Carbohydrate Polymers 102 (2014) 393–399

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Improvement of thymol properties by complexation with cyclodextrins: *In vitro* and *in vivo* studies

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ARTICLE INFO

Article history:
Received 14 February 2013
Received in revised form 16 October 2013
Accepted 29 October 2013
Available online 20 November 2013

Keywords:
Thymol
Cyclodextrin
Sealed-heating
Palatability improvement
Pharmacokinetic study

ABSTRACT

Thymol, an effective agent for microbial diseases, has a low aqueous solubility and a strong bitter/irritating taste. These physicochemical characteristics need to be improved to develop pharmaceutical preparations. This study evaluates whether β-cyclodextrin and a copolymer based on dimethylaminoethyl methacrylate (DMAEMA) interact with thymol in order to control powderization, solubilization, and taste-masking properties. The thymol–β-cyclodextrin complex was prepared by co-precipitation and sealed-heating methods. The DMAEMA copolymer was mixed with the complex using a new approach, instead of spray coating, to decrease thymol volatility. *In vivo* studies were performed. Sealed-heating is a suitable method for including thymol in β-cyclodextrin with a good loading efficiency; thymol volatility control is achieved by mixing the complex with the DMAEMA copolymer. β-Cyclodextrin accelerates the *in vivo* thymol absorption rate compared with the free drug; the thymol half-life is still long. Therefore, a low number of administrations per day are required. Although bioavailability is unchanged with respect to free thymol, high doses could be administered of a selected formulation without compromising the compliance. Furthermore, thymol that is not absorbed is held along the intestine, where it can useful in the treatment and/or prevention of intestinal bacterial diseases.

1. Introduction

Much of the attention devoted to cyclodextrins (CDs) arises from their capacity to alter the physical, chemical, and biological properties of hydrophobic substances by encapsulating molecules of a suitable size inside their annulus to form inclusion complexes (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gándara, 2009). In an aqueous solution, the slightly polar CD cavity is occupied by water molecules and therefore they can be readily substituted by appropriate "guest molecules", which are less polar than water. It is known that in order to form the complexes, water has to migrate from the interior of the cavity and be replaced by the ligand.

The inclusion of CDs exerts a profound effect on the physicochemical properties of guest molecules as they are temporarily locked or caged within the host cavity, giving rise to beneficial modifications of guest molecules, which are not achievable otherwise. These properties include: an enhanced solubility of highly insoluble guests, stabilization of labile guests against the degradative effects of oxidation, visible or ultraviolet light, and heat, control of volatility and sublimation, physical isolation of incompatible compounds, chromatographic separations, taste modification by masking off flavours, unpleasant odours, and the controlled and/or targeted release of drugs and flavours (Del Valle, 2004; Gavini et al., 2011; Loftsson & Duchêne, 2007; Spada, Gavini, Rassu, Cossu, & Giunchedi, 2012; Spada et al., 2013).

Recently, research has been conducted on the preparation and characterization of the inclusion of volatile complexes with CDs for different applications ranging from medicine, cosmetics, food, household cleaning products, and so on (Cabral Marques, 2010). Ciobanu et al. (2013) proposed the inclusion complex of β-CDs with *Mentha piperita* essential oil (EO) to perform a controlled release of aroma compounds to be used in different fields (food, cosmetics, and medicine); they also described the preparation of novel controlled release systems for the delivery of the EOs used as ambient odours using CDs and β-CD polymers (Ciobanu et al., 2012). Moreover, the efficacy of complexes of CDs and EO as antimicrobial preservative agents has been demonstrated (Hill, Gomes, & Taylor, 2013). The inclusion of β-CDs heightened the possibility of

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http://dx.doi.org/10.1016/j.carbpol.2013.10.084

increasing the solubility and stability of garlic oil and controlling its release rate, as determined by Wang, Cao, Sun, and Wang (2011).

Thymol is a volatile phenolic compound extracted from thyme, which shows a high *in vitro* activity against *Salmonella typhimurium* (Si et al., 2006). In recent years, EOs from herbal plants feed additives have attracted increasing interest as an alternative feeding strategy to replace antibiotic growth promoters (Burt, 2004; Hashemi & Davoodi, 2011). However, the low availability of raw materials, the poor yield of extracts from the raw material, and the high qualitative and quantitative variability of EOs are strong limitations on their widespread use. Natural identical Eos, which are similar to the natural molecules of herbs and are more available and cheaper, are usually employed in order to overcome these problems. Natural identical thymol (T) can be used as antimicrobial agent; nevertheless, it shows a low water solubility and low palatability due to its unpleasant taste and smell.

