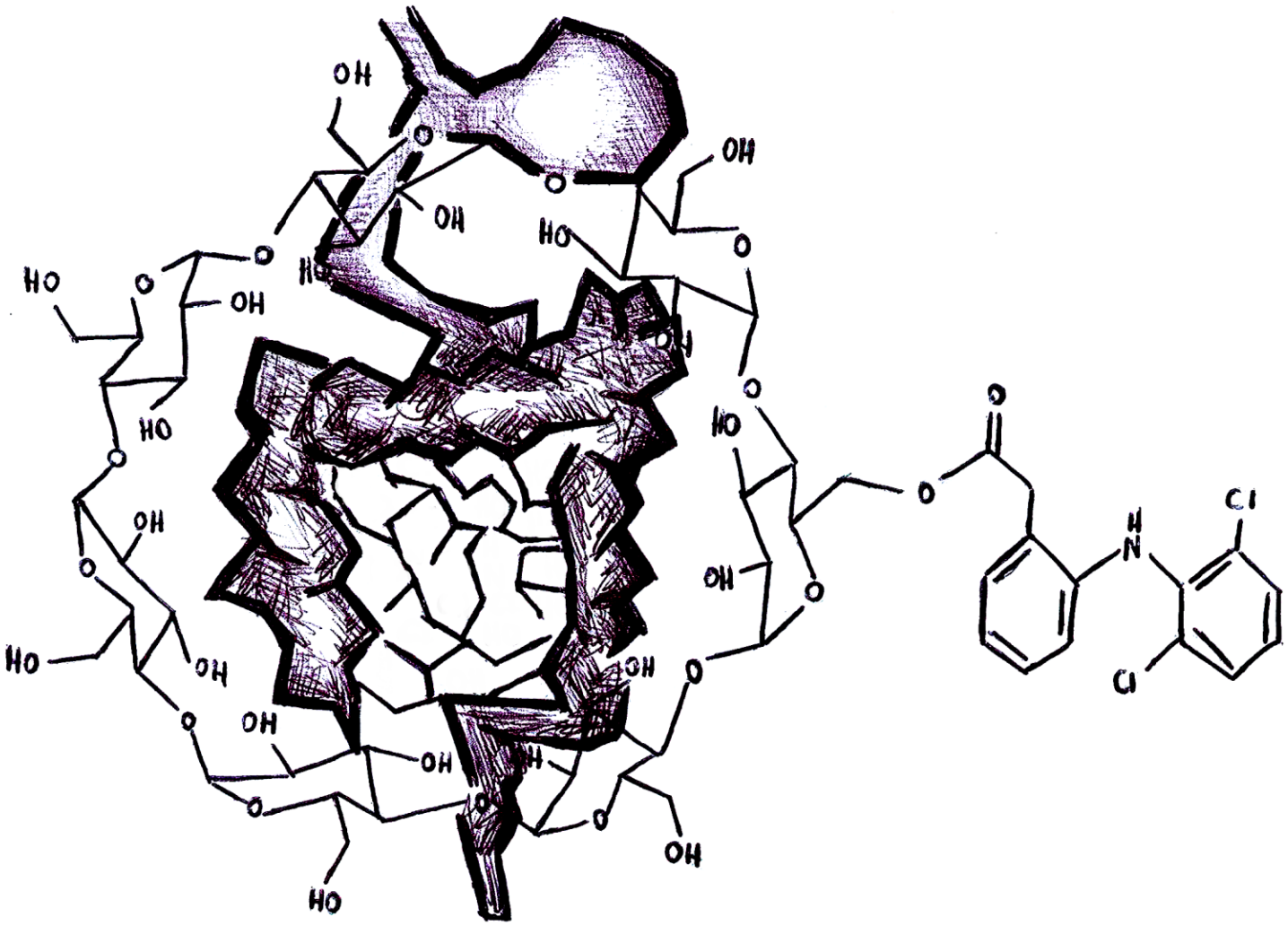

Amélia Catarina Fernandes Soares Vieira

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Tese de Doutoramento em Farmácia, na Especialidade de Tecnologia Farmacêutica, orientada pelo Professor Doutor Francisco José Veiga e pelo Professor Doutor Abdul W. Basit e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

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The work presented in this thesis has been carried out under the supervision of Professor Francisco José Baptista Veiga from the Faculty of Pharmacy, University of Coimbra and Professor Abdul W. Basit from UCL School of Pharmacy, University College London.

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and

UCL School of Pharmacy, University College London under the supervision of Abdul W. Basit.

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To my parents

Alice e Saul

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PUBLICATIONS

The results presented in this thesis allowed the following publications:

Amélia C. F. Vieira, Sudaxhina Murdan, Arménio C. Serra, Francisco J. Veiga, António M. d'A Rocha Gonsalves, Abdul W. Basit, *Influence of feeding regimens on rat gut fluids and colonic metabolism of diclofenac-β-cyclodextrin*, Carbohydrate Polymers, 112 (2014) 758–764

Amélia C. F. Vieira, Arménio C. Serra, Rui A. Carvalho, Alexandra Gonsalves, Ana Figueiras, Francisco J. Veiga, Abdul W. Basit, António M. d'A Rocha Gonsalves, *Microwaves synthesis and in vitro stability of diclofenac-β-cyclodextrin conjugate for colon delivery*, Carbohydrate Polymers, 93 (2013) 512– 517

European patent application (No. 11007013.3 – 1216) "Process to produce a diclofenac cyclodextrin conjugate" Amélia Vieira, Arménio C. Serra, Rui A. Carvalho, Alexandra Gonsalves, Ana Figueiras, António Rocha Gonsalves, Francisco Veiga

Abstract

Inflammatory diseases, namely arthritis, are prevalent worlwide, and pain is considered to be one of the most important determinants of life quality. Frequently, such conditions and the pain associated with them are managed simultaneously by the use of anti-inflammatory drugs – including both steroidal and non-steroidal – which, though readily available, have widely-recognised adverse effects and variable efficacy between individuals. In order to minimize these side effects and simultaneously increase the therapeutic efficacy associated with the use of anti-inflammatory drugs, different attempts have been made, including the potential for site-specific drug delivery to the colon. Among the useful strategies to target the colon, prodrugs are presented as a very promising solution. Cyclodextrins can be used as a carrier to develop colon-targeted prodrugs through the synthesis of a suitable covalent linkage with a drug. These molecules are known as cyclodextrin conjugates.

The work presented in this thesis was based on the knowledge aforementioned with the principal aim to successfully synthesize cyclodextrin ester prodrugs of diclofenac for colonic delivery.

The first challenge was to find a synthetic way to produce a conjugate using the most common natural cyclodextrin, β-cyclodextrin. Various strategies were attemped to establish an ester linkage between the drug and β-cyclodextrin. However, only the nucleophile substitution of mono-6-tosyl-β-cyclodextrin under microwave irradiation allowed a successful synthesis.

The application of this strategy in the synthesis of other conjugates, namely with γ- and α-cyclodextrin, was explored, but difficulties were encountered. This led us to focus our investigation on the study of diclofenac-β-cyclodextrin conjugate, which was characterized by several techniques: matrix-assisted laser desorption/ionization (MALDI) spectra, infrared (IR) spectroscopy, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and two-dimensional rotating frame nuclear overhauser effect (ROESY) spectroscopy. Its purity was confirmed by high pressure liquid chromatography (HPLC), and preliminary chemical hydrolysis assays were performed.

In vitro and *ex vivo* studies were then made in order to investigate the stability of the diclofenac-β-cyclodextrin conjugate along the gastrointestinal tract. Firstly, studies were

carried out in human faecal slurries in order to assess the ability of the conjugate to release diclofenac in the lower intestine. These stability studies were conducted by comparison with a well-known colonic prodrug, sulfasalazine. Sugar-degrading enzymes and ester-hydrolysing enzyme were used in order to find an enzymatic media able to mimic the colonic metabolism of this conjugate. Secondly, stability was studied at the level of the upper GI tract using simulated gastric and intestinal fluids as described in the Pharmacopeia. The conjugate released diclofenac at the level of the lower intestine, and exhibited good stability in the upper gastrointestinal tract. Consequently, *ex vivo* studies were conducted in fluids collected from different animal species (pig, rabbit and rat) in order to identify a suitable animal model to further perform *in vivo* studies. Rats were shown to be the animal that most closely resembled the behaviour of the conjugate in humans and therefore were used in these additional studies for modelling.

The influence of the regimen of food intake on the physiological conditions (mass contents and pH) of the gastrointestinal tract and on degradation of the conjugate *versus* sulfasalazine in the large intestine was then performed in rats in order to choose the best regimen to assess the *in vivo* bioavailability. Results showed that the feeding regimen affects gut contents (mass and pH), more specifically in the stomach and lower intestine, and also affects the rate of metabolism of diclofenac-β-cyclodextrin but not that of sulfasalazine. Fasting was shown to result in the most rapid degradation of diclofenac-β-cyclodextrin, possibly due to lack of competition (absence of food) for microbial enzymatic activity.

As a proof-of-concept, a comparative *in vivo* study in fasted rats was performed by oral administration of a suspension of diclofenac-β-cyclodextrin and sulfasalazine with parallel administration of sodium diclofenac and sulfapyridine, as a control group of rats. A lag time between oral intake of prodrugs and the appearance of the respective drugs in plasma was observed, contrarily to that observed with the oral administration of free drugs whose plasma concentrations were measurable immediately after intake. These results confirm the *in vivo* ability of this conjugate to target and release diclofenac in the colon. A reduction of 50% in bioavailability was observed for diclofenac when administered in the form of its cyclodextrin conjugate compared with the free drug.

Overall, diclofenac-β-cyclodextrin is a promising prodrug to be explored as a suitable tactic for the management of arthritis with simultaneous circumvention of the adverse gastric effects associated with the free drug.

Keywords: anti-inflammatory drugs, colonic targeting, conjugates, cyclodextrins, diclofenac, enzymes, fermentation, food regimen, gastrointestinal tract, *in vivo studies* oral delivery, microbiota, prodrugs.

RESUMO

As doenças inflamatórias, nomeadamente a artrite, são doenças que afectam a população po todo o mundo. A ausência de dor é considerada um factor determinante para a qualidade de vida. Os anti-inflamatórios, esteróides ou não esteróides são a classe de fármacos usada no controlo deste tipo de doenças. No entanto, estes estão associados a diversos efeitos adversos e a variabilidade inter-individual também afecta em termos eficácia. Diferentes estratégias foram exploradas com o objectivo de aumentar a eficácia dos anti-inflamatórios e, ao mesmo tempo, diminuir os seus efeitos adversos. Entre as várias estratégias, destaca-se a que envolve a libertação específica destes fármacos ao nível do cólon. Para este efeito, o desenvolvimento de profármacos constitui uma solução promissora. As ciclodextrinas podem ser usadas como transportadoras para o *design* de profármacos, denominados conjugados, para libertação específica no cólon, estabelecendo uma ligação covalente ao respectivo fármaco. Esta tese resulta de um trabalho desenvolvido com base neste conceito tendo como objectivo sintetisar um profármaco do diclofenac para libertação específica no colon, usando ciclodextrinas como transportadores.

