph₃P in anhydrous DMF, 70 °C

b) Thiourea 0.5% *w/v*, 70 °C

Fig. 1. Synthesis of thiolated HP-β-CD.

(DMSO-d_6): 5.8–6.1 (m, 1H, OC*H*OH), 4.3–5.2 (m, 2H, C*H*OH), 4.0–3.2 (2H, C*H₂*OH), 1.00 (s, 3H CH₃), ppm.

2.2.3. Quantitative analysis of thiol and disulfide groups

The thiol groups present on the cyclodextrin structure may be present in the free or oxidized form as disulfide bonds and have been quantified photometrically with Ellman's reagent by a method previously described (Perrone et al., 2018). Ellman's assay was carried out on Perkin Elmer 2030 Victor TM X3 multilabel plate reader spectrophotometer. For the partial Ellman's assay, a solution prepared by dissolving 1.0 mg of HP-β-CD-SH in 500 μL of 0.5 M of phosphate buffer at pH 8.0 was incubated with 500 μL of Ellman's reagent for 3 h at 25 °C. Then, the reaction mixture was centrifugation for 5 min at 13,000 rpm and 200 μL of the clear supernatant were taken and transferred into a microplate for quantification with a microplate reader by reading the absorbance at a wavelength of 450 nm. L-Cysteine was used as control.

For total Ellman's assay, a solution prepared by dissolving 1.0 mg of sample in 500 μL of 0.5 M of tris buffer, pH 7.6, was added with 500 μL of a freshly prepared sodium-borohydride solution (4.0% *m/v*) to reduce the disulfide bonds present in the compound. The reaction mixture was maintained for 1 h at 37 °C and then was cooled in ice bath and treated with 250 μL of 5 M HCl in order to deactivate the excess of reducing reagent. Finally, the resulted mixture was diluted with 1000 μL of 0.5 M phosphate buffer at pH 8.0 and incubated with 100 μL of Ellman's reagent for 90 min at 25 °C. The reaction mixture was centrifugation for 5 min at 13,000 rpm and 200 μL were withdrawn and evaluated as described above.

2.2.4. Preparation of inclusion complex

BUD/HP-β-CD and BUD/HP-β-CD-SH solid inclusion complexes were prepared by freeze-drying (Cutrignelli et al., 2019). A mixture of HP-β-CD and BUD (1 mg/mL, 1:1 *w/w*) or HP-β-CD-SH and BUD (1 mg/mL, 1:1 *w/w*) in water was stirred using an Eppendorf Thermo Mixer C (25 °C, 800 rpm) for 2 days. The obtained solution was frozen and lyophilized as described above.

2.2.5. High-Performance Liquid Chromatography (HPLC) analyses

HPLC analyses were performed with an Agilent 1260 Infinity Quaternary LC System equipped with an Agilent variable wavelength UV detector, a Rheodyne injector (Rheodyne, Model 7725i) equipped with a 20 μL loop and a OpenLAB CDS ChemStation software (Agilent, Santa Clara, CA). HPLC analyses were accomplished in reversed phase using a column Zorbax C18 column (150 mm × 4 mm; 5 μm particles) as stationary phase and a mixture of methanol and a solution of 1% TFA in deionized water (70:30 *v/v*) as mobile phase. A flow rate of 1.0 mL/min in isocratic mode and a column temperature of 35 °C were utilized continuously monitoring the column effluent at 254 nm. The samples were quantified by measuring the areas of the peaks, and references chromatographed under the same conditions. The data analysis was processed using Agilent OpenLab LC software.

2.2.6. Solubility and phase solubility studies

The phase solubility studies on inclusion complexes of BUD with HP-β-CD and BUD with HP-β-CD-SH were accompanied in accordance with the Higuchi and Connors' method (Higuchi and Connors, 1965). Solubility and phase solubility experiments were conducted at 25 °C under stirring. An excess of BUD to deionized water or to aqueous solutions of cyclodextrin (0–100 mM) was added for 72 h until solubility equilibrium was achieved. The suspensions were placed in 2 mL screw cap tubes, vortexed and maintained in a shaker water bath at the constant temperature of at 25 ± 0.2 °C for the whole time of the
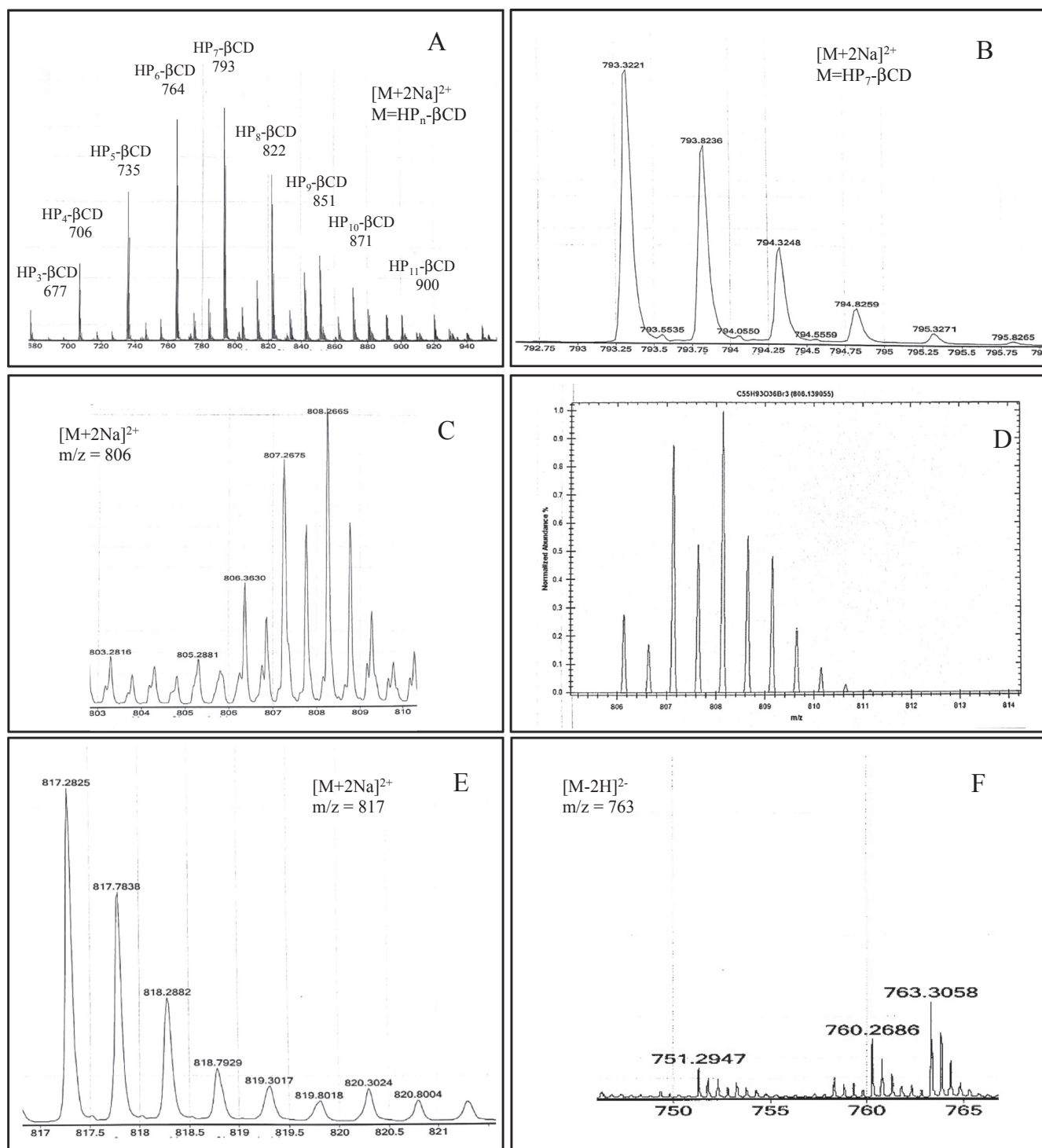
Fig. 2. LC-MS MASS Q-TOF of HP-β-CD (A-B), HP-β-CD-Br (C-D) and HP-β-CD-SH (E-F).

experiments. At appropriate time intervals, each sample was transferred into a 2 mL glass syringe and filtered through cellulose acetate membranes (0.22 μm, Advantec MFS, Pleasanton, CA, USA), and 20 μL of filtrates were immediately analyzed by HPLC, after appropriate dilution. The studies were done in triplicate. The following equation was applied to calculate the aqueous solubility at 25 °C and the 1:1 inclusion constant of the drug with cyclodextrin ($K_{1:1}$):

$$K_{1:1} = \frac{p}{S_0(1-p)}$$

(1)

where S_0 is the aqueous solubility of BUD and p is the slope of the phase solubility diagrams.