The aim of the present work is to design and characterize a delivery system containing T that is able to modify the physical, chemical, and pharmacokinetic properties of the encapsulated T by increasing its aqueous solubility, dissolution rate, and bioavailability and masking its disagreeable smell and severe irritating taste. In the first step, a complex of T with β-CD was been prepared: it is known, indeed, that the inclusion of flavours in CDs greatly reduces their volatility, oxidation, and heat-decomposition and increases their solubility, enhancing their drug absorption and bioavailability. In the present work, in order to select the most effective preparation method for T complexation, the β-CD complex was prepared by co-precipitation (Del Valle, 2004; Mulinacci, Melani, Vincieri, Mazzi, & Romani, 1996) and sealed-heating methods (Higashi, Tozuka, Moribe, & Yamamoto, 2010; Maestrelli, Gonzalez-Rodrıguez, Rabasco, & Mura, 2005), and the properties of the complexes obtained were compared. Afterwards, acid-soluble polymers such as Eudragit® E grades were added as support for the CD: the use of pH-dependent polymers offers a different approach to taste masking than the addition of artificial flavours. Eudragit® E is a copolymer based on dimethylaminoethyl methacrylate (DMAEMA) and neutral methacrylic esters and is soluble at a pH less than 5.5. This polymer can prevent the release of the delivered drug in saliva (pH 6.8–7.4) and readily dissolves in gastric fluids (pH 1.0–1.5) (Ishikawa, Watanabe, Utoguchi, & Matsumoto, 1999). Particularly, Eudragit® E PO is a cationic copolymer based on DMAEMA, butyl methacrylate, and methyl methacrylate; in combination with stearic acid and sodium lauryl sulphate, it forms a colloidal aqueous dispersion that can be sprayed on tablets and particles, providing an efficient masking of unpleasant tastes (Eudragit® Application Guidelines, 2009, chap. 7). Alternative methods to spray coating using this polymer are proposed here.

Finally, the selected formulation is orally administered to pigs and the pharmacokinetic study is performed compared with T.

2. Materials and methods

2.1. Materials

Natural identical free thymol (T) and aniline (IS) were obtained from Sigma–Aldrich (Milan, Italy). β-Cyclodextrin (β-CD) (Cavamax W7, molecular weight: 1135 kDa) was purchased from Wacker Chemie AG (Burghausen, Germany). Eudragit® E PO (EPO) was kindly gifted by Evonik Degussa (Darmstad, Germany). The dissolution media at pH 1.2 and pH 6.8 were prepared with hydrochloric acid (Riedel de-Haën, Germany) and trisodium phosphate dodecahydrate R (Sigma–Aldrich Laborchemikalien GmbH, Steinheim, Germany). Ultra-pure water was prepared with the MilliQ R4 system Millipore (Milan, Italy). Dichloromethane was purchased by Riedel-de-Haen (Milan, Italy), methanol Normapur p.a. was purchased by BDH Prolabo (Paris, France), and all the reagents were of analytical grade.

2.2. Preparation of powder formulations

Two different preparative methods, such as sealed-heating and co-precipitation, were used in order to obtain the complex between T and β-CD. Two series of formulations coded as SH and C series were prepared; both were composed of the complex T–β-CD with or without the taste–masking polymer EPO. The molecular ratios between T:β-CD were 1:1 and 1:2. Table 1 summarizes the different batches produced and their compositions.

The attempt to prepare the formulation composed by T and E PO failed because a sticky and adhesive slurry was obtained.

2.2.1. Inclusion complex preparation

Sealed-heating method: Sealed-heating, in which both the guest and the host molecules are heated within an enclosed container, is a useful technique for preparing inclusion complexes. With the sealed-heating method, crystalline inclusion complexes with poorly water-soluble drugs can be obtained without using an organic solvent (Nakai, Yamamoto, Terada, & Watanabe, 1987). Sealed-heated products were obtained by sealing physical mixtures of T and β-CD (1.28 or 2.42 g corresponding to 0.15 g of T) in a 10 ml glass ampoule where the powder was wet with 1 ml (SH1) or 1.5 ml (SH2) of acidic medium (pH 5.0) and then heated at 28 ± 2 °C for 24 h. All products were freeze-dried.

Co-precipitation method: Two solutions (C1 and C2) were prepared by dissolving β-CD into 100 ml (C1) or 150 ml (C2) of MilliQ water at 50 °C. After cooling, the exact amount of T was added and the solution magnetic stirred overnight at room temperature; then, the suspensions that were formed were further cooled for 4 h in ice bath in order to favour the inclusion complex precipitation. The β-CD–T complex was centrifuged (5702R Eppendorf AG, Hamburg, Germany) at 4400 rpm for 10 min in order to separate the solid inclusion complex. The surnatant was also stored and analyzed in order to evaluate the unloaded amount of T. All products were freeze-dried.