O primeiro desafio consistiu em explorar diferentes estratégias para a síntese do conjugado entre o diclofenac e a β-ciclodextrina. Das diversas tentativas, apenas a substituição nucleofílica do 6-mono-tosil-β-cyclodextrina sob radiação de microondas, permitiu a síntese com sucesso. A aplicação da mesma estratégia para a síntese de outros conjugados, nomeadamente com γ- e α ciclodextrina foi explorada, contudo, inúmeras dificuldades foram encontradas. Deste modo, a nossa investigação focou-se no estudo do conjugado diclofenac-β-cyclodextrina. Este novo conjugado foi caracterizado por diversas técnicas: Ionização/Dessorção de Matriz Assistida por Laser (MALDI), espectroscopia de infra-vermelho (IV), ressonância magnética nuclear de protão (^{1}H-RMN) e espectroscopia bidimensional do efeito nuclear de Overhauser (ROESY). A sua pureza foi avaliada por cromatografia de alta pressão (HPLC) tendo-se posteriormente realizado ensaios de estabilidade química.

Efectuaram-se estudos *in vitro* e *ex vivo* no sentido de investigar a estabilidade do diclofenac-β-cyclodextrina ao longo do tracto gastrointestinal. Foram realizados ensaios num sistema de fermentação com fezes humanas, de forma a descobrir a capacidade do conjugado libertar o diclofenac no intestino grosso. Estes estudos de estabilidade foram executados em comparação com um profármaco conhecido, a sulfasalazina. De forma a encontrar um sistema

enzimático que mimetize o metabolismo deste conjugado ao nível do cólon, realizaram-se estudos com enzimas capazes de degradar açúcares e enzimas com capacidade de hidrolisar ligações ester. Posteriormente, foi estudada a estabilidade ao nível do tracto gastrointestinal superior, utilizando simulados gástricos e intestinais descritos na Farmacopeia. O conjugado permitiu a libertação do diclofenac ao nível do intestino grosso e exibiu uma boa estabilidade no tracto gastrointestinal superior. Consequentemente, foram realizados estudos *ex vivo* em fluídos recolhidos de diferentes espécies de animais (porco, coelho e rato), de forma a identificar o animal adequado para a realização dos estudos *in vivo*. Os resultados obtidos no rato foram os que mais se assemelharam ao comportamento do conjugado obtido no sistema de fezes humanas. Por este motivo, o rato foi o animal escolhido para realizar os estudos subsequentes.

Com o objectivo de escolher o regime alimentar mais adequado para concretizar os estudos de biodisponibilidade, foi investigada a influência do regime de alimentação nas condições fisiológicas do tracto gastrointestinal e na degradação do conjugado *versus* sulfasalazina no intestino grosso. Os resultados mostraram que o regime de alimentação influencia os conteúdos (massa e pH), mais especificamente a nível do estomago e do intestino grosso, e também afecta a velocidade de metabolismo do diclofenac-β-cyclodextrina, mas não da sulfasalazina. O jejum permite uma libertação mais rápida do conjugado, possivelmente devido a falta de competição (ausência de alimento) para as enzimas microbianas.

Como prova do conceito, realizaram-se estudos *in vivo* em ratos em jejum, através da administração oral de uma suspensão de conjugado com sulfasalazina a um grupo de animais. Em paralelo, foi efectuada a administração de diclofenac de sódio e sulfapiridina a um grupo controlo. Observou-se um atraso entre a administração oral dos profármaco e o aparecimento do respectivo fármaco, contrariamente ao que se verificou com a administração dos fármacos livres, cujo aparecimento no plasma foi imediato. Estes resultados confirmaram a capacidade do conjugado em libertar o diclofenac especificamente no cólon. Uma redução de 50% na biodisponibilidade do diclofenac ocorre aquando da administração do conjugado comparativamente à administração do fármaco livre.

Poderemos concluir que o diclofenac-β-cyclodextrina constitui um profármaco promissor a ser explorado no combate a doenças inflamatórias, tais como a artrite, ultrapassando simultaneamente os efeitos adversos associados ao diclofenac.

Palavras Chave: administração oral, anti-inflamatórios, ciclodextrinas, conjugados, diclofenac, enzimas, estudos *in vivo,* fermentação, libertação específica no cólon, microbiota, profármacos, regime de alimentação, tracto gastrointestinal.

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LIST OF ABBREVIATIONS

AUC - area under the curve

ANOVA - Analysis of variance

BCFA - Branched-chain fatty acids

CDs - cyclodextrins

CDI - carbonyldiimidazole

DCC - *N*,*N*' dicyclohexylcarbodiimide

DMAP - 4-dimethylaminopyridine

DMF - Dimethylformamide

DMSO-d_6 - Deuterated dimethyl sulfoxide

EDAC - *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride

^{1}H NMR - proton nuclear magnetic resonance

HPLC - High Performance Liquid Chromatography

IR - Infrared spectroscopy

LC-MS - Liquid chromatography - Mass spectroscopy

MALDI - matrix-assisted laser desorption/ ionization spectroscopy

M_W - molecular weight

NaOH - sodium hydroxide

NSAIDs - non-steroidal anti-inflammatory drugs

PBS - phosphate buffer solution

RPM - rotation per minute

r.t. - retention time

SCFA - short-chain fatty acids

TEA - triethylamine

THF - tetrahydrofuran

TLC - Thin layer chromatography

UV - Ultraviolet

CHAPTER I

GENERAL INTRODUCTION

Chapter I

1 GENERAL INTRODUCTION

Prodrug

"A therapeutic agent which is inactive *per se* but is transformed into one or more active metabolites"

(Ettmayer, Amidon et al., 2004; Stella, Borchardt et al., 2007)

1.1 Overview

The oral route represents the largest market on the overall drug delivery market, herein among 84% of the 50 most-sold pharmaceutical products in the US and European are given orally (Lennernäs and Abrahamsson, 2005). Orally administered drugs are generally given in the form of immediate-release or modified-release dosage forms. In the case of immediate-release dosage forms, after oral administration, the drug is released immediately in the stomach, providing rapid absorption. Modified release dosage forms are designed to extend or delay the release of the drug in the gastrointestinal (GI) tract (Bechgaard and Nielsen, 1978; Basit, 2005). These dosage forms can be formulated as a single unit (tablets, capsules) or multiple unit (pellets, granules or minitablets) (Varum, Merchant et al., 2010).

In the area of oral delivery, targeted drug delivery specifically in the colon has been of great interest due to the importance of this region of the GI tract not only for local but also for systemic therapy (Mcconnell, Liu et al., 2009). Drug targeting to the colon is valuable in the treatment of colon diseases such as: inflammatory bowel disease (ulcerative colitis and Crohn's disease), irritable bowel syndrome (Meissner and Lamprecht, 2008), carcinomas (Lamprecht, Yamamoto et al., 2003) and infections (Mundargi, Patil et al., 2007). Moreover, the delay in drug absorption may be preferred in the treatment of diseases sensitive to circadian rhythms such as nocturnal asthma, angina, gastric ulcer and arthritis (Mcconnell, Liu et al., 2009). This region of the GI tract is considered to be more suitable for delivery of molecules that are degraded and/or poorly absorbed in the upper gut, such as peptides and proteins, improving their bioavailability (Fix, 1996; Langguth, Bohner et al., 1997; Mackay, Phillips et al., 1997; Katsuma, Watanabe et al., 2006).

Numerous strategies have been exploited to allow specific drug release in the colon (Chourasia and Jain, 2003; Basit, 2005), herein one of the major strategies of drug delivery to this part of the GI tract is prodrug based systems.

Prodrugs are designed by attachment of a specific carrier to the active molecule by a suitable covalent linkage. The prodrug should pass intact and unabsorbed in the upper GI tract and undergo biotransformation in the colon releasing the active drug molecule. This is an enzymatic process that is carried out by the inherent bacterial flora present in the colon. This enzyme-trigger mechanism in prodrugs confers a better colon-target ability (Sinha and Kumria, 2001; Sinha and Kumria, 2004; Jung and Kim, 2010) and the proof of this is the success of sulfasalazine, a azo-colonic prodrug of mesalamine (5-ASA) developed in the 1930s for the treatment of rheumatoid arthritis (Svartz, 1948) and that is still a reference on the treatment of inflammatory bowel disease (IBD) and has the greatest efficacy in ulcerative colitis (Qureshi and Cohen, 2005; Cohen, Lichtenstein et al., 2008).

However, given the toxicity associated to its carrier – sulfapyridine, other molecules have been explored as a carrier, not only for the transport of mesalamine, but also for the transport of other active molecules with potential interest to be delivery specifically on the colon. The design of an azo-prodrug requires the presence of an amine functional group on the dug molecule to establish the azo-bond linkage. However, this group is not always present on the molecules structure. Therefore, it has been explored the design of other types of linkages that allows colonic specific delivery (Jung and Kim, 2010).

Cyclodextrins are cyclic oligosaccharides known for more than 100 years, that have been studied and used to improve drug delivery of different systems (Szejtli, 2004). More recently, cyclodextrins have been explored as a carrier on the design of prodrugs for site-specific delivery of drugs. In the case of oral delivery, conjugates of cyclodextrins are presented as a strategy for colonic specific delivery of drugs (Uekama, Hirayama et al., 1998).

Diclofenac is a powerful anti-inflammatory that has been on the market for 40 years (Haas and Rossi, 1974). Due to the gastrointestinal side effects associated to it, research has been conducted in order to improve the performance of this drug. One of the approaches involves the exploration of strategies able to site-specific release of this drug into the colon (Gan,

2010). Additionally, as an anti-inflammatory it has preventive effect against colon cancer (Calatayud, Warner et al., 2001).

This dissertation accesses the ability of cyclodextrins as carriers for colonic specific delivery of a non-steroidal anti-inflammatory drug (NSAID) diclofenac.

1.2 The gastrointestinal tract: main considerations on the design of a colonic prodrug

The human GI tract starts at the mouth and ends at the anus; its length is about 8.35 meters. It is divided into sections: mainly stomach, small intestine (the duodenum, jejunum, and ileum) and large intestine (caecum, colon and rectum), allowing digestion and nutrient absorption in the proximal region to be separate from the vast microbial populations in the large intestine, thereby reducing conflict between host and microbes (Walter and Ley, 2011).

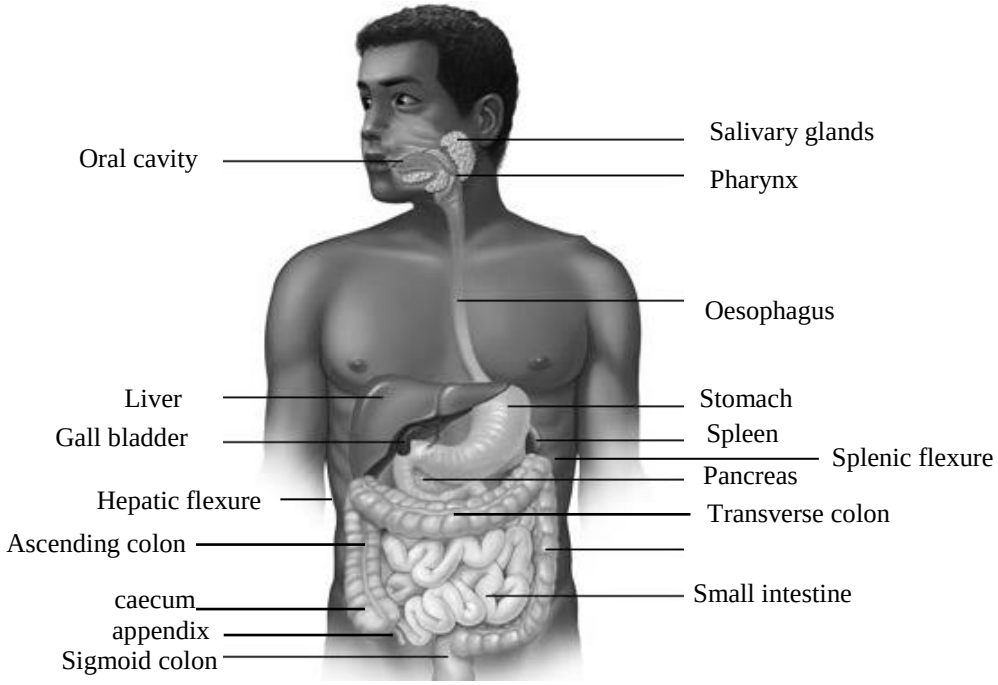


Figure 1.1 Gastrointestinal tract anatomy.

