

INCLUSION COMPLEX OF HYDROCHLOROTHIAZIDE- γ - CYCLODEXTRIN: THE EFFECT ON AQUEOUS SOLUBILITY, DISSOLUTION RATE, BIOAVAILABILITY AND THE EFFECT ON INTESTINAL PERMEABILITY USING USSING CHAMBER TECHNIQUE

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ABSTRACT

Objective: In this work the effect of γ -cyclodextrin (γ CD) on the aqueous solubility, dissolution rate, bioavailability and intestinal permeability using the Ussing chamber technique of the hydrochlorothiazide was investigated.

Materials and methods: A solid inclusion complex between HCT and γ CD was prepared by co-precipitation method and characterized by X-ray diffraction (XRD) and nuclear magnetic resonance (NMR). A randomized crossover trial was conducted on five dogs to study bioavailability. The effect of γ CD on the permeability of HCT was investigated using the Ussing chamber technique.

Results: Both the solubility and the dissolution rate of HCT- γ CD were significantly increased by its inclusion with γ CD. The in-vivo study showed significant improvement of the oral bioavailability of HCT. Compared to the control, the presence of the inclusion complex increases the area under the plasma concentration-time curve (1268.2 ± 102.64 to 2746.27 ± 164.76 , $p < 0.05$) and the maximum plasma concentration (192.85 ± 63.11 ng/ml to 353.22 ± 33.84 ng/ml, $p < 0.05$). The permeability rate of HCT was increased significantly by the formation of inclusion complex compared to the control HCT alone ($(1.96 \pm 0.27) \times 10^{-6}$ to $(3.93 \pm 0.81) \times 10^{-6}$ cm/s, $p < 0.05$).

Conclusion: The present results suggest that γ CD improves the solubility in water, the dissolution rate, bioavailability and permeability of HCT.

Keywords: Hydrochlorothiazide, γ - cyclodextrin, Inclusion complex, Bioavailability, Ussing chamber technique.

INTRODUCTION

Hypertension is the major cause of cerebral vascular accidents, hypertensive heart failure and progressive renal failure. In developed countries, heart disease and stroke are the first and the third-ranked causes, respectively, of morbidity and mortality [1]. For example, in Morocco the total prevalence of hypertension is 33.6% [2]. Diuretics and β -blocker drugs are the only two classes of antihypertensive drugs that have been tested and shown to reduce morbidity and mortality. The first line treatment recommended of hypertension is thiazide diuretics, in particular hydrochlorothiazide (HCT) (Figure 1), that has remained the most widely used for years [3].

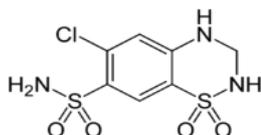


Fig. 1: Molecular structure of Hydrochlorothiazide

HCT belongs to class 4 of the Biopharmaceutics Classification System (BCS), where drugs have low solubility and low permeability [4]. These properties limit their bioavailability in an organism. In order to overcome this problem, HCT was formulated as liquisolid tablets and tested to evaluate the absorption characteristics, using six male beagle dogs [5]. On the one hand, most methods have been tested to improve HCT's aqueous solubility and bioavailability, such as the use of polymers like polyethylene glycol [6], polyvinylpyrrolidone [7], hydroxypropyl cellulose [8] and sequenced copolymers [9]. On the other hand, the complexation with beta-cyclodextrin was also studied [10,11].

Cyclodextrins (CDs) are a cyclic oligosaccharides, consisting of six α -cyclodextrin, seven β -cyclodextrin, eight γ -cyclodextrin or more glucopyranose units linked by α -(1,4) bonds [12-14]. These cyclodextrins are hydrophilic molecules outside and hydrophobic inside which allows forming inclusion complexes with the majority of hydrophobic molecules [15]. These inclusion complexes have

been shown to improve stability, solubility, bioavailability [12, 16, 17] and also known to enhance the absorption of molecules across biological barriers [18].

To our knowledge, there are no studies of the preparation of HCT- γ CD inclusion and its in-vitro, its in vivo and its intestinal permeability using an Ussing chamber technique investigation. Therefore, the aim of this work was, in the first step, to investigate the aqueous solubility and the bioavailability of HCT after its inclusion with γ CD. The former was performed by establishing kinetic dissolution profiles, while the latter was evaluated by means of a crossover trial on dogs for the complexed and uncomplexed HCT. In the second step, the intestinal permeability of both HCT and its inclusion complex with γ CD was evaluated using the Ussing chamber technique.