2.2.2. Taste-masking formulations

The inclusion complexes SH1 and C1 were used to prepare taste-masking formulations, SH EP and C EP by adding EPO, as follows:

- in the case of SH EP, the complex SH was obtained as described above; a solution of polymer EPO in 2 ml of acetone was mixed with the slurred SH in order to obtain a T:EPO weight ratio of 1:1. The mixture was dried in oven at 28 °C for 24 h and then stored in a sealed vial.
- in the case of C EP, T was added to a solution 18.5 g/l of CD. The solution was stirred overnight at 20 °C and after cooling for 4 h in an ice bath, the precipitation of the inclusion complex occurred. The components of basic taste-masking coating solution (Eudragit® Application Guidelines, 2009, chap. 7) were added to the suspension in order to obtain C EP. The basic coating formulation includes EPO, sodium lauryl sulfate (SLS, 10%, w/w respect to EPO), stearic acid (15%, w/w respect to EPO) and talc (50%, w/w respect to EPO) (Eudragit® Application Guidelines, 2009, chap. 7). The amount of EPO corresponded to a T:EPO 1:1 weight ratio (w/w). In order to avoid excessive T dilution in the powder, talc was not added. SLS, stearic acid, and EPO were added one by one in portions by homogenization at 2200 rpm for 30 min (Ultra Turrax T25 basic velox, IKA-Werke). The final mixture was freeze-dried.

Table 1
Composition of formulations obtained by using two different preparation techniques. Amounts of components are expressed in grams (g).

Formulation	T:β-CD molar ratio	Amount of T	Amount of β-CD	Amount of EPO	Preparation method of T:β-CD complex
SH1	1:1	0.3	2.27	–	Sealed heating
SH2	2:1	0.3	4.54		Sealed heating
SH EP	1:1	0.3	2.27	0.3	Sealed heating
C1	1:1	0.3	2.27	–	Co-precipitation
C2	2:1	0.3	4.54		Co-precipitation
C EP	1:1	0.3	2.27	0.3	Co-precipitation

2.3. In vitro characterization of formulations

2.3.1. T content evaluation

Formulations were submitted to drug content evaluation in order to determine the amount of T effectively loaded during the inclusion complex formation process. An exactly weighed amount of each dried formulation corresponding to 1.2 mg of T, theoretically loaded, was dissolved into 10 ml of water by sonicating at room temperature. The amount of T was determined by capillary electrophoresis by the method that is described below. In the case of C1 and C2, the analysis of the surnatant was also carried out in order to evaluate the potential unloaded amount of T.

The ratio between the effective and theoretical drug content was calculated in order to evaluate the loading efficiency percentage (*LE*(%)).

The effective drug loading (*DL*(%)) and the loading efficiency (*LE*(%)) were calculated by Eqs. (A.1) and (A.2), respectively.

$$DL(\%) = \left(\frac{rT}{tT}\right) \times tDL \quad \text{(A.1)}$$

$$LE(\%) = \left(\frac{DL}{tDL}\right) \times 100 \quad \text{(A.2)}$$

In the above equations, *rT* is the real determined amount (g), *tT* is the amount of T (g) used for the complex preparation, and *tDL* is the theoretic drug loading percentage.

Every batch was analyzed in triplicate in order to evaluate the mean result and the standard deviation (SD).

Afterwards, 1 ml was analyzed in a capillary electrophoresis-diode array detection system (Agilent Technologies, Milan, Italy).

An uncoated fused-silica capillary (50 cm × 50 μm I.D.) was used for the capillary electrophoresis separation. The running buffer consisted of 100 mM sodium phosphate with a pH of 7.4. A separation voltage of 20 kV was applied. Samples were injected hydrodynamically with a pressure of 50 mbar for 20 s. The detection was made at 210 nm. Standard curves show linearity in the range 0.01–1 mg/ml with $r^2 = 0.999$.

2.3.2. T release in the gastrointestinal environments

The release of T from powder formulations under simulated pH conditions in gastric fluid followed by intestinal fluid was investigated according to Eur. Pharm. 7.0 (2011). Tests were performed using a USP dissolution apparatus 1 (Erweka DT70, Erweka GmbH, Heusenstamm, Germany). Experiments were carried out by putting an amount of powder containing 10 mg of T into 300 ml of simulated gastric fluid (hydrochloric acid; pH = 1.2). The rotational speed was set at 100 rpm and the bath temperature at 37 ± 0.5 °C. To simulate the process of formulation moving from the stomach into the intestine, after 2 h the 0.2 M trisodium phosphate dodecahydrate solution (82 ml) was added in the vessel in order to reach pH 6.8 and the test was continued for an additional 4 h. At appropriate intervals, 1 ml of sample was taken and a fresh medium was added to maintain a constant volume and sink conditions. Then, the collected samples were analyzed by capillary electrophoresis and the amount of T released was calculated by referring to the calibration curves prepared. The drug dissolution rate was determined as a comparison.