2.2.7. Inclusion complexes stoichiometry determination (Job's plot method)

The stoichiometry of the inclusion complexes BUD/HP-β-CD and BUD/HP-β-CD-SH in aqueous solution was evaluated by the continuous variation method (Cutrignelli et al., 2014). The variations in absorbance intensity (ΔA) at 254 nm were determined for a series of BUD: cyclodextrin mixtures of different ratios from 0 to 1, keeping the total molar concentration of the species constant. The mixtures were prepared using equimolar (1.0×10^{-4} M) MeOH/H$_2$O (50/50 *v/v*)
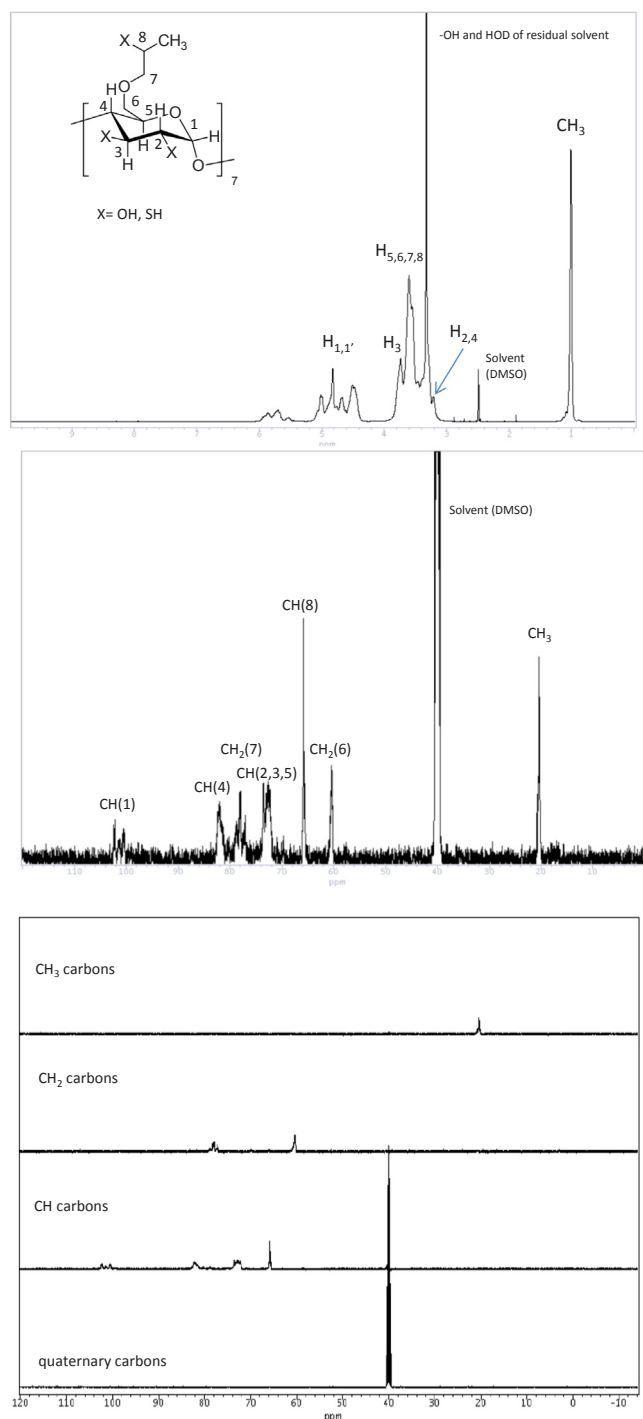
Fig. 3. ^{1}H NMR (A), ^{13}C NMR (B), and ^{13}C NMR DEPT (C) of HP-β-CD-SH.

solutions of BUD and HP-β-CD or BUD and HP-β-CD-SH. The $\Delta A \times [BUD]$ were plotted versus r, where r is the molar ratio of BUD, defined by the followed equation:

$$r = \frac{[BUD]}{[BUD] + [CD]} \qquad (2)$$

where ΔA is the difference between absorbance with and without cyclodextrin and [CD] is the molar concentration of HP-β-CD or HP-β-CD-SH.

2.2.8. Heteronuclear Single Quantum Correlation (HSQC) and Nuclear Overhauser effect Spectroscopy (NOESY) studies

The Heteronuclear Single Quantum Correlation (HSQC) and Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were carried out with an Agilent 500 MHz spectrometer, in a 5-mm NMR tube (Laquintana et al., 2016). The concentration of HP-β-CD-SH was 10 mg/mL in DMSO-d_6, for HSQC and an equimolar concentration of 2.0×10^{-3} M. Sample temperature was set to 25 °C. Chemical shifts are reported as δ values (i.e., in parts per million, ppm), relative to the residual isotopic impurity of DMSO-d_6 solvent (2.50 ppm).

2.2.9. Stability studies

The stability studies of HP-β-CD-SH were conducted at 37 °C at different pH values of 4.0, 5.0, 6.0 and 7.2, respectively. The buffers used were prepared using 50 mM acetate buffer solution, to reach pH 4.0 and 5.0, 50 mM phosphate buffer solution, for the pH 6.0 solution and, 50 mM Tris buffer solution, for the pH 7.2 solution, respectively. The thiolated cyclodextrin was solubilized with demineralized water and then incubated with the different buffer solutions, at a final concentration of 0.25%. At predetermined time intervals 500 μL of solution were taken and treated with 50 μL of a 1 M HCl solution and then analyzed for the quantification of thiol groups, using the Ellman's assay, as described above.

### 2.2.10. pKa determination by UV method

Stability of −SH groups towards oxidation is dependent on concentration of thiolate anions (−S^{-}) therefore, pK_a value of −SH groups on HP-β-CD-SH was analysed by UV spectrophotometry using a previously reported method (Shu et al., 2002). Briefly, HP-β-CD-SH (0.1% m/v) was dissolved in 1 mM HCl and to maintain stable ionic strength 0.1 M NaCl was added. NaOH (0.01%) in increasing volumes was added to HP-β-CD-SH solutions and absorbance at 242 nm and pH were measured. The pK_a was calculated by representing –log $[(A_{max} - A_i)/A_i]$ as a function of pH.

2.2.11. Cell cultures and cell viability analysis by resazurin assay

The resazurin assay was performed on human colorectal carcinoma cell lines (Caco-2) as previously described (Mahmood et al., 2015). Caco-2 cells were supplied by European Collection of Cells Cultures (ECACC, Health Protection Agency, Porton Down, Salisbury, Wiltshire, U.K.) and were cultured in Minimum Essential Medium (MEM) with Earle's balanced salts and supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were fed every day and seeded in 24-well plate at a density of ~25,000 cells/well for assays. Afterwards, the confluent cells were washed two times with pre-warmed (37 °C) phosphate buffer saline (PBS) and incubated with 500 μL of the cyclodextrin solutions in cells culture medium at a final concentration of 5 mg/mL, for 3 and 24 h. Untreated cells were employed as negative control, whereas a 4% solution of Triton X-100 in MEM served as positive control. Thereafter, cyclodextrin solutions were removed and cells were washed twice with PBS. Next, each well was loaded with 250 μL of resazurin solution in MEM (2.2 μM) and incubated for 3 h along at 37 °C. Subsequently, 100 μL of dye solution was transferred in a 96-well plate and the fluorescence was measured at a wavelength of 540 nm by microplate reader (M200 spectrophotometer, Tecan infinite, Grödig, Austria). All experiments were repeated three times and each compound's concentration was tested in triplicate.