MATERIALS AND METHODS

Materials

Hydrochlorothiazide (Cambrex, Batch350103. Italy), Ethanol (Merck, K39139083, Germany), acetonitrile (VWR, For HPLC, Batch 1120918), potassium dihydrogen phosphate (Solvachim, Batch 060999, Morocco). All organic solvents used are products for analysis.

Animals

Five male dogs weighing between 11 and 16 Kg (14.3 ± 2.7) were used in this study. During the test, the animals were kept in individual cages and were fed and watered ad libitum. The dogs were starved for 18 hours prior to the experiment. All experiments made on the animals have been approved by the ethic committee of the Faculty of Medicine and Pharmacy (Morocco).

Preparation of the solid complexes

Inclusion complex (IC): The complex of HCT with γ CD was prepared by the co-precipitation method by dissolving γ CD (109 mg) and HCT (477mg) (i.e. molar ratio 1:1) in water - ethanol (50:50, v/v). The

mixture was kept stirred for 72 hours at 40°C. Then, the suspension was filtered through a 0.45 µm Millipore filter and evaporated at 40°C until dry.

Physical mixture (PM): The physical mixture was obtained by mixing HCT with γ CD in the same ratio 1:1 in mortar with continuous stirring for 10 min.

Characterization of inclusion complexes

X-ray Diffraction

The diffractograms of X-rays of HCT, γ CD and complex samples in powder were recorded between 0 and 60° (2 θ) at room temperature (20°C \pm 2°C) with a step size of 0,11° /min using a Bruker D8 X-ray diffractometer (Karlsruhe, Allemagne) equipped with a high-speed linear detector LYNXEYE and a copper anticathode ($\lambda = 1.5406\text{\AA}$)

Nuclear Magnetic Resonance

The ¹H-NMR spectra of the samples were recorded at 25°C using the NMR spectrometer (AVANCE 300 Bruker) employing DMSO-d₆ as a solvent. The chemical shifts observed for the protons H₃ and H₅, which are located inside the cyclodextrin cavity, were ascertained and calculated according to the following formula:

$$\Delta\delta = \delta_{\text{complexed state}} - \delta_{\text{free state}}$$

Phase solubility studies

Solubility studies were performed according to the method reported by Higuchi and Connors [19]. Excess amounts of HCT (10 mg) were added to 10 ml of aqueous solutions containing various concentrations of γ CD (0-23 mM). These solutions were agitated during 72h at 25 °C. The suspensions were filtered through a 0.45 µm Millipore membrane filter. The filtrate was appropriately diluted by NaOH (0.01N) before analysis. HCT in the filtrate was determined by the UV spectrophotometric method discussed below.

The apparent constant of stability K_s according to the hypothesis of 1:1 stoichiometric ratio of complex was calculated from the phase-solubility curves using the following equation:

$$K_s = \frac{\text{Slope}}{S_0 (1 - \text{slope})}$$

The slope is obtained from the initial straight-line portion of the plot of HCT concentration against HCT- γ CD concentration, and S₀ is the solubility of HCT in an aqueous medium in the absence of the ligand.

In vitro study

Dissolution kinetic profiles were performed in 0.1N HCl medium at 37°C by the paddle method at a rotation speed of 100 rpm using a six-vessel dissolution apparatus USP XXIV. Empty capsules were filled with 25mg of HCT, one with HCT as active ingredient only and the second with HCT- γ CD inclusion complex that were added to the dissolution medium of 900 ml. 10 ml aliquots were withdrawn at 10, 20, 40, 50 and 60 min and replaced by an equal volume of the same dissolution medium. Samples were filtered through a 0.45µm membranes and their HCT concentration was assayed by the spectrophotometric method discussed below.

In vivo study

The study design performed to study the bioavailability was a single 25 mg dose, two-treatments, two periods, two sequences cross-over study with a week washout period between phase I and phase II. The drugs (25 mg) were mixed with minced meat and administered orally to each dog on two occasions. Once as HCT alone and in the second occasion as HCT- γ CD. Multiple Blood samples (1 ml) were collected in evacuated glass tubes (heparinized vacuataines, Becton and Dickinson, CA, USA) before and after administration of HCT and HCT- γ CD at 0h, 1h, 2h, 3h, 4h, 6h, 8h and 24h. The serum portion was separated by centrifugation at 3000 rpm/min for 10 min and stored frozen at -20°C until analysis by the HPLC method discussed below.