All experiments were performed in triplicate and the results were registered as average ± SD.

2.3.3. GC-HS–MS analysis

With the aim of determining the taste-masking efficacy of the formulations, the capability of powders to avoid T volatility was evaluated by GC-HS–MS analysis.

One hundred milligrams of each formulation, containing 10 mg of thymol, were directed weighed into a 2 ml-headspace vial ($n = 5$); for comparison, 10 mg of free thymol was directed weighed into a 2 ml-headspace vial ($n = 5$). The vials were sealed air tight with a silicone/polytetrafluoroethylene septum. Samples were heated for 60 min at 30 °C, while being mixed continuously. An aliquot of 200 μl was injected directly into the GC–MS apparatus with a split ratio of 10:1. The GC–MS conditions are reported in Section 2.4.3. The amount of thymol released in the head space from three formulations analyzed was compared with that released from the free thymol.

2.4. In vivo pharmacokinetics studies

Six pigs, weaned at an age of 24 days (median weight = 11 kg), were taken from a commercial piggery. The piglets were moved to an experimental farm, housed in pens with a mesh floor, and kept at a controlled temperature (28 °C at the beginning and 25 °C at the end of the experiment). The procedures carried out on the pigs were conducted in compliance with Italian laws on experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna.

2.4.1. Experimental design and diet

On the basis of the results obtained from the *in vitro* characterization, SH EP was selected to be *in vivo* tested.

Six piglets were balanced for litter and weight. The feed was prepared by carefully mixing formulation with the other feed ingredients of the standard balanced diet. The correct feed intake was calculated and the exact amount of formulation and, thus, of T administered, was determined. The actual administered dose was 0.03 g/kg. As comparison, one group received the same diet with 1% T directly in the stomach by a nasogastric tube. The administered dose was 0.05 g/kg. The piglets were cannulated to collected blood samples for pharmacokinetic studies. The timing of sampling ranged between 0.5 and 24 h. The blood samples were centrifuged to obtain plasma ad stored at −20 °C until analysis. Faecal samples were collected at 12 and 24 h. After 50 h, the pigs were anaesthetized with sodium thiopental (10 mg/kg body weight, Zoletil 100, Virbac, Milano, Italy) and euthanized by an intracardiac injection of Tanax® (0.5 ml/kg BW; Intervet Italia, Peschiera Borromeo, Italy); samples of kidney, liver, lung and muscle were collected to determine the free thymol concentration.

2.4.2. Extraction procedure from plasma, tissues, and faeces

One millilitre of plasma was deproteinized with 2 ml of acetonitrile; the suspension obtained was mixed and centrifuged at

3000 rpm for 10 min and the supernatant was then transferred into vials. One gram of tissues and faeces was extracted with methanol (2 ml), stirred in a US-apparatus for 30 min, and centrifuged at 3000 rpm (1000 × *g*) for 10 min. The supernatant was filtered with syringe filters 0.45 μm pore size and transferred into vials.

2.4.3. GC–MS conditions

GC–MS analyses were performed using an Agilent 6850 Series gas chromatograph (Agilent Technologies, Heilbronn, Germany) coupled to an Agilent 5973N Series mass spectrometer (Agilent Technologies, Heilbronn, Germany). Chromatographic separation was performed on capillary column (30 m × 0.25 mm I.D., 0.15 μm film thickness, HP-5, Agilent Technologies, Palo Alto, California, USA). Helium (purity 99.999%) was used as a carrier gas at a constant pressure of 10 psi. The temperature of the column was held at 70 °C for 5 min, and raised to 180 °C (20 °C/min).

The temperatures of the injector, detector, and ion source were set at 100, 280, and 200 °C, respectively. The electron impact was 70 eV and the flow rate of the carrier gas (helium) was 18.3 ml/min. The mass spectrometer was operated in the Selected Ion Monitoring (SIM) mode in order to quantify with increased sensitivity. The main fragments selected were 91, 135, and 150 *m*/*z* for T (t_R = 8.00 min) and 52, 66, and 93 for anyline (internal standard; t_R = 3.40 min). The quantification was carried out by triple injection.

2.5. Statistical analysis

Statistical analysis was performed with GraphPad Prism 4.0 for Windows. An unpaired *t*-test was applied to test the significance of the effect of T:β-CD molar ratio and preparation method on characteristics of formulation, as well as the reduction in T volatility. An analysis of the dissolution data was performed using the One-way ANOVA test following which individual differences between treatments were identified using a non-parametric *post hoc* test (Tukey's). The significant level was set at $P < 0.05$. Pharmacokinetic analysis was performed with PK Solutions software (version 2.0, Summit Research Services, Montrose, CO, USA).