On the development of a prodrug for colonic specific delivery, it is necessary to be aware about all the different environments from the cavity buccal until colon through which the prodrug contacts and that can influence its performance. The pH is variable along the gastrointestinal tract and a huge variability exists between people (Fallingborg, Christensen et al., 1989), but also a considerable intra-individual variability can be observed (Ibekwe, Fadda et al., 2008). In the particular case of colonic prodrugs, pH can be an obstacle to the delivery

of prodrugs as the pH changes affects the degree of ionization of weak acidic and basic produgs and, consequently, their chemical stability (Stella, Borchardt et al., 2007). Additionally, the intraluminal pH along the GI tract influences bacterial concentration in each section of the gut. This is particularly evident namely at the level of the stomach where the own pH tends to destroy most bacteria. Bacterial microbiota exist in most parts of the GI tract and become an important component of the luminal content (Hawksworth, Drasar et al., 1971; Cummings and Macfarlane, 1997a). The current total estimate number of prokaryotes that inhabits the human gut goes up to 100 trillion (10^{14}) microbes. This number makes the human gastrointestinal tract one of the most populated microhabitats on earth (Ley, Peterson et al., 2006; Sousa, Paterson et al., 2008). The main concentration site of microbiota in the GI tract is the lower intestine. At this level, microbiota is responsible for the activation of colonic prodrugs allowing the release of the drug at colonic level. However, the stability of the own drug against the colonic microbiota has also to be considered when we design a reliable prodrug as a colonic specific system.

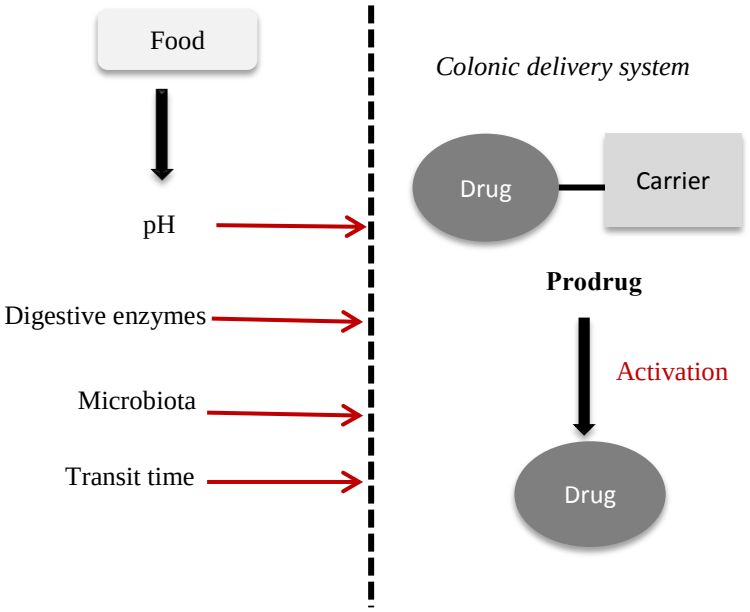


Figure 1.2 Schematic representation of the main factors to be considered on the development of a colonic specific prodrug.

Transit time through the GI tract varies depending on various factors like GI motility, quantity and quality of food ingested, feeding regimen, food timing, and also nature of the oral formulation (Yuen, 1986; Varum, Merchant et al., 2010). The average overall transit time from the mouth to the anus in humans is 24–72 hours (Stella, Borchardt et al., 2007). Considering colonic prodrugs, the time between intake of a prodrug and its arrival in the colon will affect not only the onset of the drug in the local of action and /or at the plasmatic level but also, consequently, its efficacy.

Following is described the main characteristics and functions of each sections of the GI tract that can influence the success of a colonic prodrug, including aspects related to the enzymes, pH, microbiota and gastrointestinal tract transit time

1.2.1 *Stomach*

The stomach is divided into cardia, fundus, corpus and pyloric region (antrum) and has several functions: storage of the ingested food, processing the food into a fluid chimie, mechanically and biochemically; control the rate of delivery of chimie into the duodenum and production of acid (Washington, Washington et al., 2000).

In the stomach, due to secretion of hydrogen ions by gastric mucosa (parietal cells) the pH ranges from 0.8 and 2.0 in the fasted state and the number of bacteria decreases comparably with saliva (10^7 CFU/mL) due to the acidic conditions. After ingestion of food, due to buffering and dilution effects caused by that, the pH ranges from 4 to 5 and bacteria proliferate to 10^4 -10^8 CFU/mL. Bacteria can escape due to the substantial volumes of liquid that are moving into the duodenum. At this level, microbiota is predominantly gram-positive and aerobic (Evans, Pye et al., 1988 ; Fallingborg, Christensen et al., 1989) The low pH is essential to maintain the environment sterile and regulates the production of pepsin (3.4.4.1), an exopeptidase (Dressman, Amidon et al., 1998).

Gastric emptying presents great variability, which can range from a few seconds to a number of hours and can depend on the feed status (Ibekwe, Liu et al., 2006). Solid, large and caloric meals increase gastric emptying time (Varum, Hatton et al., 2013). In general, the transit time from the mouth to the small intestine in healthy human adults is 0.5–2 hours, whereas it can be delayed to 3–6 hours and 5–8 hours after the intake of light meals and heavy meals, respectively (Stella, Borchardt et al., 2007). Digenis *et al.* verified with a multiple-unit

formulation that there is a faster gastric emptying in pre-feeding state when compared to the fasted state, due to the increase of gastric motility and gastric emptying in presence of food (Digenis, Sandefer et al., 1990).

1.2.2 *Small intestine*

Small intestine is the longest section of the digestive tube, representing 81% of the total length of the GI tract. It is divided into 3 regions: duodenum, jejunum and ileum (Desesso and Jacobson, 2001). In the proximal part of the duodenum occurs the neutralisation of the acidic contents of the stomach by the alkaline pancreatic secretions, which contains bicarbonate, lipases, amylases and proteases (Dressman, Amidon et al., 1998; Sarti, Barthelmes et al., 2011). Consequently, it is observed a burst increase of pH to 5.5. At the level of duodenum, food does not affect the pH (Kalantzi, Goumas et al., 2006). Additionally, bicarbonate secretion results in a further gradual rise in the luminal pH to 6.6 ± 0.5 at jejunum level, reaching a peak at the ileum caecal junction 7.5 ± 0.5, in health condition (Evans, Pye et al., 1988 ; Dressman, Berardi et al., 1990; Ibekwe, Fadda et al., 2008).

In the duodenum and jejunum, the relatively rapid flow of the digesta concomitantly with bile and pancreatic fluid secretions, does not allow the increase of microbial multiplication (Tannock, 1999; Sousa, Paterson et al., 2008). At this stage the predominant species are aerobic and gram positive. However, in the distal ileum gram negative bacteria outnumber the gram positive and the bacterial composition becomes higher (Sinha and Kumria, 2003).

The intestinal area is greatly enhanced by presence of plicae circulares (or Kreking folds), villis (finger like projections) and microvillis. Together, these folds provide a huge surface area for absorption, 200 m^2, whereas the area of the stomach is only about 1 m^2 (Kararli, 1995; Rowland, Tozer et al., 1995; Desesso and Jacobson, 2001). This big surface area associated with the good blood supply and the gut associated lymphoid tissue (GALT), makes the small intestine the main site of absorption (Edwards, 1993), particularly in duodenum and proximal jejunum. The material that is not absorbed suffers movement towards the large intestine. Separating the ileum from the caecum is a one-way valve, the ileocaecal junction (ICJ), which regulates the movement of material between the small intestine and the colon and prevents the reflux of colon contents, in particular, the spread of bacteria from the colon to the ileum.

Although, the mean time to cross the small intestine is of 3-4 hours (Davis, Hardy et al., 1986), the actual transit has demonstrated a range between 1 and 9.5 hours (Davis, Hardy et al., 1986; Sugito, Ogata et al., 1990; Coupe, Davis et al., 1991a). Fadda *et al.* verified that there is an acceleration in small intestinal transit in pre-feed state when compared with fasted and fed state (Fadda, Mcconnell et al., 2009).

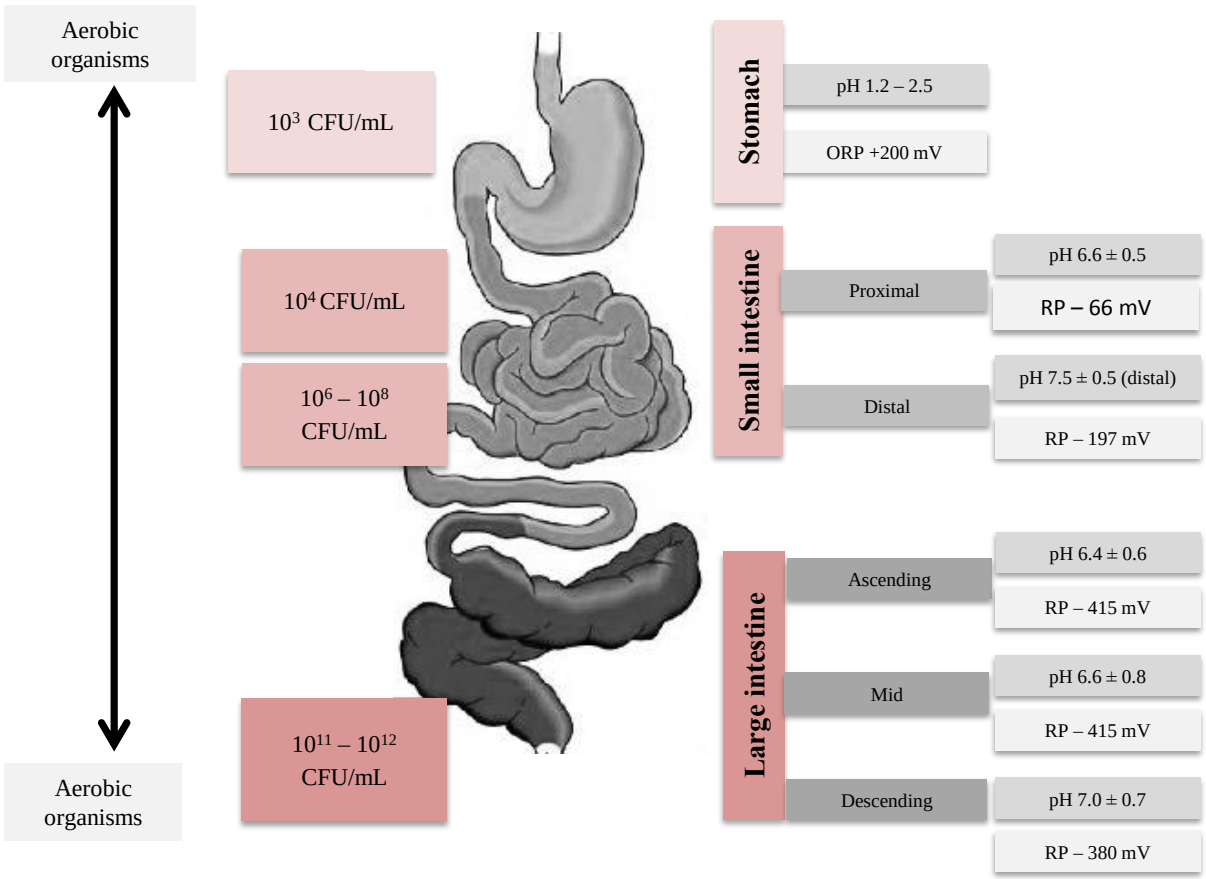


Figure 1.3 Gastrointestinal intraluminal pH, redox potential (RP) and bacterial concentration (Sousa, Paterson et al., 2008) Mean pH values (Evans, Pye et al., 1988), mean Redox potential (mV) (V Stirrup, S J Ledingham et al., 1990) and bacterial concentration (CFU/g of contents) (Simon and Gorbach, 1984; Macfarlane and Macfarlane, 2004; Sousa, Paterson et al., 2008).