Ex-vivo-bidirectional transport study

Jejunum segments (1cm) from male Wistar rats (250–300 g) were excised and mounted on an Ussing chamber system [20]. Each chamber compartment was filled with 4ml of Krebs-Ringer buffer solution (KRB, pH 7.4, NaCl 111.9, KCl 5.0, MgCl₂ 1.2, NaHCO₃ 25.0, NaH₂ PO₄ 0.4, Na₂ HPO₄ 1.6, glucose 10mM) that was continuously bubbled with carbogen O₂/CO₂ (95%/5%). A 15-min equilibration period was performed to achieve steady-state electrophysiologic conditions. After the equilibration period, 50ng/ml of HCT was introduced to the mucosal fluid (donor compartment). Aliquots of 400µl were taken from the serosal fluid (receiver compartment) every 30 minutes for 2h, with the equivalent fresh KR buffer was added each time. Concentrations of HCT were quantified by the HPLC-UV discussed below.

The apparent permeability (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

Where dQ/dt is the flux of HCT from the mucosal to the serosal side, C₀ is the initial concentration of HCT in the donor compartment and A is the area of the membrane (1 cm²). All experiments were assessed during sink conditions and conducted in triplicates.

Analytical methods validation

In-vitro analysis by spectrophotometry

The standard solution is prepared by dissolving HCT in 0.1N NaOH. Then dilutions were prepared to obtain five levels of concentrations of 0.006 to 0.014 mg/ml. Three repetitions were prepared for each concentration level. The whole preparation step was prepared for three days. These solutions were used as calibration standards. Each solution was analyzed three times. The method was validated according to ICH Q2R1 guidelines [21].

The calibration curve is linear over the concentration range of 6–14 µg/ml with R²=0.998. The precision intra-day (repeatability) and inter-day (intermediate precision) for HCT were satisfactory with CV% values less than 2%. The mean percent recovery falls inside the interval confidence [98%, 102%], which demonstrates the accuracy of the method. The limit of quantification and detection was 0.26 and 8.56 10⁻³ µg/ml respectively.

Bioanalysis by HPLC

The plasma concentrations of HCT were determined by a high liquid chromatographic method. The HCT was separated on a C₁₈ column (150mm \times 4,6mm i.d. \times 5µm particle size, Beckman, Derwood, Maryland, USA) equipped with a pre-column C₁₈ (4 \times 3mm, phenomenex, Torrance, CA, USA). The mobile phase consisted of a mixture of acetonitrile and phosphate buffer (containing 10mM of potassium dihydrogen phosphate, pH adjusted to 3 with orthophosphoric acid) in the ratio (88%,12%). The LC system was operating isocratically at a flow rate of 1 ml/min. The detection was carried out at 273 nm.

A Dionex 680 series HPLC system (Dionex Corporation, Sunnyvale, CA, USA) was used in this study. More precisely, the chromatographic system consisted of a quaternary P680 pump, an Auto sampler ASI-100, a TCC100 column oven and 340U Dionex UV-Detector.

Plasma aliquots of 900µl were mixed with 100µL of hydrochlorothiazide (10µg / ml) and 100µl of Chlorothiazide as an internal standard. The sample was extracted with 3ml of diethyl ether-ethyl acetate (50:50, v/v) by mechanical agitation. After centrifugation at 3000 rpm, the upper organic phase was evaporated at 60°C under a current of nitrogen. The residue was reconstituted with 400µL of mobile phase and injected into the HPLC system.

The method was validated over a concentration range of 75-5000 ng/ml. The calibration curve is linear in the whole range with a coefficient of correlation more than 0,998. The precisions intra and inter-day for HCT were satisfactory with values between 4.85 and 6.86%. The percent recovery of 91 \pm 5% demonstrates the accuracy

of the method. The detection and quantification limits were 9 and 18 ng/ml respectively.

Transport study analysis by HPLC

The HCT concentration in a Krebs ringer buffer was analyzed by high performance liquid chromatography. The chromatographic separation was achieved by a C₁₈ column (150mm ×4,6mm i.d.×5µm particle size, Beckman, Derwood, Maryland, USA) at a flow rate of 1ml/ml. The mobile phase consisted of a mixture of acetonitrile and a phosphate buffer (containing 10 mM of potassium dihydrogen phosphate, pH adjusted to 3 with orthophosphoric acid in the ratio (90 %,10 %)). The detection was set at 273 nm.