3. Results

3.1. Preparation of powder formulations

The literature reveals different techniques for the preparation of the inclusion complex between T and β-CD, such as supercritical carbon dioxide (Locci, Lai, Piras, Marongiu, & Lai, 2004) and co-precipitation methods (Mourtzinos, Kalogeropoulos, Papadakis, Konstantinou, & Karathanos, 2008; Ponce Cevallos, Buera, & Elizalde, 2010). We set the parameters to obtain the inclusion complex of T and β-CD by the sealed-heating method, for the first time. The powders were characterized in terms of the loading efficiency and the T release and compared with formulations obtained by the co-precipitation method.

The results show that the amount of T loaded in the formulation depends on the preparation method used and the composition of the formulation: SH1 and SH2 show real values of LE% consistent with the theoretical ones, indicating that sealed-heating is a suitable technique for loading of T in β-CD. There are no statistical differences between SH1 and SH2, meaning that the molar ratio does not influence the T loading when the sealed-heating method is used ($P > 0.05$).

Differential scanning calorimetry (DSC) was used to determine the complex formation (data not shown); measurements were performed by the Mettler STARe system (Mettler Toledo, Novate Milanese, Italy) in the temperature range −10 to 300 °C curves of powders are compared with the spectra of the single substances (T or β-CD). The β-CD thermogram shows water loss as a broad endothermic event with a peak at 140 °C, indicating heat sorption likely caused by water evaporation. The thermogram corresponding to T shows an endothermic peak at nearly 50 °C corresponding to its melting point. The curve corresponding to the T–β-CD complex does not show any sharp endothermic peak in the range of the melting point of the pure compound. The fact that T does not melt is due to its encapsulation in the host β-CD; this is in agreement with thermograms from Ponce Cevallos et al. (2010) and Mourtzinos et al. (2008). After the CD complexes are formed, the total or partial disappearance of thermal events (melting point) corresponding to guest molecules is generally taken as a proof of complex formation (Pralhad & Rajendrekumar, 2004; Williams Robert, Mahaguna, & Sriwongjanya, 1998).

The addition of taste-masking polymer does not affect the loading efficacy of β-CD ($P > 0.05$).

On the contrary, analyses of the formulations C1 and C2 show that T is distributed between the solid precipitated after centrifugation (42% and 77%, respectively) and surnatant (58% and 23%, respectively); as reported by several authors, this could be due to the equilibrium between the insoluble and soluble complex, where the latter is in equilibrium with its components (Brewster & Loftsson, 2007; Cabral Marques, 2010; Del Valle, 2004). The molar ratio between T and CD affects the amount of complex formed: in fact, by increasing the amount of β-CD, the loading of T increases as higher LE values of the precipitate are obtained ($P < 0.05$).

The addiction procedure of EPO to SH1 and C1 does not cause a loss of T from the formulations ($P > 0.05$).

Table 2
Reduction of T volatility.

Formulation	Reduction of T volatility (% ±SD)
SH1	11.5 ± 4.9
C1	10.9 ± 3.4
SH EP	96.6 ± 0.5
C EP	95.0 ± 0.7

3.2. Thymol release in the gastrointestinal environments

Results from the *in vitro* release test are illustrated in Fig. 1. The formulations show similar T dissolution profiles ($P > 0.05$): between 60% and 80% of T is released in the first 15 min in the acidic medium and remains stable, while the remainder is released within 30 min of the change in pH. The presence of EPO in formulations causes significant differences in the T release profile of SH EP and C EP ($P < 0.05$) in the first 60 min at an acidic pH and the formulation produced by sealed-heating is able to release T amounts higher than the formulation obtained by the co-precipitation method. Nevertheless, they show profiles that are consistent with the correspondent formulations SH1 and C1 ($P > 0.05$).

All formulations are able to increase the release rate of T in the simulated gastrointestinal (GI) environment in the first 30 min regardless of the pH; data that are not reported show that only 35% of free T amount tested is released at 0.1 M HCl after 30 min and 70% after 120 min, whereas about 90% after 360 min is found at a pH of 6.8. Again, formulations obtained by sealed-heating provided better results.

3.3. Evaluation of T volatility in formulations

The capability of powders to control T volatility, evaluated by GC-HS–MS analysis, is shown in Table 2. Inclusion of the complex T:β-CD slightly decreases the volatility of T amounting to about 11%, regardless of the preparative method ($P > 0.05$). On the contrary, the addition of EPO to SH and C leads to significant control of