2.2.12. In vitro mucoadhesion studies

Mucoadhesive properties of inclusion complexes of BUD with HP-β-CD and with HP-β-CD-SH were investigated using an *in vitro* model (*ex-vivo*) as previously reported (Bhatt et al., 2014). Freshly excised porcine oesophagus mucosa was supplied from a slaughterhouse (Innsbruck, Austria). The collected oesophagus tissue was cleaned, divided into petri dishes and frozen at −20 °C until use. Then, they were used
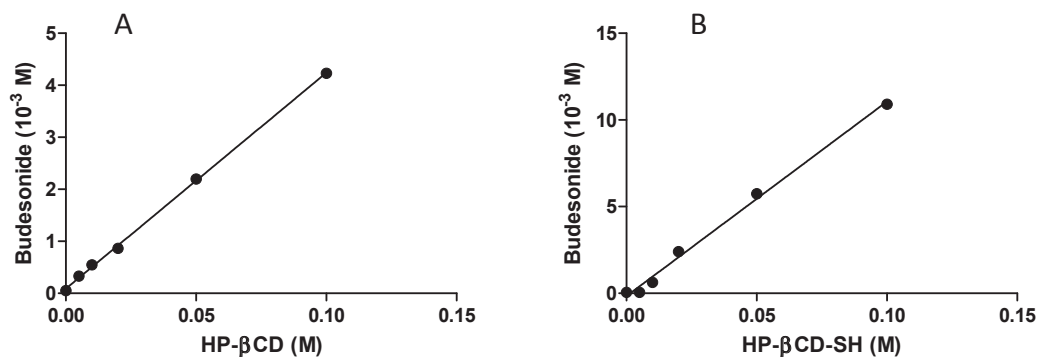
Fig. 4. Phase solubility studies of inclusion complexes of BUD with HP-β-CD (A) and HP-β-CD-SH (B). Each value is the average of three different experiments ± standard deviation.

Table 1
Solubility (S) and Phase solubility studies at 25 °C in water of BUD/HP-β-CD and BUD/HP-β-CD-SH complexes. Results are expressed as mean ± S.D of three experiments.

Sample	S (10^{-3} M)	$K_{1:1}$ (M^{-1})	Slope (p)	R^2
HP-β-CD	4.3[a]	861.11	0.041 ± 0.005	0.9993
HP-β-CD-SH	10.9[a]	2513.52	0.112 ± 0.004	0.9962
BUD	0.05028	–	–	–

[a] Water solubility of BUD with 100 mM of oligomer.

shortly after reconstitution of the thawed tissue and conditioned with 100 mM PBS at pH 6.8. For the experiments of mucoadhesion, the small tissue scraps were incubated in thermostatic chamber at 37 °C and 100% of relative humidity and placed over a half cut 50 mL falcon tubes inclined at 45° for wash-off. Solutions of BUD/HP-β-CD and BUD/HP-β-CD-SH inclusion complexes in 100 mM phosphate buffer solution (pH 6.8) at a concentration of 1 mg/mL were separately placed on each tissue scraps and after 30 min the mucosa were continuously rinsed with 100 mM PBS pH 6.8. The mucosa was continuously rinsed with phosphate buffer pH 6.8 at a flow rate of 1 mL/min. The phosphate buffer flowing down the mucosa was collected at following pre-determined time points: 10, 20, 30, 40, 50 and 60 min and 100 µL of them was filtered through 0.22 µm CA filters and analysed by HPLC as described above. BUD (1 mg/mL) alone was dispersed in100 mM phosphate buffer solution (pH 6.8) used for comparative purposes.

2.2.13. Statistical data analysis

The Bonferroni's Multiple Comparison Test (GraphPad Prism version 5 for Windows, GraphPad Software, San Diego, CA) was used for analysis of variance (ANOVA) and the statistical significance was assigned to $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Preparation and characterization of thiolated HP-β-CD

The thiolated HP-β-CD (HP-β-CD-SH) was prepared following a two-steps synthesis shown in Fig. 1. In particular, in the first step the bromine intermediate HP-β-CD-Br was prepared using the NBS reagent and the TPP dehydrating agent. Next, the HP-β-CD-Br was converted into the HP-β-CD-SH thiomer using a 0.5% (w/v) thiourea solution. Both the intermediate and the final product were purified by dialysis and then dried by lyophilization. Degassed deionized water was used in the dialysis purification procedures in order to avoid the oxidation of the thiol groups. The dialysate containing the purified thiolated cyclodextrin was then immediately frozen and freeze-dried. The bromination is the limiting step of the entire process and the number of bromine residues on the cyclodextrin template depends on the weight ratios between the reagents, as previously reported (Ijaz et al., 2015). Different w/w ratios of NBD and TPP were tested to optimize the appropriate proportion of the reagents to be used to generate the HP-β-CD-Br (HP-β-CD: TPP: NBD = 1:0.1:0.2 w/w). Then, in the second step the bromine atoms are completely substituted with the thiol groups using a thiourea solution. The two compounds were fully characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, and MS Q-TOF.

In particular, from the analysis of the MS spectra it was possible to determine the exact number of the bromine atoms inserted and then replaced by thiols per cyclodextrin. Fig. 2 shows the mass spectra of HP-β-CD, HP-β-CD-Br and HP-β-CD-SH in panels A-B, C-D and E-F, respectively. The spectra were acquired in the range between 0 and 2000 Da and are double-charged spectra (m/z, z = 2) for the addition of two sodium ions. Panels A-B display the mass-spectra of the pristine HP-β-CD. The bell shaped signals (Fig. 2, panel A) is due to the degree of substitution of the pristine cyclodextrin and allows to calculate an average HP substitution degree of 7. The set of peaks of each signal is
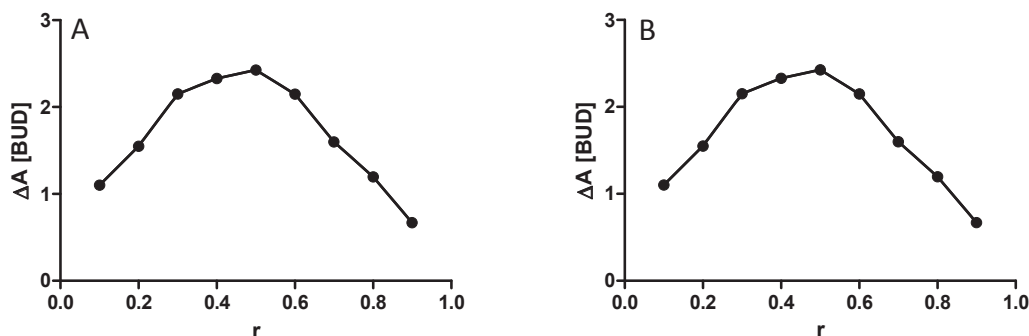
Fig. 5. Job's plot diagrams for determination of the stoichiometry of the inclusion complexes of BUD with HP-β-CD (A) and HP-β-CD-SH (B). Each value is the average of three different experiments ± standard deviation.

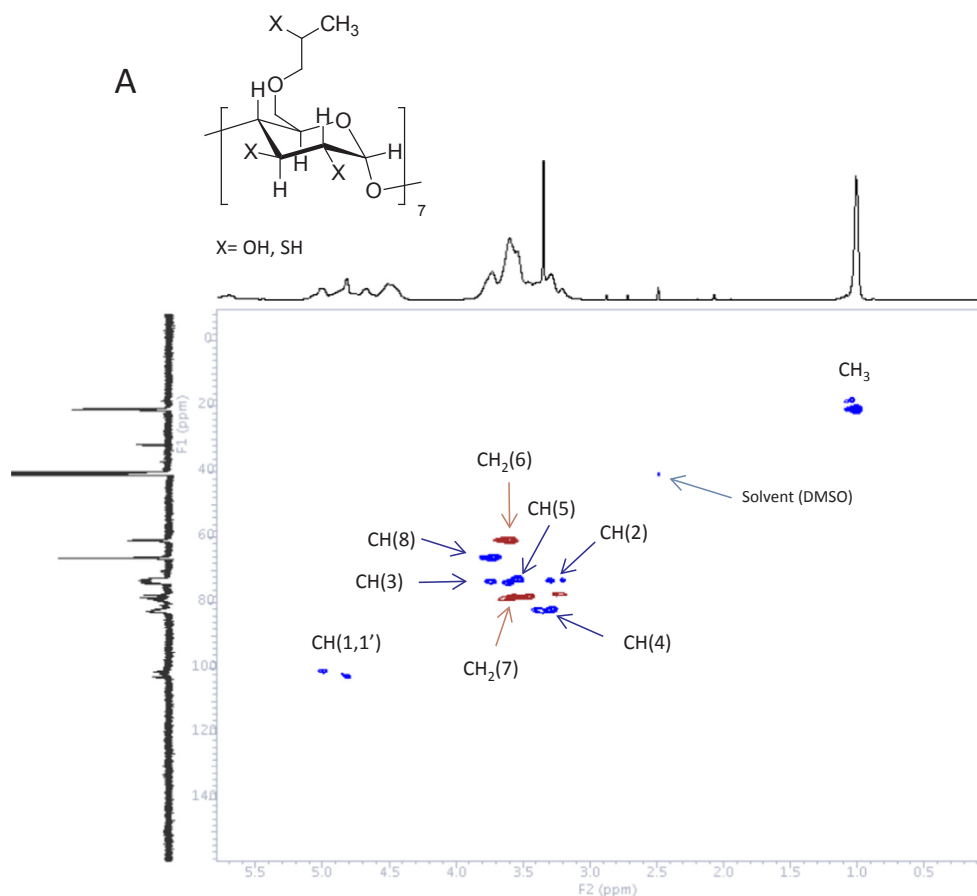**Fig. 6.** ^{1}H, ^{13}C HSQC of HP-β-CD (A) and BUD/HP-β-CD-SH complex (B). The primary carbons (blue) and the secondary ones (red) were highlight, while the arrows indicated the assignments of individual H-C cross peaks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