The method was validated over a concentration range of 0.15–2.25 µg/ml. the calibration curve is linear in fixed range with a coefficient of correlation more than 0.998. The precisions intra and inter-day for HCT were lower than 2%. The value of the percent recovery is within the confidence interval (97.28-102.43 %), which demonstrates the accuracy of the method.

Statistical analysis

All analyses of data were performed with a statistical software package (SPSS 13, USA). The results are expressed as means and standard deviations. Comparative statistical studies on the bioavailability between HCT and HCT-CD inclusion complex were performed by ANOVA and the non-parametric Wilcoxon test. The differences between two related parameters were considered statistically significant for p value < 0.05.

RESULTS

Phase Solubility studies

The profile of the phase solubility of HCT is shown in Figure 2. The solubility of HCT increases as a function of cyclodextrin concentration, showing a typical A_L type profile [19]. The determination coefficient (r^2) for the solubility curve can be calculated to distinguish between A_P and A_L types. The γ CD system gave r^2 value of 0.0996 > 0.0990, then the profile can be regarded as A_L type. The stability constant K_s of HCT- γ CD inclusion complex is 284.27 mM. At a concentration of 25mM of γ CD the solubility of HCT was increased from 0,39 to 2,5mM/l, which represents an increase of the solubility almost 6 times more than HCT in water.

Characterization of the inclusion complex

XRD analysis

The X-ray diffract gram of HCT showed in Figure 3a. presents different crystalline peaks at a diffraction angle of 16.8°, 18.8°, 19.1°, 29° and 36°(2 θ), suggested that the drug was present as a crystalline material. The diffraction profile of physical mixture (Figure 3c) was found to be the simple superposition of each component (HCT and γ CD); this justifies the difficulty of these two products to react in powder form. Whereas the diffractogram of the inclusion complex (Figure 3d) showed the disappearance of the diffraction peaks of HCT which confirms that an inclusion complex between HCT and γ CD was formed by the co-precipitation method.

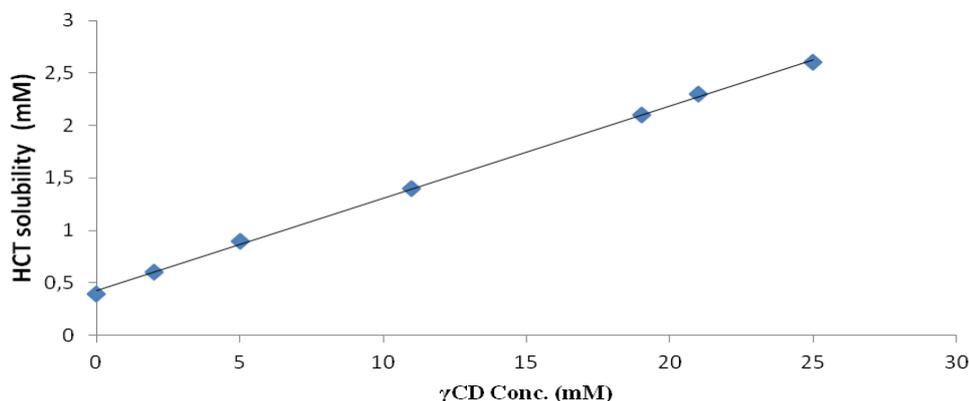


Fig. 2: HCT phase solubility diagram in the presence of γ -CD.