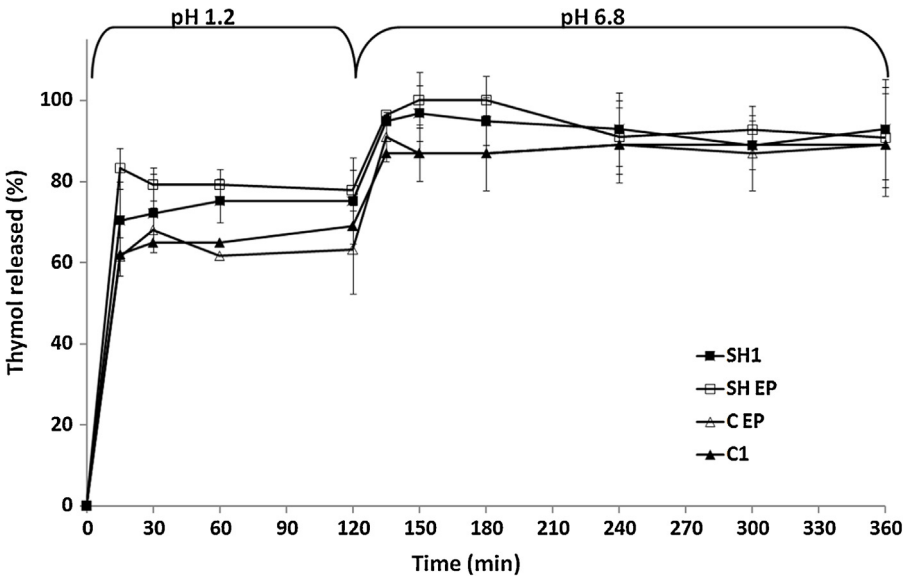

Fig. 1. *In vitro* drug release kinetics in the GI-simulated fluid. T dissolution profiles from SH1 (■), SH EP (□), C1 (▲), and C EP (△) at a pH of 1.2 and a pH of 6.8 ($n = 3 \pm SD$).

Table 3
Pharmacokinetic Parameters with standard deviations (±SD).

	$t1/2$ (h)	T_{max} (h)	C_{max} (μg/ml)	AUC_{0-24} (μg h/ml)	$AUMC_{0-24}$ (μg h/ml)	MRT[a] (h)	CL (area)[b] (ml/h)	$t1/2$ elimination phase[c] (h)	$t1/2$ absorption phase[d] (h)
T	51.9 ± 3.0	1.3 ± 0.5	3.6 ± 0.4	26.4 ± 3.1	216 ± 11.0	64.5 ± 9.1	715.8 ± 40.3	51.87 ± 4.6	0.9 ± 1.0
SH EP	22.3 ± 6.3	0.5 ± 0.0	3.8 ± 0.5	27.0 ± 5.9	195.0 ± 21.9	23.8 ± 11.5	733.6 ± 86.4	22.0 ± 6.2	0.3 ± 1.1

[a] *MRT*: Mean Residence Time (time for 63.2% of administered dose to be eliminated).
[b] CL: Clearance. Systemic clearance based on observed data points: $AUC_{(0-24)}$.
[c] Elimination phase (terminal phase regression analysis, 13–24 h).
[d] Absorption phase (first residual regression analysis, 0.5–6 h).

the T volatility over 95% ($P < 0.05$). There are no significant differences between the SH EP and C EP behaviours ($P > 0.05$).

3.4. *In vivo* pharmacokinetic study

The pharmacokinetic profile of the drug after *in vivo* oral administration of the formulation SH EP, chosen on the basis of results obtained from *in vitro* characterization, is evaluated and compared with that of free T. The thymol plasma concentration profile *versus* time is shown in Fig. 2; the pharmacokinetic parameters related to these curves were calculated and are listed in Table 3.

The blood concentration profiles of free T and T loaded into SH EP are nearly consistent; analysis the pharmacokinetic parameters, however, shows prominent differences. Results from pharmacokinetic analysis of SH EP, indeed, show that a single dose of T, orally administrated, is rapidly absorbed but slowly eliminated within 24 h. The time needed to reach maximum concentration (T_{max}) in fact, is very short (30 min) and the half-life of absorption phase, $t1/2$ value, is very low (about 0.3 h). Nevertheless, an extensive $t1/2$ elimination phase is found (22 h). As a consequence, *T* shows a very long half-life of about 22 h. This is attested also by the values of Mean Residence Time (MRT) and Clearance listed in Table 2. The maximum concentration achieved in the blood (C_{max}) is 3.8 μg/ml and Area Under Curve (AUC_{0-24}) is 27 μg h/ml, which are not significantly different from the correspondent values of free T ($P > 0.05$).

Nevertheless, the formulation seems to increase the rates of absorption and elimination processes compared with free T: after the administration of free T, indeed, drug absorption is slower (T_{max} and $t1/2$ absorption = 1.3 h and 0.91 h, respectively, compared with 0.5 h and 0.3 h for SH EP; $P < 0.05$). Moreover, the elimination rate is increased and the $t1/2$ value changes from 52 h (in the case of free T) to 22 h (in the case of formulation). This behaviour leads to a notable decrease in the half-life of T absorbed from SH EP (22 h), compared with T, whose half-life is almost two times shorter than that of the formulation (52 h) ($P < 0.05$). These data are confirmed by the MTR values, which are higher in the case of T, meaning that free T elimination slowly occurs. On the other hand, no difference is detected in the AUC values and thus bioavailability remains unchanged.