1.2.3 *Large intestine*

The large intestine comprises the caecum, colon, rectum and the anal canal. It represents 19% of the total length of the GI tract, herein the caecum, forms only 5% of the length of the human large intestine (Desesso and Jacobson, 2001; El-Kattan and Varma, 2012). The colon is subdivided into the ascending (right), transverse, descending (left) and sigmoid colon (the proximal colon usually refers to the ascending colon and transverse colon). In the adult, it is

approximately 150 cm long, significantly shorter than the small intestine. The 'large' refers to the diameter which varies from approximately 9.0 cm in the caecum to 2.0 cm in the sigmoid colon (Mrsny, 1992). The large gut receives material from the ileum, which has already been digested and the contents are then mixed and retained for 6-12 hours in the caecum and right colon. After the hepatic flexure, the chimie is processed into faeces, pass through the transverse to the left colon for storage and eventual excretion concomitant with epithelial cell turnover and bacteria (Cummings and Macfarlane, 1991; Hastewell, Williamson et al., 1991; Basit, 2005). The ascending colon is the preferential region in the lower intestine regarding to action of drugs, since the substantial residence time in this part and also because has more free water when compared to the transverse colon (Diakidou, Vertzoni et al., 2009).

As compared with the small intestine, the large intestine shows much reduced surface area for absorption. This is due to the presence of semilunar folds instead of circular Kerkring's valves and due to the lack of villi and less developed microvilli (Hastewell, Williamson et al., 1991; Kararli, 1995). Additionally, the narrow ‘tighter junctions’ between epithelial cell, restrain the paracellular permeability of hydrophilic compounds (Hastewell, Williamson et al., 1991).

Colon participates in the maintenance of fluid and electrolyte balance through reabsorption of water, sodium cation, chloride and secretion of potassium and bicarbonate (Dawson, 1991). Additionally, colon contains lower levels of luminal and mucosal digestive enzymes than stomach and small intestine (Gibson, Mcfarlan et al., 1989).

However, the human colon is the major site for growth and microbial digestion. The colonic microbial community is very complex due to the diversity of species and, moreover, due to the difficulty to distinguish between permanent and transient members (Yatsunenko, Rey et al., 2012). The studies surrounding gastrointestinal microbiota have been extended for over a century and nowadays, it is estimated that the total number of procaryotes that inhabit the human gut goes up to 10^{14}, herein aerobic species outnumber (Savage, 2001; Ley, Peterson et al., 2006). It is estimated that the gut microbiome contain 150-fold more genes than the human genome (Qin, Li et al., 2010).

Current knowledge allows to recognize that the lower gastrointestinal tract contains over 400 distinct species of bacteria, which belongs to relatively few phyla (Yang, 2008; Jung and Kim, 2010). The predominant microbiota belong to the phylum Firmicutes (gram positive)

and Bacteroidetes (gram negative), which account for more than 90% of the overall phylogenetic types. Additionally, Actinobacteria (gram positive) has also been found in human gut, normally with lower abundances of Verrucomicrobia and Proteobacteria and Fusobacteria (Eckburg, Bik et al., 2005; Wu, Chen et al., 2011; Yatsunenko, Rey et al., 2012; Aziz, Doré et al., 2013; Scott, Gratz et al., 2013).

In the ascending colon, microorganisms having plentiful supply of dietary nutrients, tend to grow rapidly, while in the transverse and descending colon substrate availability is lower and bacteria growth is slower (Sousa, Paterson et al., 2008). Nevertheless, aproximately 1/3 of the faecal dry weight consists of bacteria (Sinha and Kumria, 2003).

The complete definition of microbiota requires further studies, which may attend the combination of conventional and molecular microflora analysis. Moreover, the impact of microbiota on host physiology is still unclear (Mai and Morris, 2004).

It is known that microbiota exists in relative stable conditions that rely on nutrient availability (carbohydrates, proteins and fats), pH, temperature, redox potential, degree of anaerobiosis and healthy state (Cummings and Macfarlane, 1991; Ojetti, Gigante et al., 2009) (Scott, Gratz et al., 2013).

Primarily, these colonic bacteria rely on carbohydrate dietary components that are undigested by human enzymes in the upper GI tract for energy and growth, including resistant starch (RS), non-starch polysaccharides (NSP), and oligosaccharides (including prebiotics and cyclodextrins). Anaerobic fermentation of these subtracts results in the production of volatile fatty acids called short-chain fatty acids (SCFA), CO_2, H_2, CH_4 and H_2S. (Scott, Duncan et al., 2008; Scott, Duncan et al., 2011). Gram-negative anaerobes belonging to the genus *Bacteroides* are the principal polysaccharide-degrading bacteria (Cummings and Macfarlane, 1991). When the carbohydrate sources are depleted, which occurs namely at the level of the distal colon, residual proteins are used as a source of energy. The fermentation of proteins results in the formation of SCFA but also of branched chain fatty acids (BCFA) and other metabolites (Scott, Gratz et al., 2013).

Short chain fatty acids are the major products of fermentation, which accounts for up to 10% of the human energy source (Frank, St. Amand et al., 2007). Butyrate, in particular, is the major source of energy for the colonocytes (the epithelial cells that line the colon), propionate

is transported to the liver where it has a role in gluconeogenesis, whilst acetate enters systemic circulation and is used in lipogenesis (Cummings and Macfarlane, 1997b; Scott, Duncan et al., 2008).

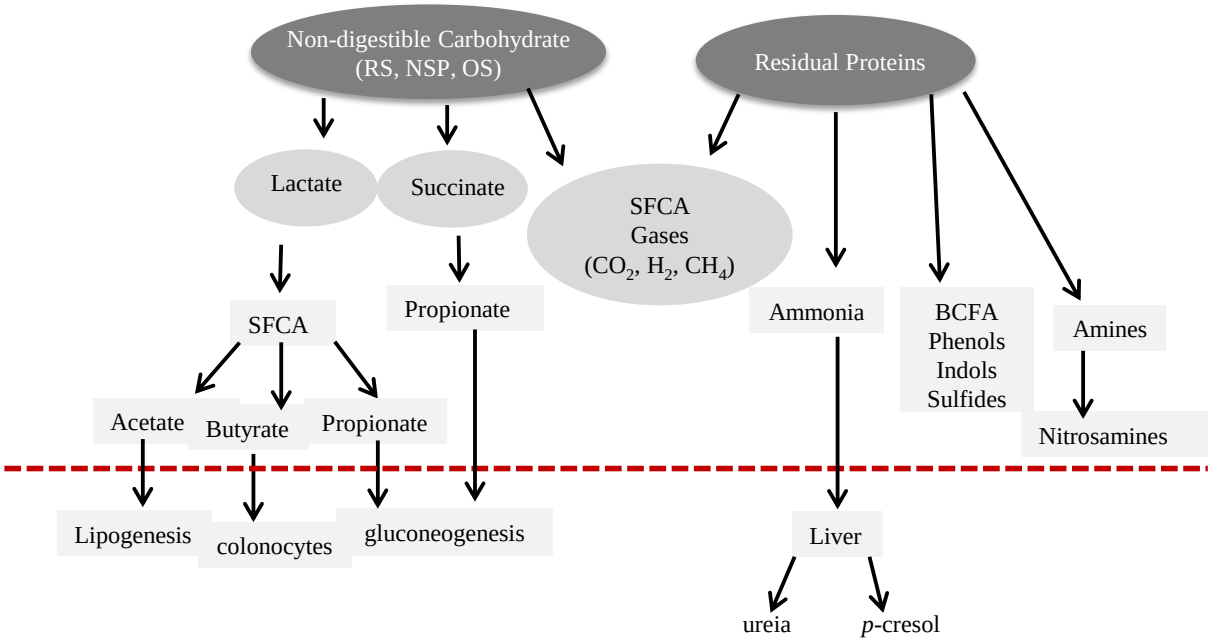


Figure 1.4 Summary of bacterial metabolites derived from non-digestible carbohydrate and from residual protein fermentation. RS – resistant starch, NSP – non-starch polysaccharide, OS – oligosaccharides (including cyclodextrin), SCFA – short chain fatty acids, BCFA – branched chain fatty acids (isobutyrate, 2-methylbutyrate and isovalerate). Adapted from (Scott, Gratz et al., 2013).

Due to the acidification of the contents by short-chain fatty acids resulted from fermentation by colonic bacterial, in the passage from ileum to caecum, a drop in the pH is observed (Evans, Pye et al., 1988 ; Scott, Gratz et al., 2013) Therefore, values of pH in contents from ascending colon were 7.8 and 6.0 in fasted and fed state, respectively (Diakidou, Vertzoni et al., 2009). The pH increases along the colon due to the progressively lower carbohydrate fermentation by bacteria, continuous bicarbonate secretion and metabolism of protein and urea to ammonia, particularly in the distal colon (Nugent, Kumar et al., 2001). On the other hand, short chain fatty acids are electronically charged at the gastrointestinal pH, and therefore a lower redox potential is expected in the large intestine (V Stirrup, S J Ledingham et al., 1990; Sousa, Paterson et al., 2008).

1.2.4 *Colon as a site for drug delivery*

The high viscosity of fluids and the lower availability of fluids, especially after the hepatic flexure, turn the dissolution, particularly of poorly soluble drugs, difficult and limit the diffusion of drugs from the lumen to the site of absorption. Additionally, the wider lumen diameter reduces the exposure of drug molecules to the site of absorption (Basit, 2005). However, a greater part of the gut transit time is spent in this part of the GI tract (almost 80%), which makes this site of the gut favorable not only for absorption of some drugs but also favorable for activation of colonic prodrugs with release of the respective drug (Tuleu, Basit et al., 2002; Stella, Borchardt et al., 2007; Sousa, Paterson et al., 2008). Actually, some drugs that are considered to have good absorption at colonic level including, diclofenac, 5-fluorouracil, glibenclamide, ibuprofen, lamirocoxib, metoprolol, nifedipine, salicylic acid, simvastatin between others listed by Emma *et al.* (Mcconnell, Liu et al., 2009).

Moreover, the colon is a relatively inert site for the metabolism of drugs by cytochrome P450 (Biechea, Narjozb et al., 2007), and therefore drugs that are substrates for this enzyme have higher absorption from the colon, as demonstrated for simvastatin (Tubic-Grozdanis, Hilfinger et al., 2008). Additionally, the levels of efflux transporters (e.g. P-glycoprotein) are lower in the colon, which may also contribute to improve oral drug bioavailability of drugs that are substrates for these transporters (Englund, Rorsman et al., 2006; Berggren, Gall et al., 2007).