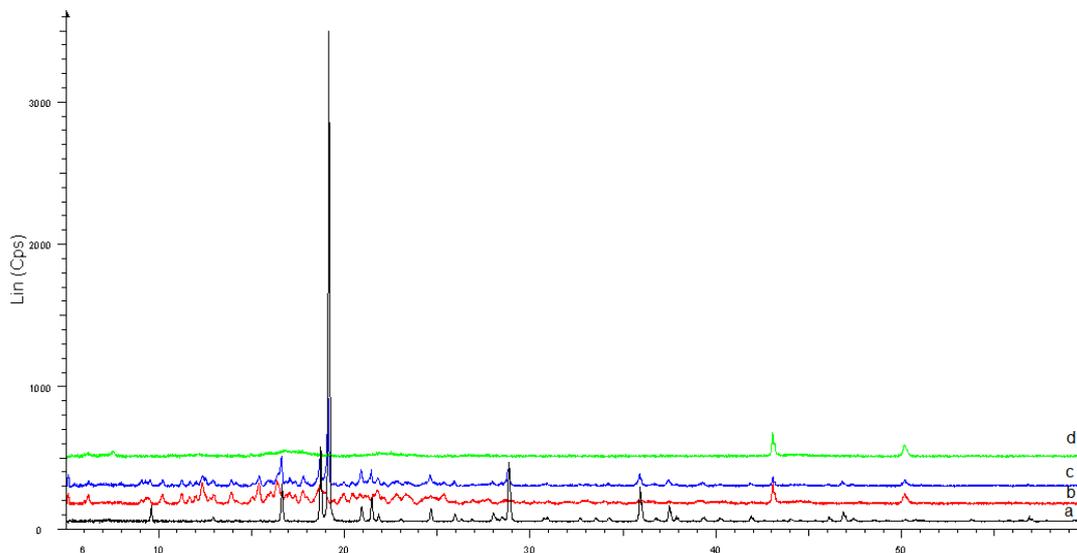


Fig. 3: RX diffractograms of (a)HCT, (b) γ CD, (c)physical mixture,(d) inclusion complex (IC)

NMR Analysis

Proton NMR allows to identify a molecule from the chemical displacement of its protons. Any modification of the nucleus environment, such as the approach of another molecule, will cause changes to its chemical displacement. This is particularly interesting for cyclodextrins for which it is possible to identify changes in the chemical displacement of protons located inside the cavity (H_3 , H_5). When there is inclusion, they are the most affected, while the protons located outside were little affected [22].

The H^1 -NMR spectra of γ CD, HCT- γ CD physical mixture and inclusion complex were presented in Figure 4, Figure 5 and Figure 6 respectively. The chemical shifts (ppm) for the protons of the free γ CD and HCT- γ CD complex are reported in Table 1. Analysis of upfield changes in the H^1 -NMR chemical shift values for the protons H_3 and H_5 with ($\Delta\delta=0.034$ ppm and $\Delta\delta=0.021$ ppm) respectively, can be interpreted as a good indication of the formation of inclusion complex by the co-precipitation method, while no change was observed at the chemical displacements of the two protons H_3 and H_5 ($\Delta\delta=0.002$ ppm and $\Delta\delta=0.001$ ppm) for the physical mixture compared to the free γ CD.