Table 4 illustrates the amount of T recovered in organic matrices: the T concentrations found in organs such as the liver, lungs, kidneys, and muscles are very low. On the other hand, a high T concentration is found in the analyzed fractions of intestines, both on mucosa and content, indicating that T is not completely absorbed and that it is distributed along the intestinal tract and on the intestinal mucosa. In particular, the GI transit is influenced by the formulation: when free T is administered, a low amount is found in the jejunum while the highest concentration is recovered in the colon content. Moreover, higher T amounts are found in faeces after 12 h than after 24 h, indicating that a more rapid GI transit occurs.

Table 4
T concentration recovered in organic matrices analyzed (ng/g ± SD, $n = 6$).

Matrix analyzed	Free T	T from SH EP
Liver	37.85 ± 1.26	39.40 ± 0.17
Kidney	57.59 ± 0.94	41.20 ± 0.09
Lung	–	–
Muscle	–	–
Jejunum mucosa	Traces	22.74 ± 0.57
Jejunum content	68.91 ± 0.54	333.46 ± 1.34
Colon mucosa	36.36 ± 1.51	23.85 ± 0.19
Colon content	570.00 ± 2.44	158.29 ± 0.83
Faeces 12 h	779.95 ± 2.48	115.82 ± 2.66
Faeces 24 h	92.18 ± 1.02	121.18 ± 2.11

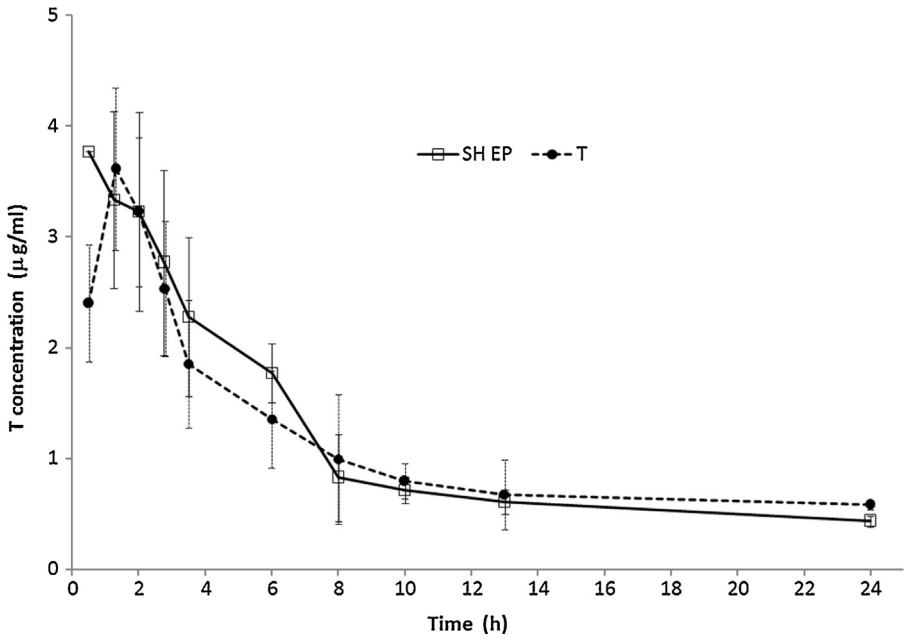

Fig. 2. Blood thymol (T) concentration (μg/ml) after oral administration of drug free (0.05 g/kg) and formulation SH EP (0.03 g/kg). Data are expressed as mean values (±SD, $n = 6$).

On the contrary, when SH EP is administered, a prolonged GI transit takes place, as demonstrated by the high jejunum and colon content and also by the presence of T in faeces after 24 h (121.18 ng/g; Table 4).

4. Discussion

The application of the sealed-heating method for loading T into β-CD is proposed here for the first time. It is suitable to include this volatile substance with a very good loading efficiency. This technique is simple and it requires a manufacturing time that is as long as that of the co-precipitation method frequently used (Mourtzinos et al., 2008; Ponce Cevallos et al., 2010). The inclusion complex prepared by sealed-heating, using a 1:1 molar ratio between two components (SH1), is able to increase the dissolution rate of T, which dissolves slowly in GI-simulated fluid (data not shown); this effect is due to the well-known effect of β-CD acting as a solubilizer of substances that are poorly water soluble (Brewster & Loftsson, 2007). All formulations show this behaviour, regardless of the preparation method used.