The metabolic potential of colon is equal or greater than of the liver. In general, while oxidation and conjugation are the predominant reactions metabolism in the liver, reduction and hydrolyses are the preferred ones in the large intestinal lumen (Jung and Kim, 2010). Colonic microbiota is capable of metabolizing a wide variety of drug molecules; over 30 drug molecules have been identified as substrates for colonic bacteria (Sousa, Paterson et al., 2008). The main reductive enzymes include nitroreductase, azoreductase, deaminase and urea dehydroxylase (Scheline, 1973). On the other hand, between hydrolytic enzymes, glycosidases are particularly important once they are involved in the activation of polyssacarides based systems. The prevalent glycosidases are amylase, pectinase, xylanase, α-L-arabinofuranosidase, β-D-xylosidase, β-D-galactosidase and β-D-glucosidase (Englyst, Hay et al., 1987). The presence and capacities of these enzymes produced by the microbiota at colonic level are the key to the design of colonic delivery systems activated by

microbiota and also colonic prodrugs (e.g. sulfasalazine) able to site-specific release of drugs into the colon (Mooter, 2006). Further description about colonic enzymes is present in Chapter III, namely related enzymes that are possibly involved on the degradation of cyclodextrins.

The colon is a site of interest to delivery drugs that are medicated to colonic diseases, inflammatory bowel diseases (Chron's diseases and ulcerative colitis), constipation/diarrhoea, colorectal cancer, irritable bowel disease and intestinal infections. Drug is released directly in the affected area, not only resulting in an increase of the therapeutic potency by reduction of the dose administered, but also decreasing the occurrence of adverse effects, which results from the release of drugs in the upper GI tract or unnecessary systemic absorption (Chourasia and Jain, 2003; Gupta, Bhandari et al., 2009). Glucocorticoids, mesalazine, antimicrobial agents (nalidix acid and sulfa drugs), antispastic agent (mentol), anti-constipation (naloxone and nalmefene), chemopreventive agents (ursodeoxycholic acid), anti-cancer agents (9-aminocampothecin, 5-fluorouracil), immunosuppressive agents, vermicides (mebendazole), cyclooxygenase-2 (COX-2) selective inhibitors to prevent colon rectal cancer, peptides and proteins drugs have high potential to be delivered to the colon.

Moreover, therapeutic agents for the management of arthritis, asthma and gastric ulcer, which commonly have a peak symptom at bedtime, are potential candidates for colonic release. The treatment according to the circadian rhythm is called chronopharmacology, which includes anti-inflammatory drugs, histamine receptor 2 blokers, proton pump inhibitors and antiasthmatic agents (Jung and Kim, 2010).

1.2.4.1 Colonic delivery of non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are the most commonly prescribed therapeutic agents (Jones, 2001) and are good candidates for the development of controlled release preparations, particularly through the oral route. Their anti-inflammatory action is mainly based in the inhibition of cyclo-oxygenase (COX), which is an enzyme that converts arachidonic acid into inflammatory mediators such as prostaglandins, prostacyclins, and thromboxanes (Kim, Brar et al., 2009). Two important COX enzymes have been described: COX-1, which is constitutively expressed in all tissues is involved in the synthesis of prostaglandins that protect the stomach and kidney from damage, and COX-2 which is almost undetectable in most tissues under normal

physiological conditions, but is induced by inflammatory stimuli, such as cytokines, being responsible for prostaglandins that contribute to the pain and swelling of inflammation (Vane and Botting, 1998). These drugs are widely used as anti-pyretics, analgesics, and in the treatment of chronic inflammatory diseases, such as arthritis.

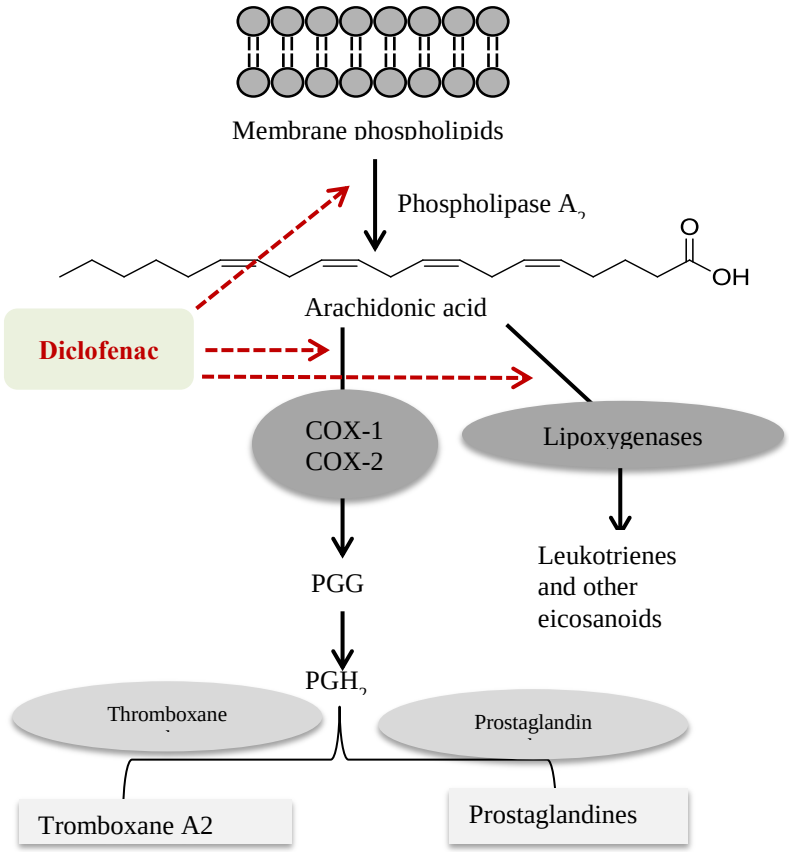


Figure 1.5 The main sites of action of the anti-inflammatory drugs, adapted from the reference (Ulrich, Bigler et al., 2006).

Beyond these traditional therapeutic indications, nowadays NSAIDs are also used as agents able to protect against Alzheimer's disease (Veld, Ruitenberg et al., 2001; Etminan, Gill et al., 2003). Other new applications of these drugs are being pursued, particularly in the cure and prevention of adenomas and colon tumors. Inflammation is now considered a well-established cancer risk factor, and a number of inflammatory conditions predispose to cancer, including ulcerative colitis (which increases about 20-fold the risk of developing colon cancer) (Coussens and Werb, 2002). Cancer prevention studies suggest that there may be a 40–50% reduction in mortality from colorectal cancer in people who take aspirin or other NSAIDs on a regular basis (Smalley and Dubois, 1997). NSAIDs are effective chemopreventive agents against colorectal neoplasia and are considered effective as single agents because they work

early in carcinogenesis across multiple pathways (Ulrich, Bigler et al., 2006; Cuzick, Otto et al., 2009). Second, NSAIDs reduce the number and size of colon adenomas (Hanif, Pittas et al., 1996). However, the gastrointestinal toxicity associated with the conventional NSAIDs may limit their long-term use for cancer prevention Long-term NSAIDs therapy has been associated with an increased risk of gastric erosion, ulcers and bleeding (Go, 2006). NSAIDs disrupt the normal gastric mucosal barrier of bicarbonate and hydrophobic mucus by two mechanisms. The principal mechanism, independent of route of administration, results from disturbance of prostaglandin synthesis by inhibition of the constitutive cyclo-oxygenase (COX) isoenzyme, COX-1. In the stomach, prostaglandins maintain mucosal blood flow, stimulating secretion of bicarbonate and mucus and regulating mucosal cell turnover and repair. Loss of protection by inhibition of prostaglandins renders the stomach vulnerable to damage by gastric acid. The second mechanism arises from the fact that NSAIDs become lipid soluble at low pH. Taken orally, they can cross the lipid barrier into gastric mucosal cells. At intracellular pH, they lose lipid solubility and become trapped, disrupting cell function, perhaps by inhibiting mitochondrial oxidative phosphorylation. Damage to surface mucosal cells compromises the normal protective mechanisms, reducing resistance to acid damage (Hayllar and Bjarnason, 1995; Hawkins and Hanks, 2000). It has been estimated that about 60% of all patients chronically treated with NSAIDs present dyspeptic symptoms and around 30% have gastroduodenal ulcers (Silverstein, Graham et al., 1995; Laine, 2001).

In order to decrease gastrointestinal effects associated with NSAIDs various approaches have been adapted to formulate systems to specifically release drugs in the colon (Luppi, Cerchiara et al., 2003; Akhgaria, Garekania et al., 2005; Orlu, Cevher et al., 2006; Maestrellia, Zerroukb et al., 2008; Piao, Lee et al., 2008).

1.2.4.2 Diclofenac

Diclofenac [2-(2, 6-dicloranilino) phenyl acetic acid] is a non-steroidal anti-inflammatory drug, usually found as sodium or potassium salt. As a representative of the phenylacetic acid derivatives group, diclofenac is widely used in the long-term treatment of inflammation and painful conditions of rheumatic and non-rheumatic origin (Sallmann, 1986). This molecule inhibits preferentially COX-2 (Warner, Giuliano et al., 1999; Hinz, Rau et al., 2003; Yamazaki, Muramoto et al., 2006), and also inhibits the lipoxygenase pathway, reducing the

formation of leucotriens (Elder, Halton et al., 1997). In addition, diclofenac can inhibit phospholipase A_2 which may explain the high anti-inflammatory activity of this drug.

Much interest involves the specific release of diclofenac in the colon, given that such an approach would allow for circumvention the adverse gastric effects notoriously associated with drugs of this class. It is also widely believed that the colonic delivery of diclofenac would provide a suitable way for the management of arthritis pain by chronotherapy (Lin and Kawashima, 2012). Furthermore, diclofenac is known to be well-absorbed in the colon (Gleiter, Antonin et al., 1985) and has demonstrated to have an effective chemopreventive action against colon cancer (Saini, J. Kaur et al., 2009; Jasmeet and Nath, 2010).

Several strategies have been studied with the purpose to release diclofenac in the colon, namely a time-based system that consisted in a polymeric matrix tablet (González-Rodríguez, Maestrelli et al., 2003), a time- and pH-dependent colon-specific tablet (Cheng, An et al., 2004), a combination of pH dependent polymer coating and a matrix able to release the drug by an ionic-exchange mechanism (Ambrogi, Perioli et al., 2008), Eudragit® L 100-55 nanofibers – a pH dependent system (Shen, Yu et al., 2011), poly(methyl acrylates)-coated chitosan‑diclofenac sodium nanoparticles (Huanbutta, Sriamornsak et al., 2013). Below is described the main systems for colonic delivery of drugs.

1.3 Systems to colonic target delivery of drugs to the colon

Because of the distal location of the colon in the GI tract, an ideal system for colonic delivery should prevent drug release in the stomach and small intestine, and provide an abrupt onset of the drug upon entry into the colon (Yang, 2008). Rectal administration offers the shortest route for targeting drugs to the colon. Rectal formulations such enemas, foams and suppositories seem to be a very simple way for local drug delivery. However, it allows only reaching the rectum and lower parts of the distal colon. Reach the proximal part of colon via rectal administration is difficult. Moreover, this route of administration can be uncomfortable for patients and compliance may be less than optimal (Mooter, 2006). Therefore, the oral delivery is presented as the most convenient and preferred way to target the colon.