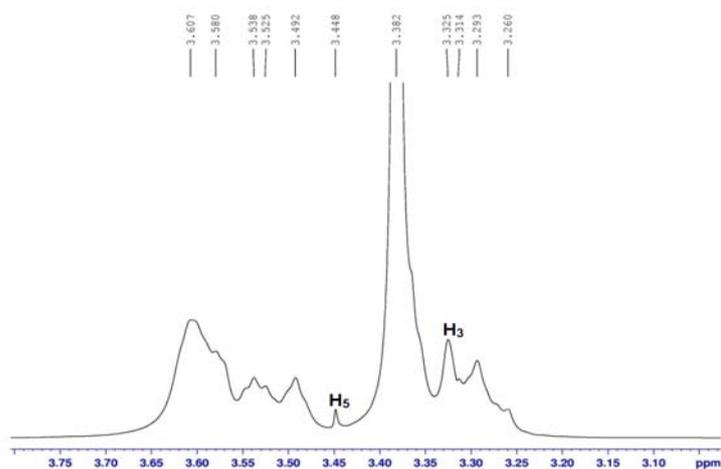


Fig. 4: 1H -NMR Spectra of γ CD

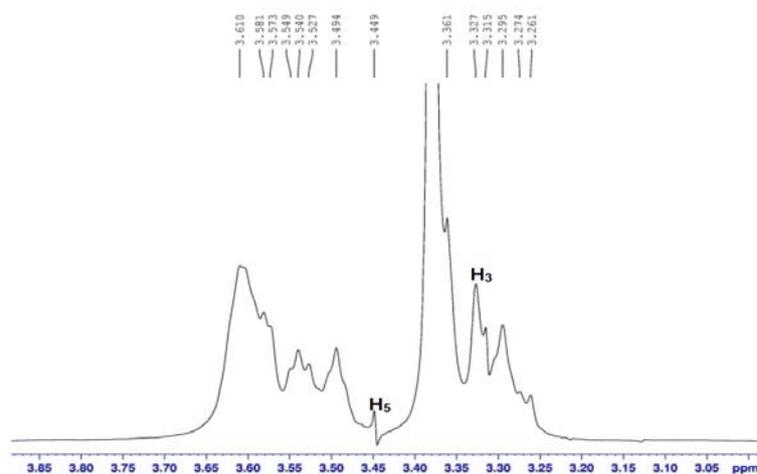


Fig. 5: 1H -NMR Spectra of physical mixture

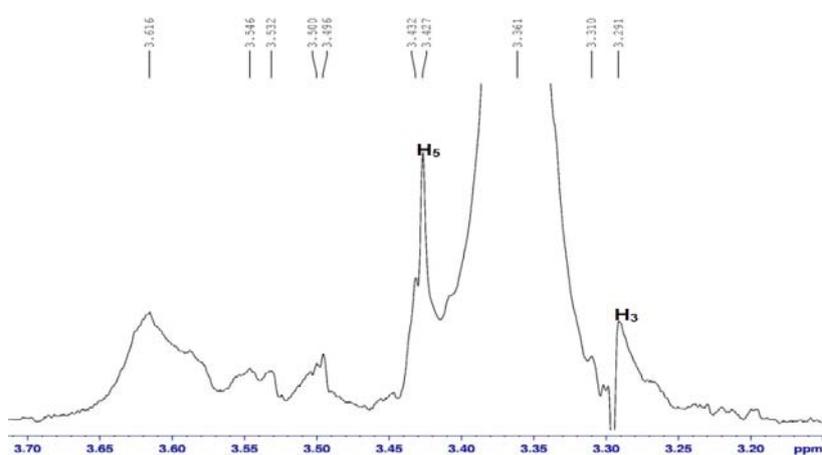


Fig. 6: 1H -NMR Spectra of inclusion complex (HCT- γ CD)

Table 1: ¹H-NMR Chemical shift (ppm) of the protons of free γ CD, γ CD-HCT physical mixture and γ CD-HCT inclusion complex

Protons	H ₃ $\Delta\delta$	H ₅ $\Delta\delta$
γ CD	3.325	3.448
γ CD-HCT physical mixture	3.327 0.002	3.449 0.001
γ CD-HCT complex	3.291 0.034	3.427 0.021

$\Delta\delta$: change in chemical shift

In vitro study

The dissolution profiles of free HCT and HCT- γ CD inclusion complex were shown in Figure 7. As can be seen from the figure, it was evident that the HCT- γ CD complex exhibited faster dissolution than the corresponding free HCT. Whole forms started with immediate

release at $t = 10$ min, with 20% and 75% dissolved of free HCT and the inclusion complex respectively. These dissolved percentages reached 90% for the HCT- γ CD complex after 60 min. The enhancement effect of HCT- γ CD on the dissolution rate of HCT could be explained from the enhanced aqueous solubility of HCT after the formation complex.

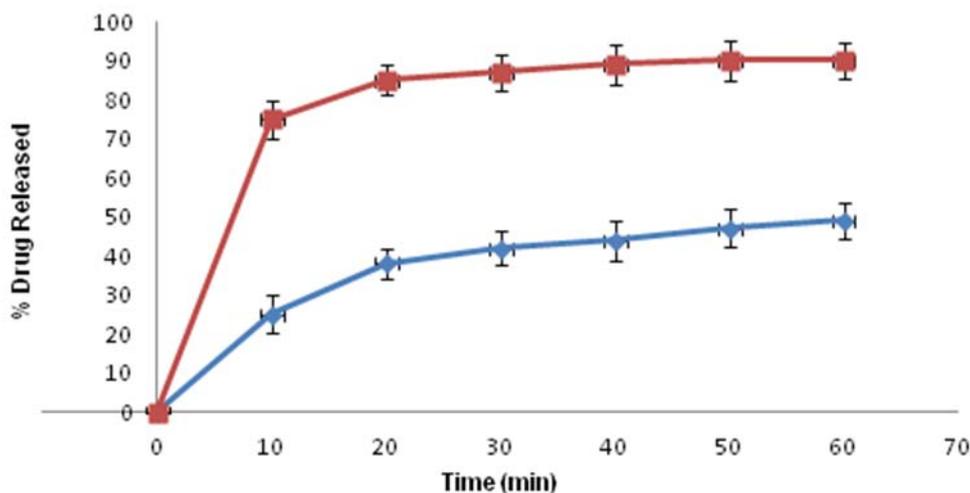


Fig. 7: Dissolution profiles for HCT (■), HCT- γ CD inclusion complex (◆)

In vivo study

Based on the in-vitro results (enhancement of solubility and dissolution rate in water), we hypothesized that the HCT- γ CD complex can improve the oral bioavailability of HCT, and thus the in vivo bioavailability study in dogs was performed.