due to the isotopic abundance (Fig. 2, panel B), which allowed to determine, with precision, the number of bromine atoms per cyclodextrin. As reported in Fig. 2, panels C and D, the number of bromine atoms introduced per cyclodextrin is equal to three, as demonstrated by the calculation, performed with the software supplied (MassHunter B0600) with the instrument, of the isotopic abundance related to a compound of molecular formula $C_{55}H_{93}O_{36}Br_3$. Furthermore, the spectrum analysis of HP-β-CD-SH (Fig. 2, panel E) shows that all the bromine atoms have been replaced with thiol groups. It is also possible to evidence in the spectra reported in Fig. 2, panel F, the presence of the disulfide compound ($m/z = 763$) due to the partial oxidation of the intramolecular SH constituents. These evidences have been confirmed by Ellman's assay on thiol groups (partial assay) and disulfides (total assay), as described below.

The IR and NMR spectra are consistent with the structures of the products (data not shown). The IR spectra of the HP-β-CD-SH shows additional bands at 2650 (w), 1177 cm^{-1} (s) and 635 cm^{-1} (m), compared to the unmodified cyclodextrin. The peaks were attributed to the SH (2650 cm^{-1}) or CS (1177 cm^{-1}) stretching vibrations. The ^{1}H NMR ^{13}C NMR and ^{13}C-DEPT experiment, shown in Fig. 3 allowed to correctly assign the signals of the HP-β-CD-SH. ^{1}H NMR (Fig. 3, panel A) spectrum allows to attribute the signals of the methyl protons of the hydroxypropyl residues at 1.00 ppm, while the protons bound to the C2-8 carbon atoms of the cyclodextrin ring are not easily attributable. ^{13}C NMR spectrum (Fig. 3, panel B) allows to attribute the signals of CH$_3$ of the hydroxypropyl residues at 20 ppm and the signal at 60 and 65 ppm which have been attributed to carbon atoms CH$_2$ (6) and CH$_2$ (8), respectively. ^{13}C-DEPT experiment (Fig. 3, panel C) was designed to improve the sensitivity of ^{13}C NMR spectra. This experiment consents

the precise separation of primary (CH), secondary (CH$_2$), tertiary (CH$_3$) and quaternary (C) carbons, permitting to correctly assign the chemical shift of each carbon atom. In particular, the secondary carbon atoms, CH$_2$ (6) and CH$_2$ (7) were identified and separated from the primary ones. Furthermore, the absence of quaternary carbons in the ^{13}C NMR spectra indicates that the adopted synthesis and purification processes did not involve degradation and opening reactions of the cyclodextrin ring.

3.2. Quantitative analysis of thiol and disulfide groups

The mucoadhesive properties of thiolated cyclodextrins is strongly influenced by the presence of thiol residues and by the number of these free groups exposed on the oligomer ring (dos Santos et al., 1859). In fact, to be mucoadhesive the thiol moieties of the new modified cyclodextrin HP-β-CD-SH, must form disulfide bonds through oxidative coupling of the exposed free thiol groups with cysteine-rich subdomains of mucus glycoproteins. Hence, it is important to quantify the thiol and disulfide groups exposed on the modified cyclodextrin. The free thiol groups present on HP-β-CD-SH were detected with the so called partial Ellman's assay. In order to prevent oxidation of the free thiol groups, exposure to the air was avoided by storing freeze-dried HP-β-CD-SH samples in a vacuum dryer and freshly analyzed within 24 h after freeze-drying. While, the oxidized thiols groups were calculated by difference from total thiol moieties (reduced and oxidized groups) recorded by total Ellman's assay. The result of Ellman's analysis show an average value of free thiol groups of 826.06 ± 31.35 μmol/g of oligomer, and total thiol groups of 975.28 ± 42.01 μmol/g of oligomer (oxidized thiol groups 149.21 ± 36.68 μmol/g of oligomer). The
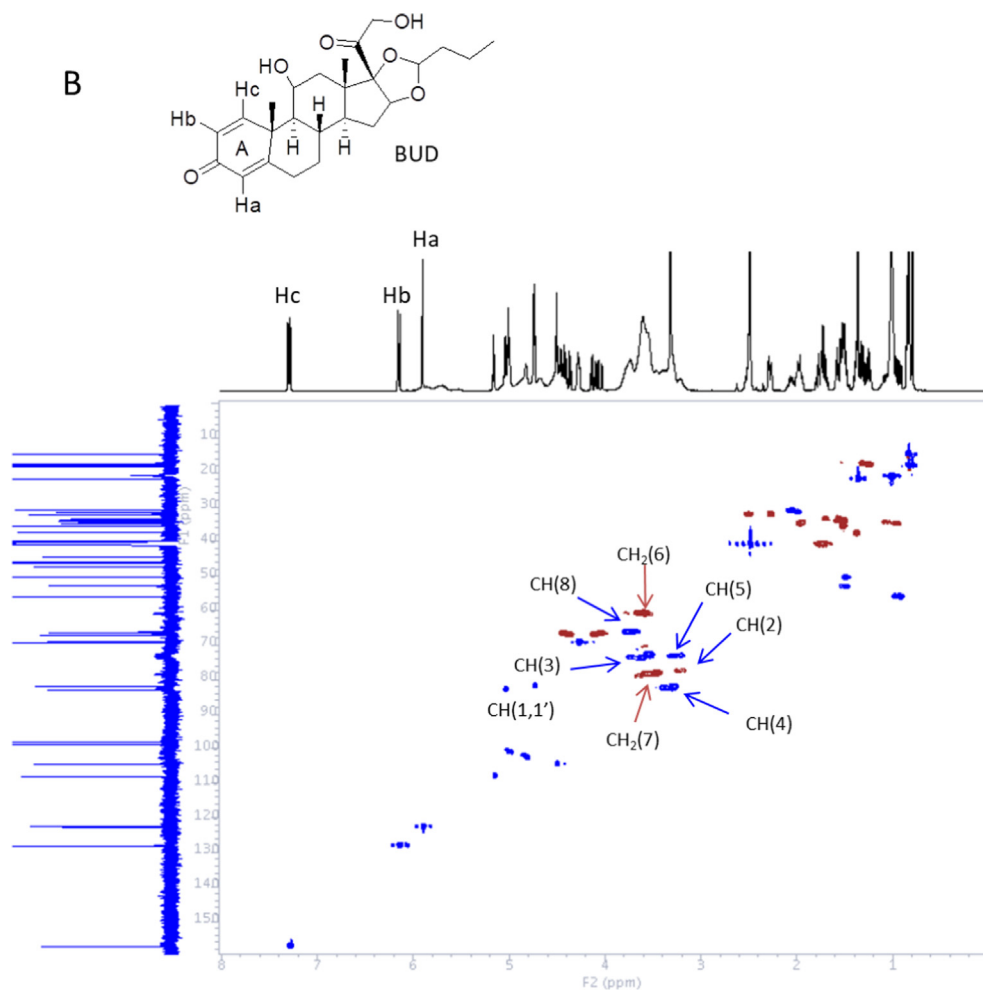
Fig. 6. (*continued*)

degree of substitution (DS) of thiol groups per oligomer was equal to 3.1% and their quantity is equal to 84.7% of the total thiol groups present on the ring of the HP-β-CD-SH and with a low content of disulfide groups. The substitution degree is in accordance with the value recorded with the mass analysis.