Many different colon-specific drug delivery systems have been investigated based on the exploration of selected physiological parameters that exhibit particular features only in the

colon. However, due to the similarity between distal small intestine and the proximal colon, there are not many options that allow drug release only in the colon (Basit, 2005). Systems have been developed considering the next aspects: variation of pH along the GI tract (pH-based systems), transit time in the GI tract (time-based systems), intra-luminal pressure (pressure-based systems), composition of the microbiota (microbiota-based systems) or the combination of two of these anterior factors (**Table 1.1**).

- pH-based systems

pH-dependent systems are designed based on the difference in pH-levels along the GIT. These formulations use pH sensitive polymer-based coatings that are insoluble at low pH levels, preventing dissolution and drug release in the upper gut but dissolving in the elevated pH conditions of the distal gut (Mcconnell, Liu et al., 2009). The thickness of the coating is the determinant factor for the success of these systems. The most commonly used pH-dependent polymers are methacrylic acid copolymers usually known as Eudragit®. The major drawback of these systems is associated with the fact that pH is not predictably stable and therefore the site-specificity of formulations can be poor. pH is influenced by diet, disease (e.g. pH is lower in inflammatory bowel disease and in ulcerative colitis) and intra and inter-individual differences observed along the GI tract (Leopold and Eikeler, 2000; Mooter, 2006; Mcconnell, Fadda et al., 2008). It was reported that release of drug from these systems may start in the proximal small intestine (Singh, 2007) and, on the other hand, some of these formulations pass intact through the gut, due to failure of the coating to dissolve (Sinha and Kumria, 2003; Ibekwe, Fadda et al., 2006; Ibekwe, Liu et al., 2006; Mcconnell, Liu et al., 2009).

- Time-dependent systems

Time-dependent systems are designed to release the drug after a predetermined lag time of 5 or 6 hours, which is expected to be the time required for the dosage form to reach the colon (Basit, 2005). These systems use erodible or a swelling coating polymers (Table 1.1).

These formulations are susceptible to variable gastrointestinal transit, in particular, variable gastric emptying due to the size, shape and density of the dosage form and/or state of feeding and health condition of the subject (accelerated transit time is observed in IBD, carcinoid syndrome, diarrhea and ulcerative colitis) (Coupe, Davis et al., 1991a; Coupe, Davis et al.,

1991b; Vassallo, Camilleri et al., 1992; Ohe, Camilleri et al., 1993; Mcconnell, Liu et al., 2009). Therefore, the colon arrival time of a dosage form cannot be accurately predicted and, consequently, it is considered that this time-based systems cannot afford a reliable drug release in the colon (Mcconnell, Fadda et al., 2008).

- Pressure controlled systems

Presssure controlled systems are designed to release the drug by rupture of the dosage form in response to the raised luminal pressure in the distal gut. This is caused by the increase of viscosity and quantity of luminal contents (Yang, Chu et al., 2002). The performance of this system appears to be dependent on thickness of the coating and the capsule size of the capsule (Yang, Chu et al., 2002; Meissner and Lamprecht, 2008). The data related to the pressure along the GI tract are limited.

- Microbiota activated systems

These systems are designed taking in account the abrupt increase of bacteria population and associated enzymatic activities in ascending colon. These systems can be divided into: coating/matrices systems, which use polymers susceptible to fermentation in the lower intestine and prodrugs activated by colonic enzymes. This approach seems to be more site-specific since the biodegradable enzymes produced by the vast microbiota only exists in the colon and, therefore, this is the most promising strategy for colonic delivery of active agents.

The coating/matrices systems can be formulated using natural polymers namely polysaccharides (plant (e.g., pectin, guar gum, inulin, amylose), animal (e.g. chitosan, chondroitin sulphate), algal (e.g., alginates) or microbial (e.g., dextran)), or synthetic or semi-synthetic polymers. These natural polymers are found in abundance, are inexpensive, non-toxic and biodegradable. However, some of them are hydrophilic leading to premature release of drugs. Hence, these natural polymers can be chemically modified to increase their hydrophobicity (semi-synthetic polymers). Additionally, these systems can exploit the use of synthetic polymers (Fadda, Mcconnell et al., 2009; Shukla and Tiwari, 2012). With respect to safety, natural polymers are more favourable. On the other hand, the derivatization of natural polymers can result in decrease of biodegradability (Mooter, 2006).

Prodrugs approach provides site-specificity delivery and has been intensively explored. In the market, produgs of 5-aminosalicilic acid are commercially available (Table 1.1). The reduction of the azo bond, with the aid of azoreductase enzymes produced by the large intestinal microbiota, has been the basis of the development of colonic prodrugs, including those available in the market. The first example of this is sulfasalazine, which consists of mesalazine (5-ASA) linked via an azo bond to a sulphonamide, sulfapyridine (Zoetendal, Akkermans et al., 1998). Due to the undesirable side effects caused by sulfapyridine, an effort has been made to replace sulfapyridine by carrier molecules thought unlikely to produce side effects (Sousa, Paterson et al., 2008). Therefore, olsalazine, which consists of 2 molecules of 5-ASA linked by an azo-bond, was developed. However, this molecule did not demonstrate a proportionally greater effect. Other azo prodrug of mesalazine is balsalazide which uses an inert carrier to transport mesalazine into the colon (Basit, 2005). A drawback of azo prodrugs is that the reduction process might be too slow, especially when diarrhoea occurs and leads to fast elimination of the delivery system (Meissner and Lamprecht, 2008). Other carriers have been explored for site colonic delivery of prodrugs (section 1.3.1).

- Combined systems

Complementary strategies considering the combination of different mechanisms of colonic drug release have been developed in order to overcome the limitations associated with systems that take in account only one of the mechanisms to trigger the colon.

1.3.1 *Prodrugs as a strategy for site colonic delivery of drugs*

Currently, it is estimated that about 10% of worldwide marketed drugs can be classified as prodrugs (Zawilska, Wojcieszak et al., 2013). For a drug to become a prodrug, the drug should have an appropriate functional group able to conjugate by a covalent linkage with a colonic specific carrier. The prodrug should pass intact through the upper gastrointestinal tract, but once in the large intestine, the covalent link should be cleavable via a reduction or a hydrolysis process activated by the resident microbiota, releasing the active drug molecule from the carrier (Stella, Borchardt et al., 2007; Jung and Kim, 2010; Dhaneshwar and Vadnerkar, 2011) To pass intact the upper gastrointestinal tract without significant absorption, it should be hydrophilic or large, which prevents passive transport through the intestinal epithelial cell layer (Ungell, Nylander et al., 1998; Chae, Jang et al., 2005). Therefore, the choice of the carrier is critical.

Table 1.1 Oral formulations for colonic site-specific delivery in the market.

Colon Delivery System	Market Brand	Description	Reference
pH dependent systems	Asacol® (mesalazine)	Coating polymer soluble at pH > 7 (Eudragit S)	(Ibekwe, Liu et al., 2006)
	Salofalk® (mesalazine)	Eudragit®L-coated tablet (release at pH >6)	(Singh, 2007)
	Budenofalk® (budenoside)	Eudragit S and L - coated granules (slow release at pH>6.4)	(Ibekwe, Fadda et al., 2006; Ibekwe, Liu et al., 2006)
	Entocort® (budesonide)	Eudragit® L100-55-coated ethylcellulose granules (slow release at pH >5.5)	(Edsbäcker, Bengtsson et al., 2003)
Time dependent systems	Pentasa®	Ethylcellulose-coated granules in tablet or sache form (slow release of drug through membrane)	(Wilding, Kenyon et al. 2000)
Microbial triggered delivery	Dipentum® (olsalazine)	Azo prodrugs of 5-aminosalicic acid.	(Roldo, Barbu et al., 2007)

Among hydrophilic small molecules, aminoacids, glycosides and sulphate have been explored as carriers to target drugs to the colon. Aminoacids have polar groups, like the $-NH_2-$ and $-COOH-$ that reduce the membrane permeability, and are additionally free of adverse effects. Amide prodrugs can be hydrolyzed by the amidase originated from microbes in the colon (Scheline, 1973). Conjugation of a drug with a sugar moiety (glucose, galactose, cellulose, glucoronic acid) forms glycosides (glucoside, galactoside, cellobioside, glucoronides). Glycoside prodrugs are hydrophilic and poorly absorbed from the small intestine, but once they reach the colon, they can be effectively cleaved by bacterial glycosidases to release the free drug or to be absorbed by the colonic mucosa (Sinha and Kumria, 2001). Conjugation with sulphate is another approach that has been explored since these prodrugs are activated through the action of sulphatases at colonic level (Jung, Doh et al., 2003).

Table 1.2 Colonic prodrugs designed using small carrier and the *in vitro*/*in vivo* performance.

	Carrier	Drug	Performance of the prodrug	Reference
Aminoacids conjugates	Tyrosine Methionine Alanine Glutamic acid	Salicylic acid	Amide prodrugs tested in rabbits: methionine prodrug absorved in the duodenum; tyrosine prodrug was hydrolyzed in the small intestinal mucosa; L-alanine and glutamic acid prodrugs were hydrolyzed only in the contents of the lower GI tracts such as caecum and colon; D-alanine with negligible hydrolyses (tested in rabbits).	(Nakamura, Shiota et al., 1988; Nakamura, Kido et al., 1992; Nakamura, Tagami et al., 1992)
	Glycine Aspartic acid Glutamic acid Taurine Cysteine	5-aminosalicylic acid	Amide prodrugs are stable in the upper intestinal; glycine prodrug releases 65% and 27% of 5-ASA in caecal and colonic contents in 8 hours of incubation, respectively; aspartic prodrug releases 37% of 5-ASA, while glutamic prodrug releases 8% of 5-ASA in caecal contents in 16 h of incubation; taurine prodrug release 45% and 20% of the drug in caecal and colonic produg in 8 hours; cysteine prodrug releases 92% of 5-ASA after 24 hours.	(Jung, Lee et al., 2000; 2001; Jung, Kim et al., 2003; Jung, Kim et al., 2006) (Leopold and Friend, 1995a; Leopold and Friend, 1995b)
Glucosides conjugates	Glucose Galactose Cellobiose	Dexamethasone Prednisolone Hydrocortisone Fludrocortisone	Prodrug hydrolyses is low in the stomach and the proximal small intestinal contents and increases in the contents of distal small intestine, and is maximum in the caecal content homogenates. Galactoside prodrugs were found to be more rapidly hydrolyzed than the corresponding glucosides, which are hydrolyzed faster than the cellobioside.	(Friend and Chang, 1985)
	Glucose	Dexamethasone Prednisolone .	Dexamethasone glucoside showed that nearly 60% of the oral dose reached the caecum between 4–5 hours, while the free drug is absorbed from the small intestine. Contrarily, the prednisolone prodrug was not that site-specific, with 15% of dose administered reaching the caecum in 4–5 h and only 11% recovered unabsorbed from the caecum.	(Friend and Chang, 1984; Friend and Tozer, 1992)
	Glucoronic acid	Dexamethasone budesonide	A similar study conducted in normal and colitic rats showed a high level of drug delivery to the colon using glucuronide prodrugs of dexamethasone and budesonide.	(Haeberlin, Rubas et al., 1993) (Cui, Friend et al., 1994; Nolen, Fedorak et al., 1997)
		Naloxone and Nalmefene	These prodrugs are activated in large intestine, followed by a resultant diarrhea. These prodrugs do not interfere with the central analgesic effects of narcotics.	(Simpkins, Smulkowski et al., 1988)
Sulphate conjugates	Sulphate	Prednisolone Dexamethasone	Sulfate ester can serve as a novel colon-specific pro-moiety which limits the absorption in the upper intestine and release the drug by action of microbial sulfatse.	(Jung, Doh et al., 2003) (Kim, Kong et al., 2006)

The large molecules used as a carrier are natural or synthetic polymers which undergo metabolism by the colonic microbiota. Examples of biodegradable molecules are poly(L-aspartic acid) (Leopold and Friend, 1995b), polyssacarides including dextran, starch, starch, alginates, glycogen, pullullan, agarose, cellulose, chitosan, chitin and carrageenan (Vandamme, Lenourry et al., 2002; Dhaneshwar and Vadnerkar, 2011) and oligossacarides namely cyclodextrins (section 1.2.2) (Kamada, Hirayama et al., 2002). In the case of large carriers used to design prodrugs, the respective drug should have high efficacy in order to provide effective dose on administration of a practical dose of prodrug. The setback of polymeric materials is the large quantity required to be taken orally, which is not suitable when the drug has low efficacy (e.g. 5-ASA whose dose is 0.5 to 3 g/day). The problem of the large quantity of polymeric prodrug can be circumvent increasing the degree of substitution of the polymer, this is, by increasing the number of active molecules linked to each unit of polymer (Mooter, 2006; Jung and Kim, 2010).