Plasma concentration-time profiles of HCT after an oral administration of a single dose of 25mg of free HCT and the HCT- γ CD inclusion complex are shown in Figure 8. As shown in this

figure, the HCT was much better absorbed from the HCT- γ CD inclusion complex solution than from the free HCT. The plasma concentration of HCT increased rapidly and reached C_{max} within 4 h in all dogs. The C_{max} and the $AUC_{0-\infty}$ increased significantly (192.855 ± 6311 ng/ml to 353.229 ± 33.841 ng/ml, $p < 0.05$) and (1268.2 ± 102.649 to 2746.270 ± 164.761 , $p < 0.05$) (Table 2). But there was no significant change in T_{max} . Consequently, the bioavailability of HCT in dogs was improved by its inclusion complex with γ CD.

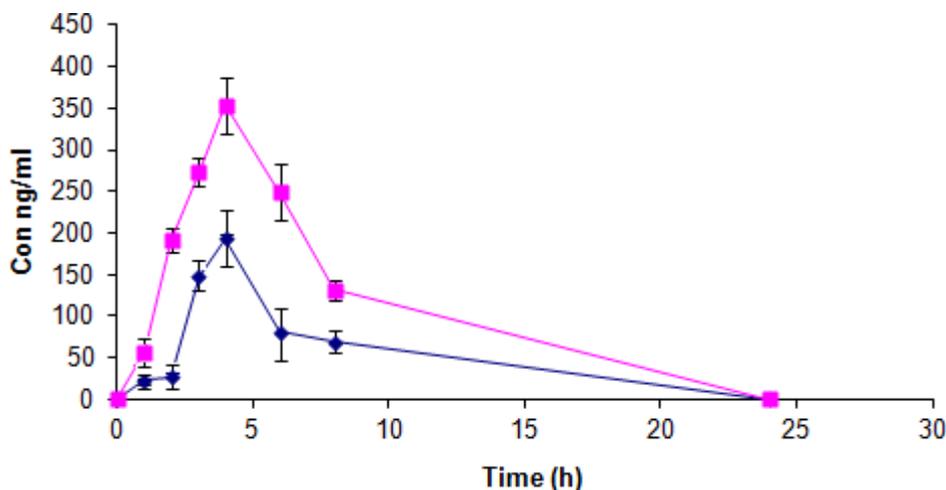
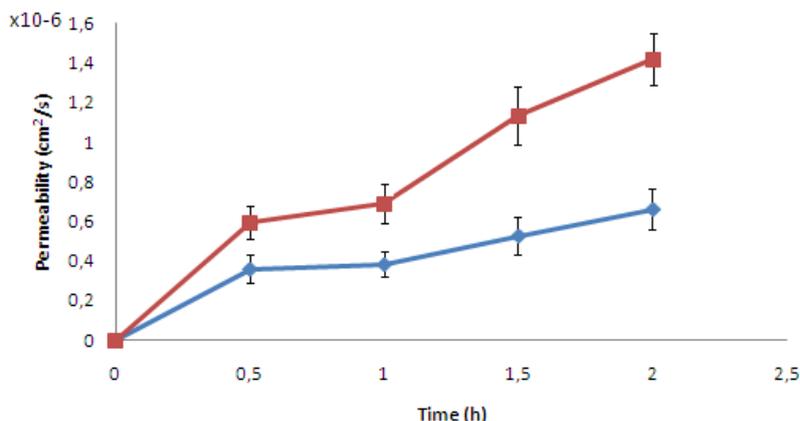


Fig. 8: Mean (\pm S.D.) plasma concentration-time profiles of HCT (◆) and HCT- γ CD inclusion complex (■) after oral administration (25 mg) in dogs ($n = 5$).