The addition of the taste-masking EPO (SH EP and C EP) does not cause a loss of T from formulations whereas significant differences in the T release behaviour are determined in acidic media. Nevertheless, the presence of β-CD is absolutely necessary to prepare formulations containing EPO, which is, however, important as an agent because it is able to decrease T volatility, as demonstrated by the volatility test. This result demonstrates the capability of formulations containing EPO to mask the unpleasant smell and taste of T and thus enhance the palatability of the powders.

Regarding the use of EPO, it is known that it is applied as coating agent. Here, the addition of the polymer was proposed, with success, directly into the T:β-CD complex. In particular, the preparation of SH EP is more favourable as it is simpler, faster, and cheaper than that of C EP; in addition, we found that preparing large amounts of powders for *in vivo* experiments was easy.

The data reported in the literature show that the *in vivo* absorption of T occurs in the stomach and depends on the solubilization of EOs. The amounts that were recovered in the small intestine represented only a minor fraction of the ingested quantity of T and were probably the parts that escaped solubilization and absorption in the stomach due to adsorption by feed ingredients (Michiels et al., 2008).

In vivo data confirm that T is absorbed mainly into the stomach; it shows low plasma concentrations compared with the dose administered and thus a high distribution volume. This result indicates that T is inclined to distribute into the body. The clearance value is low and therefore it is also slowly eliminated. The half-life, indeed, is long. As a consequence, a reduced number of administrations should be required. However, the amount absorbed is very low as the AUC value demonstrates. Therefore, high doses are necessary. This could be a problem due to the unpleasant smell and taste of T.

On the contrary, a rapid absorption of T occurs when it is administered by the formulation SH EP. The T_{max} value is 30 min, whereas T_{max} of free T, administered to pigs, is 1.3 h. The improvement in the absorption rate of T is due to β-CD; it is known, indeed, that CDs interact with compounds that are poorly water soluble to increase their apparent solubility. Increasing the apparent solubility of a drug can increase the drug dissolution rate. For compounds whose oral bioavailability is limited by solubility or dissolution rate, increasing the apparent solubility can also increase the oral bioavailability (Brewster & Loftsson, 2007). Moreover, many papers testify to the absorption penetration enhancement effect of CDs by different administration routes (Al-Hilal, Alamb, & Byunc, 2013; Spada et al., 2013). Nevertheless, T is slowly eliminated, as the $t1/2$ elimination value demonstrates. The half-life, even if it is almost halved, is still remarkably long and the number of administrations *per day* is still low. The C_{max} and AUC_{0-24} values, the areas under the curve from time 0 to 24 h, are very similar to that of free T, indicating, thus, that the formulation does not modify the bioavailability of T. Such a long half-life could be due to an accumulation of T in the body; but as the amount of T recovered in organic matrices is very low, it could also be due to a high percentage of bonding with plasma proteins.

A high amount of T is found in the analyzed fractions of the intestine, both on mucosa and content, indicating that SH EP prolongs the GI transit time of T and T not completely absorbed; it is distributed along the intestinal tract and on the intestinal mucosa,

particularly in the colon. Data regarding the amount of T found in faeces after 12 and 24 h confirm this hypothesis: when free T is administered, indeed, high residues in faeces were found after 12 h but when T is administered formulated in SH EP, higher amounts of T are detected after 24 h. Very small amounts (1–2%) of β-CD are absorbed in the upper intestinal tract after oral administration; no metabolism in the upper intestinal tract occurs but it is metabolized by bacteria in the caecum and colon (Del Valle, 2004), where most of the T residuals from the SH EP formulation are recovered and where they could exert a topical therapeutic effect. Moreover, as Janczyk, Trevisi, Souffrant, and Bosi (2008) found that supplementing the pigs' diet with high doses of thymol caused clear changes in jejunum microbacterial community, the SH EP formulation, due to the presence of β-CD, could protect jejunum flora.

5. Conclusions

This work demonstrates that the unpleasant organoleptic properties of T can be masked by including T in a formulation based on β-CD and containing EPO; this formulation can be added to feed ingredients of a standard diet.

In vivo results demonstrate that formulated T is rapidly absorbed, due to the presence of CD, and slowly eliminated. Accordingly, it maintains a long half-life (even if halved, compared with free T), which could allow for a very low number of daily administrations. Although its bioavailability is not improved with respect to free T, high doses could be administered of a selected formulation SH EP without compromising its compliance. Furthermore, T that is not absorbed T after oral administration is distributed along the intestine; particularly, it is detained on the surface of the intestinal mucosa and thus can exert a topical effect on the intestinal tissue, useful in the treatment and prevention of intestinal bacterial diseases.

Acknowledgements

This work was financially supported by "Regione Autonoma della Sardegna-Progetto di Ricerca CRP1_404 (Legge Regionale 7 agosto 2007, n. 7)". The authors are also grateful to Mrs. Paola Manconi for technical support. The authors would like to thank Dr. Milena Sorrenti for assisting in the DSC analysis.

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