Cyclodextrins (section 1.2.2) are cyclic oligosaccharides that revelead to be promising in targeting drugs to the colon and are explored in this thesis as carriers for colonic delivery (Kurkov and Loftsson, 2013). Following, it is presented a brief description of the use of cyclodextrins in the field of oral drug delivery, including their application on colonic drug delivery.

1.3.2 *Cyclodextrins*

The first written record on cyclodextrins was published in 1891 by the French scientist A.Villiers. He described the isolation of a bacterial digest from starch, which was stable towards acid hydrolysis and, like starch, did not display reducing properties. It is now believed that Villiers had isolated a mixture of α- and β-cyclodextrin (α-CD and β-CD).

Later, an Austrian microbiologist, Franz Schardinger, described two compounds that he had isolated from bacterial digest of potato starch, which he designated α-dextrin and β-dextrin. It was not until the 1940s, however, that the structure and physicochemical properties of cyclodextrins (CDs) were described in detail. The first cyclodextrin-related patent was issued in Germany in 1953 (Loftsson and Duchêne, 2007; Loftsson and Brewster, 2010).

Cyclodextrins, also named Schardinger dextrins, cycloglucans or cycloamyloses make up a family of oligosaccharides comprised of five or more D-glucose attached by α-1-4 glycosidic

linkages. Cyclodextrins are produced from starch by the action of an enzyme, cyclodextrin glycosyltransferase (CDTGase; ED 2.4.1.19), which can be produced by a variety of bacteria.

The three most common cyclic oligosaccharides are α-, β- and γ-CD consisting of six, seven and eight glucose units, respectively (Szejtli, 1998). Cyclodextrins consisting of more glucose units also exist, but they are currently too expensive to be utilized in the development of practical applications.

As can be seen in the Figure 1.6**,** glucose repeating unit of a cyclodextrin contains three different hydroxyl groups. The glucose repeating unit contains one primary hydroxyl group at position 6 and two secondary hydroxyl groups connected to C-2 and C-3 (Wenz and Höfler, 1999).

The whole molecule assumes a rigid structure because of the formation of a belt of intramolecular hydrogen bonds between hydroxyl groups at the 2- and 3-positions of adjacent glucose units. The rotation of the secondary hydroxyl groups is thus restricted, whereas rotation of the primary hydroxyl groups is free, thus reducing the effective diameter of the cavity on the primary side of the molecule (Khan, Forgo et al., 1998).

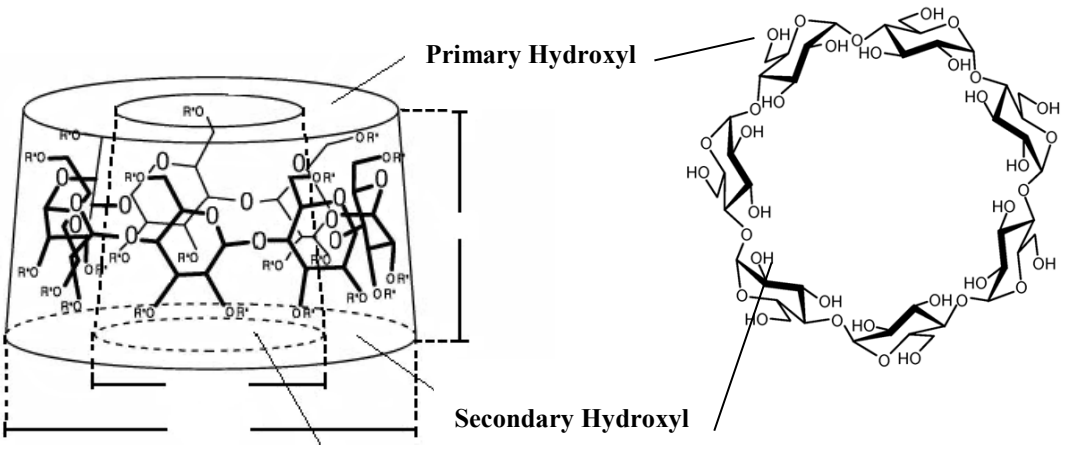


Figure 1.6 Schematic representation of the cone-shaped structure of the cyclodextrin.

Therefore, CDs possess doughnut-like annular structures with wide and narrow hydrophilic ends delineated by secondary 2-OH and 3-OH and primary 6-OH groups, respectively. The presence of the hydroxyl groups makes the upper and lower end of the molecule hydrophilic.

The cavity of the cyclodextrin rings consists of a ring of C-H groups, a ring of glycosidic oxygen atoms and again a ring of C-H groups. This renders the interior of the cyclodextrin rings hydrophobic, providing a cavity for the inclusion of non-polar guest compounds.

Encapsulation of a molecule within cyclodextrin depends upon the size of the cavity and the drug. α-Cyclodextrin is generally too small, γ-CD is not stable enough, and hence β-cyclodextrin has been extensively used in this area (Khan, Forgo et al., 1998). However, β-cyclodextrin has a solubility lower than the others cyclodextrins. The unusual low water solubility of β-cyclodextrin may be explained by the very rigid structure resulting from the H-bonding of the C-2 hydroxyl of a glucopyranose unit with the C-3 hydroxyl of an adjacent unit**.** Interference with the hydrogen bonding of the hydroxyl groups increases the water solubility of the cyclodextrins. At high pH, ionization of hydroxyl groups increases water solubility; at pH 12.5 the solubility of β-cyclodextrin is 750 g/L (Coleman, Nicolis et al., 1992).

Chemically modified cyclodextrin derivatives have been synthetized with a view to extend the physiochemical properties and inclusion capacity of the parent CDs. In general, natural cyclodextrins have low aqueous solubility. Modifications of the natural cyclodextrins allow to overcome its low solubility and, therefore, improves its applicability.

Table 1.3 Physical properties and molecular dimensions of the natural cyclodextrins (Valle, 2004).

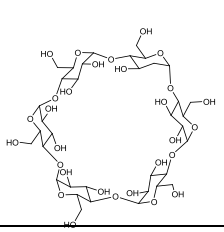
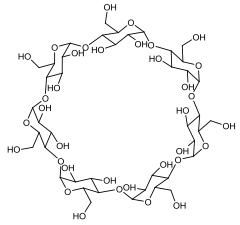
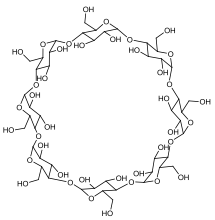
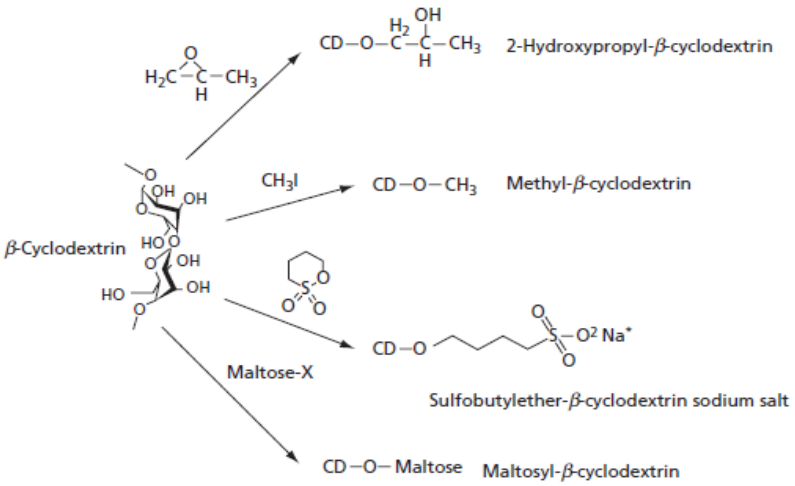
Cyclodextrin	**α**	**β**	**ϒ**
Structure			
Number of glucose Units	6	7	8
M_W (g/mol)	972	1135	1459
Water Solubility (g/100ml) at 25°c	14.5	1.85	23.2
Cavity Diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3

Table 1.4 Structural and physiochemical properties of selected cyclodextrin of pharmaceutical interest (Marcus E. Brewster and Loftsson, 2007; Loftsson and Brewster, 2010).



Cyclodextrin	MS	M_W (Da)	Solubility in water (mg/mL at 25ºC)
2-Hydroxypropyl-α-cyclodextrin (HPαCD)	0.65	1199	>500
2-Hydroxypropyl-β-cyclodextrin (HPβCD)	0.65	1400	>600
Sulfobutylether-β-cyclodetrin sodium salt (SBEβCD)	0.9	2163	>500
Randomly methylated β-cyclodextrin (RMβCD)	1.8	1312	>500
	0.57	1191	200
Maltosyl β-cyclodextrin (MβCD)	0.14	1459	>500
2-hydroxypropyl-γ-cyclodextrin	0.6	1576	>600

MS:molar degree of substitution; M_w:

Chemically modified cyclodextrins of pharmaceutical interest are classified in hydrophilic, hydrophobic and ionizable cyclodextrin derivatives. In the hydrophilic derivatives, the hydroxypropyl derivatives of β- and γ-cyclodextrin, the randomly methylated β-cyclodextrin, sulfobutylether β-cyclodetrin hydroxyalkylated and branched cyclodextrin derivatives are the most relevant ones (Loftsson, Brewster et al., 2004) The introduction of ionizable groups on the hydroxyl groups of cyclodextrins offers a pH-dependent hydrophilic activity on such derivatives. In this group, carboxymethyethyl cyclodextrins are the most important ionizable derivatives.

The physicochemical properties of the CD derivatives, including their aqueous solubility and complexation capabilities, not only depend on the structure of the appended substituent but

also on their location within the CD molecule and the number of substituents per CD molecule. The molar degree of substitution (MS) is defined as the average number of substituents that have reacted with one glucopyranose repeat unit (Loftsson and Brewster, 2010).

1.3.3 *Cyclodextrins in oral drug delivery*

The lipophilic microenvironment of the central cavity of CDs gives the ability to form inclusion complexes by taking the whole molecule or rather some nonpolar parts in its hydrophobic cavity. Taking this in advantage, cyclodextrins can enhance the bioavailability of hydrophobic drugs by increasing drug solubility, dissolution and/or drug permeability.