### 3.3. Solubility and phase solubility studies

The aqueous solubility (S_0) of BUD at $25 \pm 0.2\,°C$ was recorder in ultrapure water and measured by HPLC analysis, quantifying the concentration of a saturated solution of the drug. In accordance with the data already reported, the aqueous solubility of BUD is equal to 0.021 g/L ($S_0 = 5.028 \cdot 10^{-5}$ M). The phase solubility study is a widely accepted method to estimate the complexation effect of cyclodextrin on the drug solubility (Cutrignelli et al., 2014). The phase solubility diagrams on inclusion complexes of BUD with HP-β-CD and HP-β-CD-SH are shown in Fig. 4, panels A and B, respectively. Linear regression analysis of the plotted data, in according to the Higuchi and Connors equation (Table 1, Fig. 4, panel A), gives complexation constant ($K_{1:1}$) for the BUD/HP-β-CD complex equal to 861.11 M^{-1}. This diagram shown the development of a water-soluble complex, which can be classified as A_L type complex, according to Higuchi and Connors' model. Moreover, a *p* lower than unit is related to a complex that has a 1:1 host: guest stoichiometry (Higuchi and Connors, 1965). These results are in good agreement with the data described above (Dufour et al., 2015). Likewise, the linear regression analysis for BUD/HP-β-CD-SH complex (Fig. 4, panel B), gives a $K_{1:1}$ equal to 2013.52 M^{-1}. The drug solubility increased linearly in the range of 0–100 mM for both

HP-β-CD and HP-β-CD-SH and the water solubility of BUD in the presence of 100 mM of HP-β-CD or HP-β-CD-SH increased about 85 times (1.85 mg/mL, $4.3 \cdot 10^{-3}$ M), and 220 times (4.69 mg/mL, $10.9 \cdot 10^{-3}$ M), respectively. The higher solubility of BUD in the presence of HP-β-CD-SH is probably due to the thiol residues on the cyclodextrin scaffold, which might influence in positive the lipophilic character of the cavity, increasing the fitting of the guest drug, as demonstrated by the higher value of the complexation constant. The proposed formulation is a liquid preparation containing the soluble complex of BUD with HP-β-CD-SH, whose complexation/dissociation constant ($K_{1:1}$) was equal to 2013.52 M^{-1} indicating an interaction that allows the release of the drug even under minor dilution as occurring in the esophagus.

3.4. Inclusion complexes stoichiometry determination (Job's plot method)

The Job's plot method, also known as the continuous variation method is widely used for the determination of the stoichiometry of inclusion complexes. The value of XG for which the plot presents the maximum deviation gives the stoichiometry of the inclusion complex. The stoichiometry of the complexes BUD/HP-β-CD and BUD/HP-β-CD-SH in aqueous solution was evaluated using equimolar (2×10^{-3} M) methanol–water solutions of the drug and cyclodextrins, and measuring the change of the absorbance (ΔA) of the guest during addition of the host, as described in experimental section. The value of ΔA × [BUD] for which the plot shows the maximum deviation gives the stoichiometry of the inclusion complex. As reported in Fig. 5, for each complexes BUD/HP-β-CD and BUD/HP-β-CD-SH a maximum value at r = 0.5 with a symmetrical shape proved the presence of complexes with 1:1
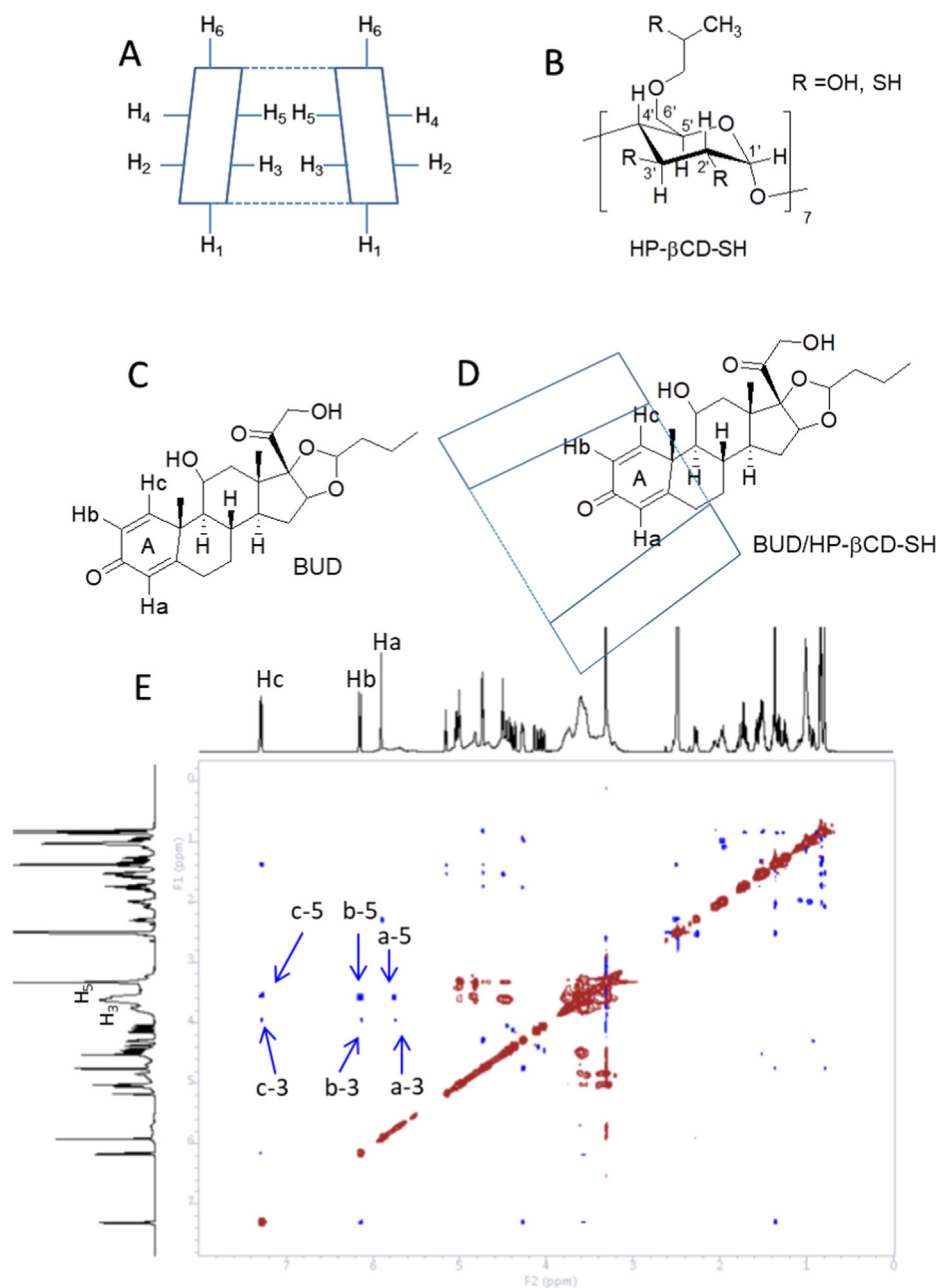
Fig. 7. Scheme of the CD internal and external protons (A). Chemical structure of HP-β-CD-SH (B). Structure of BUD (C). Schematic drawing of BUD/HP-β-CD-SH inclusion complex with the entrance of the BUD into the cavity of the CD through the narrow rhyme (D). 2D NOESY of BUD/HP-β-CD-SH (E). The arrows indicated the assignments of individual NOE cross peaks from H_a, H_b and H_c protons of BUD and H-3 protons of HP-β-CD-SH (a-3, b-3 and c-3) or H_a, H_b and H_c protons of BUD and H-5 of HP-β-CD-SH (a-5, b-5 and c-5).

stoichiometry, within the range of evaluated concentrations.