Table 2: Pharmacokinetics parameters of HCT in dogs (n=5) that were administered a single oral dose of 25mg as a control and as inclusion complex

Parameter	Unit	HCT	HCT- γ CD *P
AUC _{0-∞}	(ng h/ml)	1268.2 ± 102.64	2746.27 ± 164.76 < 0.05
C _{max}	(ng/ml)	192.85 ± 6.31	353.22 ± 33.84 < 0.05
T _{max}	(h)	4.2 ± 0.25	3.8 ± 0.11 > 0.05

Each value represents the mean ± S.D of five experiments

**Fig. 9: Intestinal permeability VS time using Ussing chamber: ◆ HCT; ■ inclusion complex HCT- γ CD****Table 3: Apparent permeability coefficient of HCT/ HCT- γ CD on jejunum tissue (n=6)**

Condition	Control (HCT)	HCT- γ CD inclusion complex	*P
P _{app} × 10 ⁻⁶ (cm ² /s) ± S.D.	1.96 ± 0.27	3.93 ± 0.81	< 0.05

Ex-vivo-bidirectional transport study

The effect of the HCT- γ CD complex on the permeability of HCT from the apical to basolateral side of jejunum was evaluated in comparison to the effect of free HCT using the Ussing chamber system. As shown in Figure 9, the intestinal permeability of the HCT- γ CD inclusion complex increased significantly as a function of time compared to the intestinal permeability of free HCT. The Papp of the complex is two times greater than the uncomplexed drug ((1.96 ± 0.27) × 10⁻⁶ cm/s to (3.93 ± 0.81) × 10⁻⁶ cm/s) (Table 3).

DISCUSSION

All toxicity studies of the oral administration of cyclodextrins, have shown its safety; this is because their very low gastro-intestinal absorption (0.1 to 3%) [23,24]. The Cyclodextrins belong to the family of molecule "cages", these molecules, hydrophilic outside and hydrophobic inside, have the tendency to form complexes of inclusion with the majority of the hydrophobic molecules [25]. They are known by their property to increase the solubility and bioavailability of organic molecules by the formation of inclusion complexes [26,18] such as albendazole [27,28], saquinavir [29] and insulin [30].

We used this approach to evaluate the effect of gamma-cyclodextrin on the solubility, bioavailability and permeability of HCT. We chose γ CD because the solubility of HCT was increased satisfactorily by inclusion with γ CD compared to that obtained with the beta-CD (data not shown).

The solubility results of HCT show that HCT- γ CD inclusion complex solubility is 6 times greater than free HCT (at a fixed concentration of 25 mM of γ CD, the solubility of HCT was increased from 0,39 to 2,5 mM).

The inclusion complex of HCT with γ CD was prepared by two methods, co-precipitation and physical mixing in the molar proportions 1: 1. The co-precipitation method is the most used in

studies with the disadvantage of the high consumption of time and energy, which affects the cost of synthesis; furthermore the complexation by physical mixing was incomplete [12].

The characterization of the binary mixtures was performed using XRD and NMR. Other analysis methods may be used, such as isothermic titration calorimetry [31], thermogravimetric analysis [32].

The dissolution profile of HCT alone was compared with the HCT- γ CD inclusion complex. According to norms demanded by USPXXII, the inclusion complex HCT- γ CD showed a better percentage release than HCT alone, exceeding 60% of HCT liberated after 60min.

In vivo study results showed an increase in bioavailability of HCT. This enhancement was reflected in the values of C_{max} and AUC parameters. The maximum plasma concentration of HCT is 192,855 ± 6311, while that corresponding to the complex is 353,229 ± 33.841. The absorption was significantly improved (p < 0,05). This improvement was related to the increase in the solubility and dissolution rate of HCT, which led to a better absorption.

A transport study showed that γ CD improved the permeability of HCT. That can be explained by the act of CD as permeation enhancers by direct action on the intestinal mucosal membrane permeability [33].

CONCLUSION

The HCT- γ CD inclusion complex was prepared successfully by the co-precipitation method and characterized by XRD and NMR. The solubility and the dissolution rate of the HCT- γ CD were much better than the HCT alone as a consequence of the enhanced solubility and decrease in crystallinity caused by the inclusion complex. It was also demonstrated that the bioavailability of HCT was improved significantly by its inclusion with γ CD. The Ussing chamber results demonstrated the enhancement of the permeability of HCT through the intestinal segment via complexation.

The whole results of this study suggest the potential use of γ CD for improving the bioavailability and the gastrointestinal tract absorption of HCT as oral preparations.

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