3.5. Heteronuclear Single Quantum Correlation (HSQC) and Nuclear Overhauser effect Spectroscopy (NOESY) studies

The ^{1}H, ^{13}C HSQC and 2D NOESY experiments were achieved to provide more information on the geometry of inclusion complexes. The description of structure of an inclusion complex in solution (e.g. host-guest geometry) can be experimentally obtained by 2D-NMR. The drug can include the CD cavity in several ways, through the wide or the narrow rim. ^{1}H, ^{13}C-HSQC experiment (Fig. 6) provides the correlations between a carbon and its attached protons. The resulting spectrum is two dimensional (2D) with one axis for proton (^{1}H) and the other for ^{13}C from which it is possible to separate the peak of each unique proton

attached to the heteronucleus, crossing the signals of the proton (^{1}H) and carbon (^{13}C). In Fig. 6 are highlight the primary carbons (blue) and the secondary ones (red), while the arrows indicate the assignments of individual cross peaks. This experiment allowed to explain the over-lapping signals between 3.0 and 4.0 ppm for both HP-β-CD (Fig. 6, panel A) and BUD/HP-β-CD-SH complex (Fig. 6, panel B). In particular, the cross peaks at 3.6 and 3.8 ppm were assigned to the protons bound to the C5 and C3 carbon atoms of the cyclodextrin ring (H_3 and H_5, respectively) that interact with the drug, as shown below in the 2D-NOESY spectrum. The 2D ^{1}H NMR NOESY (Fig. 7) provides the in-formation of inter and intra molecular interactions between three aro-matic protons from budesonide (Ha, Hb and Hc) and the proton H_5 of HP-β-CD-SH highlighting the inclusion of 2.5-cyclohexadiene-1-one (ring A) inside the cyclodextrin cavity. Fig. 7 shows the scheme of the
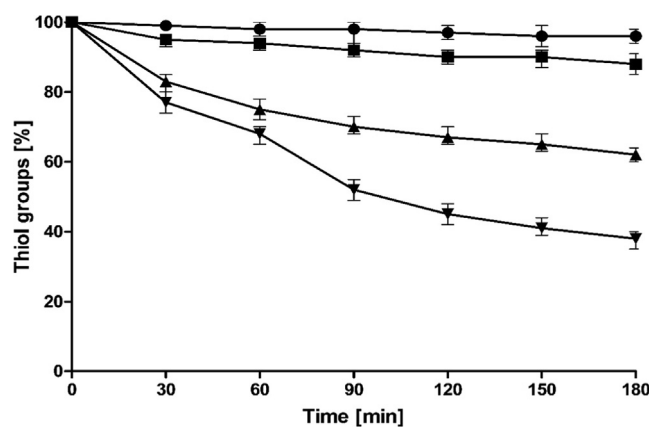
Fig. 8. Stability of thiol groups towards oxidation at 37 °C at different pH conditions. pH 4 (●), pH 5 (■), pH 6 (▲) and pH 7.2 (▼). Indicated values are means ± standard deviation of three experiments.
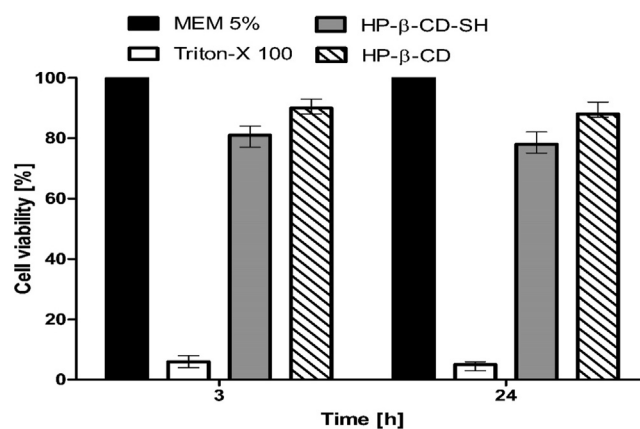

Fig. 10. Cell viability of Caco-2 cells exposed for 3 and 24 h to 5 mg/mL HP-β-CD and HP-β-CD-SH. Untreated cells were used as control and a 4% (*m/v*) solution of Triton X-100 in MEM served as positive control. Indicated values are means ± standard deviation of three experiments.
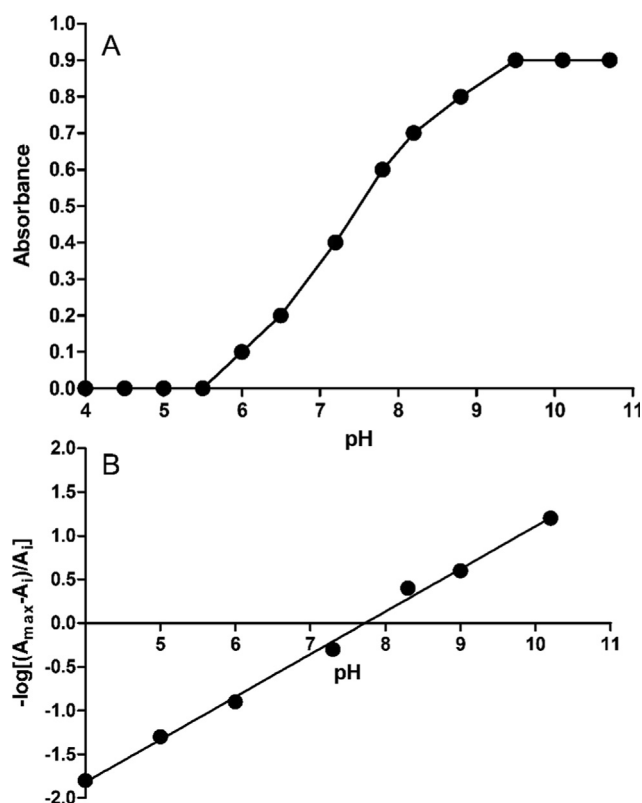

Fig. 9. Absorbance of the HP-β-CD-SH as a function of pH (A) and logarithmic representation of the absorbance ($-\log[(A_{max} - A_i)/A_i]$) as a function of pH, where the pKa values correspond to the interception with the abscissa (B).

Fig. 11. Residence time of BUD in BUD/HP-β-CD complex (●), BUD/HP-β-CD-SH complex (■), and BUD as control (▲) on porcine esophagus mucosa being continuously rinsed with 100 mM phosphate buffer pH 6.8 at 37 °C and 100% relative humidity.

between the H_3 protons of HP-β-CD-SH with Ha, H_b and H_c. In all cases the interaction of BUD with only internal protons of HP-β-CD-SH were observed. In addition the H_6 protons of HP-β-CD-SH were not affected by the inclusion process. Thus, it is possible to confirm that the BUD is included in to the HP-β-CD-SH cavity *via* wider rim. This analysis confirms the results previously described for the inclusion of BUD with HP with a key difference (Dufour et al., 2015). In fact, we observed that the inclusion of BUD in the thiolated cyclodextrin occurs through the narrow rhyme deeper into hydrophobic cavity, as evidenced by the presence of additional NOE peaks between the H_a, H_b and H_c protons and the H_3 protons of the CD. The latter are expressed inside the cavity, like the H_5 protons, but they are deeper, if we consider the entrance from the narrow rhyme. The entry through the wide rhyme is less probable because the latter NOE peaks are weak, compared to the first NOE group (H_3-H_a,H_b, and H_c), confirming the phase solubility studies.

3.6. Stability studies

The mucoadhesive capacity of a thiomer is due to the presence of thiol residues. These groups must be in the no-oxidized form to bind to the mucosal surfaces through the formation of covalent bonds, disulfide bridges, with the thiol groups of cysteine residues of mucins. The thiol groups are pH-sensitive and their conversion into the conjugated base form at high pH values facilitates disulfide oxidation. For this reason

CD protons of the internal lipophilic cavity and external protons (panel A), the structure of HP-β-CD (panel B) and the structure of BUD (panel C). Moreover was reported the diagram with the entrance of the BUD into the cavity of the CD, through the narrow rhyme (Fig. 7, panel D) and ¹H-2D NOESY of BUD/HP-β-CD-SH (E). Therefore, the presence of NOE cross-peaks between H_a, H_b and H_c protons of BUD in 2D NOESY spectrum is an indication that they are in spatial contact through space within the cavity of HP-β-CD-SH. Fig. 7 shows the 2D spectrum of BUD/HP-β-CD-SH systems, with the intermolecular NOE cross-peaks observed. The first group of NOE peaks (a-5, b-5 and c-5) are strong interaction, and show the interaction between the H5 protons of HP-β-CD-SH with the H_a, H_b and H_c proton of BUD. The second group are weak NOE peaks (a-3, b-3 and c-3) that were assigned the interaction
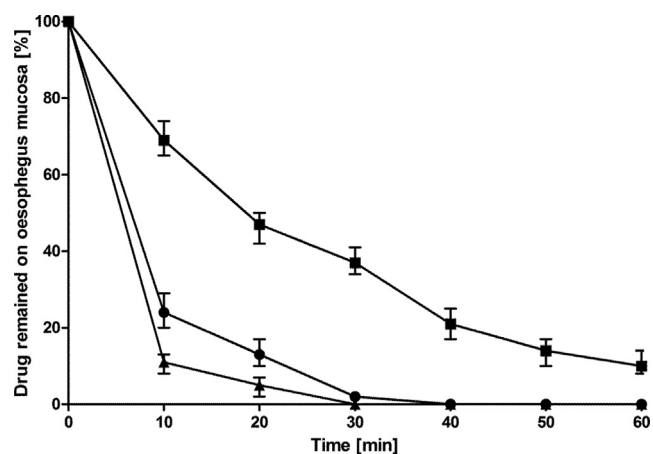
the stability of the HP-β-CD-SH thiomer was studied at different pH values of 4.0, 5.0, 6.0 and 7.2, and the resultant behaviors are shown in Fig. 8.

At pH 4.0, as well as at pH 5.0, there is no significant decrease of the initial thiol groups content. Whereas, when the pH value increases, the thiolated cyclodextrin undergoes a progressive oxidative process with reduction of the free thiol groups to about 60% and reaches 40% of the initial value after three hours at pH 6.0 and 7.2, respectively. Oxidation, as expected, is caused by the dissociation of the SH group to thiolate, which reacts to produce the disulfide bridges. Moreover, the pKa of thiol groups was also determined as in disulfide bond formation thiol groups are converted to thiolate ions. Thiol group (−SH) has a non-polar covalent bond between sulfur and hydrogen and sulfur loses its proton (H$^+$) due to a small difference in electronegativity to form a thiolate anion (−S$^-$). The increase in pH increases the absorbance due to the formation of thiolate anions (−S$^-$) as shown by the sigmoidal curve of absorbance vs pH in Fig. 9 (A). The pKa value of HP-β-CD-SH was calculated by intercept with abscissa in a graphical representation of $-\log[(A_{max} - A_i)/A_i]$ against pH as represented in Fig. 9 (B). The pKa value measured by UV spectrophotometry was found to be equal to 7.8 ± 0.5 at 25 °C, evidencing that above pH 5.8 the dissociation of the thiol group to thiolate occurs, which is in agreement with the results of the stability study.

3.7. Cytotoxicity assay

Cytotoxicity studies of HP-β-CD and HP-β-CD-SH were performed on the CaCo-2 cell line, chosen as an in vitro biocompatibility model. Cell viability experiments were conducted using the resazurin assay because it seems to be more sensitive than the MTT test. This assay is based on the ability of mitochondrial enzymes of living cells to reduce the soluble dye in resorufin by the transfer of electrons from NADPH$^+$ to resazurin. The results shown as histograms in Fig. 10 evidenced that both HP-β-CD and HP-β-CD-SH are not cytotoxic for CaCo-2 cells exposed for a short (3 h) and long period (24 h) at the concentration of 5 mg/mL. The amount of −SH groups bound to polymers backbone had marginal effect on their toxicity (Loretz and Bernkop-Schnürch, 2007) and cell viability of $^>$80% was considered as non-cytotoxic under the defined experimental conditions (Shahzadi, 2019). Therefore, newly synthesized thiolated HP-β-CD was considered safe for in-vivo application. Based on the phase solubility studies, a concentration of 5 mg/mL of HP-β-CD and HP-β-CD-SH guarantees a water solubility of BUD of 0.022 mg/mL. Hence, as for HP-β-CD, HP-β-CD-SH can be considered a biocompatible material, which in a small volume solubilizes the daily dose of BUD.

3.8. In vitro mucoadhesion studies

Mucoadhesion ability of the new HP-β-CD-SH thiomer was measured using a porcine esophageal mucosa by wash-off method as previously reported (Denora et al., 2016), and the results are shown in Fig. 11. The complexes BUD/HP-β-CD and BUD/HP-β-CD-SH, and free BUD used as a reference are fixed on the initial portion of the tissue, which is then continuously washed-off by the buffered solution due to the 45° inclination of the support. The mucosa was continuously rinsed with phosphate buffer pH 6.8 at a flow rate of 1 mL/min. The phosphate buffer flowing down the mucosa was collected at following pre-determined time points: 10, 20, 30, 40, 50 and 60 min. The wash-off samples are then collected and analyzed to quantify the drug. The results of the experiment shown that both complexes, compared to BUD alone, are able to keep the drug longer in contact with the mucosal layer of the tissue. In particular, the BUD/HP-β-CD-SH complex is more efficient, due to the presence of the thiol groups, which make the HP-β-CD-SH carrier more mucoadhesive than the pristine HP-β-CD.

4. Conclusions

Due to the lack of commercial medicines for the treatment of EoE, currently, there is a need for BUD mucoadhesive compounded oral liquid formulations, which can be easily swallowed by children or adults affected by dysphagia. Therefore, an effective formulation should be based on MDDSs that extend the time of contact between the administered drug and the oesophageal mucosa, increasing its bioavailability at the target site and reducing the ingested dose and the systemic side effects as well. In the present study, a new thiolated cyclodextrin, namely HP-β-CD-SH, was developed with the dual function to solubilize BUD, by forming a reversible BUD/HP-β-CD-SH water soluble inclusion complex, and to increase its residence time on the oesophageal mucosa. HP-β-CD-SH was fully characterized and phase solubility studies demonstrated that in a small volume, easily swallowed by both children and adults, this new biocompatible excipient solubilize BUD at the recommended doses. Mucoadhesive studies on porcine oesophagus mucosa proved that HP-β-CD-SH extend the permanence of BUD on the target site and so it might be a useful MDDS for the pharmacist to prepare BUD oral liquid formulations instead of suspensions and satisfying the absence of commercial medicines.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Asim, M. Hussain, Moghadam, A., Ijaz, M., Mahmood, A., Xaver Götz, R., Matuszczak, B., Bernkop-Schnürch, A., 2018. S-protected thiolated cyclodextrins as mucoadhesive oligomers for drug delivery. J. Colloid Interface Sci. 531, 261–268.

Attwood, S.E., 2019. Overview of eosinophilic oesophagitis. Br. J. Hosp. Med. (Lond.) 80 (3), 132–138. https://doi.org/10.12968/hmed.2019.80.3.132.

Bernkop-Schnürch, A., 2005. Thiomers: a new generation of mucoadhesive polymers. Adv. Drug Deliv. Rev. 57, 1569–1582.

Bernkop-Schnürch, A., Steininger, S., 2000. Synthesis and characterisation of mucoadhesive thiolated polymers. Int. J. Pharm. 194, 239–247.

Bhatt, H., Naik, B., Dharamsi, A., 2014. Solubility enhancement of budesonide and statistical optimization of coating variables for targeted drug delivery. J. Pharmaceut. https://doi.org/10.1155/2014/262194. number 262194.

Bhatt, H., Naik, B., Dharamsi, A., 2014. Solubility enhancement of budesonide and statistical optimization of coating variables for targeted drug delivery. J. Pharmaceut. 2014, 1–13. https://doi.org/10.1155/2014/262194.

Burjonrappa, S., et al., 2007. Mucin expression profile in Barrett's, dysplasia, adenocarcinoma sequence in the esophagus. Indian J. Cancer 44 (1), 1.

Cutrignelli, A., Lopedota, A., Denora, N., Iacobazzi, R.M., Fanizza, E., Laquintana, V., Perrone, M., Maggi, V., Franco, M., 2014. A new complex of curcumin with sulfobutylether-β-cyclodextrin: characterization studies and in vitro evaluation of cytotoxic and antioxidant activity on HepG-2 cells. J. Pharm. Sci. 103 (12), 3932–3940.

Cutrignelli, A., Sanarica, F., Lopalco, A., Lopedota, A., Laquintana, V., Franco, M., Boccanegra, B., Mantuano, P., De Luca, A., Denora, N., 2019. Dasatinib/HP-β-CD inclusion complex based aqueous formulation as a promising tool for the treatment of paediatric neuromuscular disorders. Int. J. Mol. Sci. 20 (3). https://doi.org/10.3390/ijms20030591. pii: E591.

Dellon, E.S., Sheikh, A., Speck, Olga, Woodward, Kimberly, Whitlow, Ann B., Hores, Jessica M., Ivanovic, Marija, Chau, Allen, Woosley, John T., Madanick, Ryan D., Orlando, Roy C., Shaheen, Nicholas J., 2012. Viscous topical is more effective than nebulized steroid therapy for patients with Eosinophilic esophagitis. Gastroenterology 143, 321–324. https://doi.org/10.1053/j.gastro.2012.04.049.

Dellon, Evan S., Woosley, John T., Arrington, Ashley, McGee, Sarah J., Covington, Jacquelyn, Moist, Susan E., Gebhart, Jessica H., Tylicki, Alexandra E., Shoyoye, Shiyan O., Martin, Christopher F., Galanko, Joseph A., Baron, John A., Shaheen, Nicholas J., 2019. Efficacy of budesonide vs fluticasone for initial treatment of eosinophilic esophagitis in a randomized controlled trial. Gastroenterology 157, 65–73.

Denora, N., Lopedota, A., Perrone, M., Laquintana, V., Iacobazzi, R.M., Milella, A., Fanizza, E., Depalo, N., Cutrignelli, A., Lopalco, A., Franco, M., 2016. Spray-dried mucoadhesives for intravesical drug delivery using N-acetylcysteine- and glutathione-glycol chitosan conjugates. Acta Biomater. 1 (43), 170–184.

dos Santos, Andreia G., Bayiha, Jules César, Dufour, Gilles, Cataldo, Didier, Evrard, Brigitte, Silva, Liana C., Deleu, Magali, Mingeot-Leclercq, Marie-Paule, 1859. Changes in membrane biophysical properties induced by the Budesonide/Hydroxypropyl-β-cyclodextrin complex. Biochimica et Biophysica Acta (BBA) – Biomembranes 2017, 1930–1940. https://doi.org/10.1016/j.bbamem.2017.06.010.

Dufour, G., Bigazzi, W., Wong, N., Boschini, F., de Tullio, P., Piel, G., Cataldo, D., Evrard, B., 2015. Interest of cyclodextrins in spray-dried microparticles formulation for sustained pulmonary delivery of budesonide. Int. J. Pharmaceut. 2, 869–878.

Grace, M.P., et al., 1985. Keratin expression in normal esophageal epithelium and squamous cell carcinoma of the esophagus. Cancer Res. 45 (2), 841–846.

Gupta, S.K., Vitanza, J.M., Collins, M.H., 2015. Efficacy and safety of oral budesonide suspension in pediatric patients with eosinophilic esophagitis. Clin. Gastroenterol. Hepatol. 13, 66–76. https://doi.org/10.1016/j.cgh.2014.05.021.

Higuchi, T., Connors, K.A., 1965. Phase solubility technique. Adv. Anal. Chem. Instrum. 4, 117–212.

Hopwood, D., Coghill, G., Sanders, D., 1986. Human oesophageal submucosal glands. Histochemistry 86 (1), 107–112.

Ijaz, M., Matuszczak, B., Rahmat, Deni, Mahmood, A., Bonengel, S., Hussain, S., Huck, C.W., Bernkop-Schnürch, A., 2015. Synthesis and characterization of thiolated -cyclodextrin as a novel mucoadhesive excipient for intra-oral drug delivery. Carbohydr. Polym. 132, 187–195.

Laffleur, Flavia, Bacher, Lukas, Menzel, Stefan Vanicek Claudia, Muhammad, Ijaz, 2016. Next generation of buccadhesive excipient: Preactivated carboxymethyl cellulose. Int. J. Pharmaceut. 500, 120–127.

Laquintana, V., Denora, N., Cutrignelli, A., Perrone, M., Iacobazzi, R.M., Annese, C., Lopalco, A., Lopedota, A.A., Franco, M., 2016. TSPO ligand-methotrexate prodrug conjugates: design, synthesis, and biological evaluation. IJMS 17 (6). https://doi.org/10.3390/ijms17060967. pii: E967.

Loretz, B., Bernkop-Schnürch, A., 2007. In vitro cytotoxicity testing of non-thiolated and thiolated chitosan nanoparticles for oral gene delivery. Nanotoxicology 1 (2), 139–148.

Lucendo, A.J., Miehlke, S., Schlag, C., Vieth, M., von Arnim, U., Molina-Infante, J., Hartmann, D., Jan Bredenoord, A., Ciriza de los Rios, C., Schubert, S., Brückner, S., Madisch, A., Hayat, J., Tack, J., Attwood, S., Mueller, R., Greinwald, R., Schoepfer, A., Straumann, A., 2019. Efficacy of budesonide orodispersible tablets as induction therapy for Eosinophilic esophagitis in a randomized placebo-controlled trial. Gastroenterology 157, 74–86.

Mahmood, A., Bonengel, S., Laffleur, F., Ijaz, M., Leonaviciute, G., Bernkop-Schnürch, A., 2015. An in-vitro exploration of permeation enhancement by novel poly-sulfonatethiomers. Int. J. Pharmaceut. 496, 304–313. https://doi.org/10.1016/j.ijpharm.2015.10.013.

Meyer, W., Luz, S., Schnapper, A., 2009. Lectin histochemical aspects of mucus function in the oesophagus of the reticulated python (Python reticulatus). Anatomia Histol. Embryol. 38 (4), 316–318.

Moghadam, A., Ijaz, M., Asim, M. Hussain, Mahmood, A., Jelkmann, M., Matuszczak, B., Bernkop-Schnürch, A., 2018. Non-ionic thiolated cyclodextrins – the next generation. Int. J. Nanomed. 13, 4003–4013.

Oliva, S., Rossetti, D., Papoff, P., Tiberti, A., Rossi, P., Isoldi, S., Amil Dias, J., Lucarelli, S., Cucchiara, S., 2017. A new formulation of oral viscous budesonide in treating paediatric Eosinophilic oesophagitis: a pilot study. J. Pediatr. Gastroenterol. Nutrit. 64 (2), 218–224. https://doi.org/10.1097/MPG.0000000000001281.

Perez-Vilar, J., Hill, R.L., 1999. The structure and assembly of secreted mucins. J. Biol. Chem. 274, 31751–31754.

Perrone, M., Lopalco, A., Lopedota, A., Cutrignelli, A., Laquintana, V., Douglas, J., Franco, M., Liberati, E., Russo, V., Tongiani, S., Denora, N., Bernkop-Schnürch, A., 2017. Preactivated 2thiolated glycogen as mucoadhesive polymer for drug delivery. Eur. J. Pharm. Biopharm. 119, 161–169. https://doi.org/10.1016/j.ejpb.2017.06.011.

Perrone, M., Lopedota, A., Liberati, E., Russo, V., Cutrignelli, A., Laquintana, V., Pereira de Sousa, I., Franco, M., Tongiani, S., Denora, N., Bernkop-Schnürch, A., 2017. Natural dendrimers: synthesis and in vitro characterization of glycogen-cysteamine conjugates. Eur. J. Pharm. Biopharm. 115, 168–176.

Perrone, M., Lopalco, A., Lopedota, A., Cutrignelli, A., Laquintana, V., Franco, M., Bernkop-Schnürch, A., Denora, N., 2018. S-preactivated thiolated glycol chitosan useful to combine mucoadhesion and drug delivery. Eur. J. Pharm. Biopharm. 132, 103–111. https://doi.org/10.1016/j.ejpb.2018.09.015.

Shahzadi, I., et al., 2019. Arginine-based cationic surfactants: biodegradable auxiliary agents for the formation of hydrophobic ion pairs with hydrophilic macromolecular drugs. J. Colloid Interface Sci. 552, 287–294.

Shu, X.Z., Liu, Y., Luo, Y., Roberts, M.C., Prestwich, G.D., 2002. Disulfide cross-linked hyaluronan hydrogels. Biomacromolecules 3 (6), 1304–1311.

Vinit, C., Dieme, A., Courbage, S., Dehaine, C., Dufeu, C.M., Jacquemot, S., Lajus, M., Montigny, L., Payen, E., Yang, D.D., Dupont, C., 2019. Eosinophilic esophagitis: Pathophysiology, diagnosis, and management. Archives de Pédiatrie 26 (3).