The inclusion complexes between poorly soluble drugs and hydrophilic cyclodextrins allow the increase of water solubility of the drug and consequently the dissolution rate. The increase in the dissolution performance can improve the oral bioavailability of class II and IV drugs of BCS (Biopharmaceutical Classification System), improving the pharmacological effect and allowing a reduction in the dose of the administered drug (Valle, 2004; Loftsson, Vogensen et al., 2007; Rebecca L. Carrier, Lee A. Miller et al., 2007; Vyas, Saraf et al., 2008). Otherwise, CDs increase bioavailability by increasing the permeability of hydrophobic drugs, by making the drug available at the surface of the biological barrier of the intestine. In case of water-soluble drugs, CDs increase drug permeability by direct action on mucosal membranes (Rajeswari Challa, Alka Ahuja et al., 2005; Loftsson, Vogensen et al., 2007).

In addition, CDs are mainly used to improve other desirable properties of drugs such as stability (Rajeswari Challa, Alka Ahuja et al., 2005), gastrointestinal tolerability (Loftsson, Brewster et al., 2004), and can be used to mask the undesirable taste of drugs (Rajeswari Challa, Alka Ahuja et al., 2005; Loftsson, Vogensen et al., 2007).

This ability to form complexes makes CDs applicable as functional drug carriers controlling rate and/or time profile of oral drug release. The hydrophilic derivatives (methylated, hydroxyalkylated, branched 6-glucosyl and maltosyl) and ionizable derivatives (carboxyalkyl, carboxymethyl, sulfates, alkylsulfonates) of CDs can serve as potent drug carriers for immediate release and delay release formulations, respectively, while the release rate of water soluble drugs can be retarded by hydrophobic derivatives of CDs (ethylated and peracylated) (Hyrayama and Uekama, 1999; Uekama, 2002; Uekama, 2004).

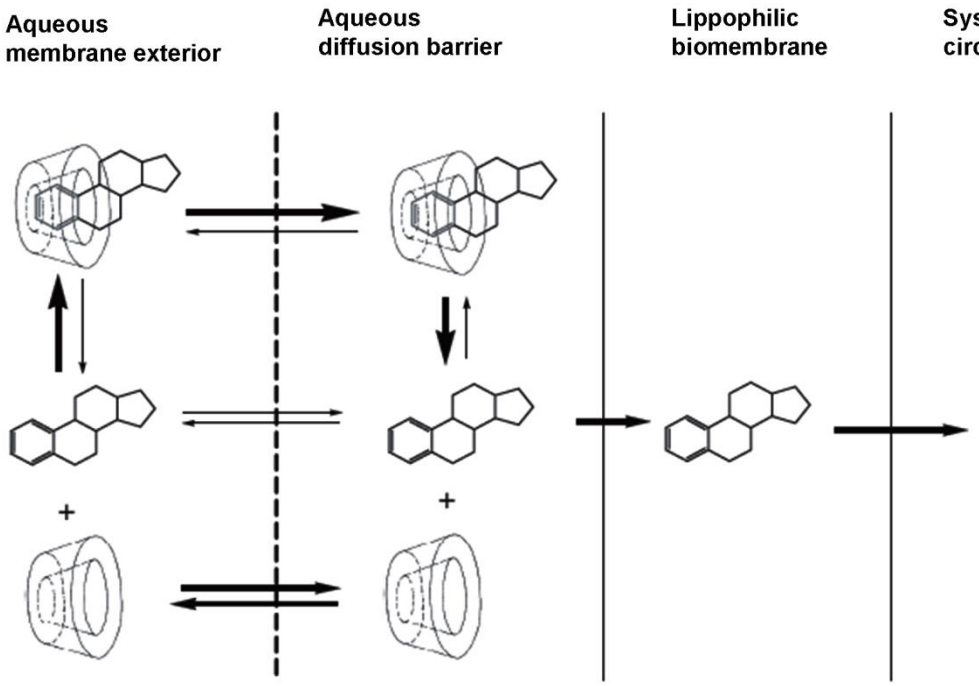


Figure 1.7 Effect of cyclodextrin complexation on the drug bioavailability after non-parental administration. (Loftsson, Jarho et al., 2005).

Table 1.5 The biopharmaceutics classification system and the effect of drug/cyclodextrin complexation on the oral bioavailability of drugs (Loftsson, Brewster et al., 2004).

FDA Class*	Drug properties		RDS to drug absorption[3]	Effect of drug complexation
	Aqueous solubility[1]	Permeability[2]		
I	Highly soluble	Highly permeable	(good bioavailability)	Can decrease absorption
II	Poorly soluble	Highly permeable	Aqueous diffusion	Can enhance absorption
III	Highly soluble	Poorly permeable	Membrane permeation	Can decrease absorption
IV	Poorly soluble	Highly permeable	Aqueous difusion and membrane permeation	Can enhance absorption

*FDA Biopharmaceutics Classification System of orally administered drugs. [1]Intrinsic solubility of the drug in the aqueous membrane exterior. [2]Passive drug permeation through lipophilic biomembrane such as the gastrointestinal mucosa. [3]RDS of drug delivery from the aqueous exterior into the body.

FDA: Food and Drug Administration; RDS: Rate determining step

1.3.3.1 Cyclodextrin conjugates for colonic drug delivery

When administered orally, a CD complex is in equilibrium with the molecule and the empty CD. In GI tract fluids, the degree of dissociation is dependent on the magnitude of the stability constant of the complex (Hyrayama and Uekama, 1999).

This form of drug carrying as an inclusion complex is not suitable to deliver the drug to the targeted site, since the drug complex can dissociate and the drug releases, by dilution process and/or competitive inclusion effects before reaching the colon. To overcome this problem the drug can be conjugated with a cyclodextrin. In this conjugation the drug bonds covalently to cyclodextrin (Uekama, 2004).

CDs are not absorbed through biological membranes such as the GI tract because of their bulky and hydrophilic nature. Natural α-CD, β-CD and γ-CD are more resistant towards non-enzymatic hydrolysis than the linear oligosaccharides. They are chemically stable under neutral and basic conditions and the hydrolyse rate increases in the order α-CD < β-CD, γ-CD (Szejtli, 1998; Loftsson and Brewster, 2010) Moreover, natural α- and β-cyclodextrins, unlike γ-cyclodextrin, cannot be hydrolysed by human salivary and pancreatic α-amylases. However, the vast microbiota present in the colon, especially *Bacteroides*, breaks these into small saccharides that are absorbed in the large intestine (Flourié, Molis et al., 1993; Irie and Uekama, 1997; V. R. Sinha and Kumria, 2001; Chourasia and Jain, 2003).

Therefore, CDs are useful as colon-targeting carriers, and various cyclodextrin prodrugs have been synthesized in order to obtain site-specific delivery of drugs to the colon. In addition, in the USA, natural CDs (α-, β-, γ-) are 'Generally Regarded As Safe' (GRAS) which allows safety assurance of these prodrugs as new entities (Astray, Gonzalez-Barreiro et al., 2009).

Moreover, fermentation of cyclodextrins leads to production of short-chain fatty acids (SCFA) that can contribute to the maintenance of the health and integrity of the colonic epithelium, as described previously (Giardina and Inan, 1998).

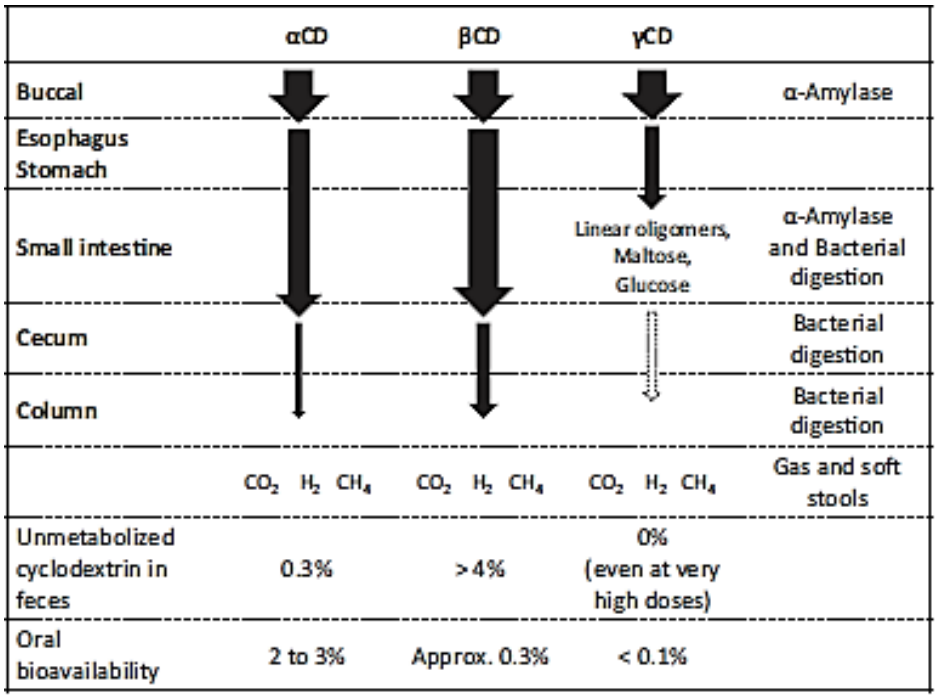


Figure 1.8 Schematic comparison of the digestion of natural α-CD, β-CD and γ-CD after oral administration. Cyclodextrins are slowly hydrolysed by α-amylases but relatively rapidly digested by bacteria. Adapted from (Kurkov and Loftsson, 2013).

Drug molecules were selectively conjugated using the primary or secondary hydroxyl groups of CDs through an ester or amide linkage. Therefore, a limited number of prodrugs can be formed due to the chemical linkage required in their formation.

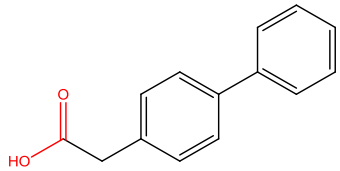
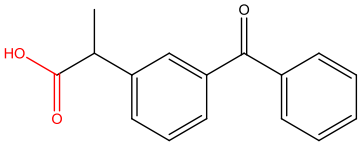
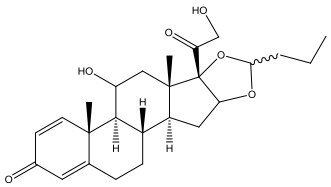
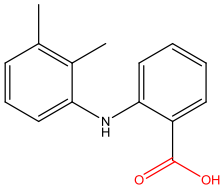
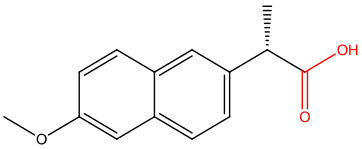
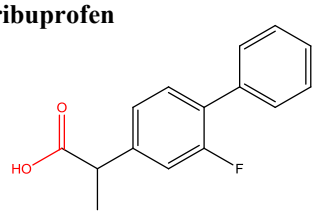
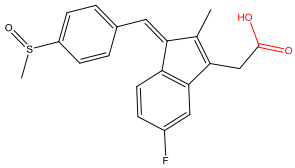
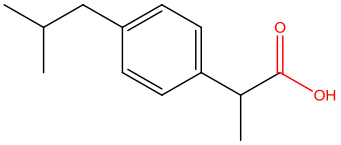
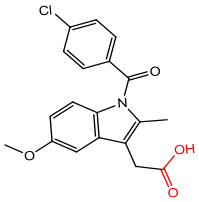
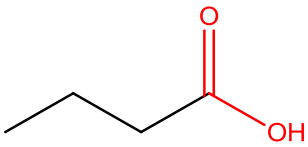
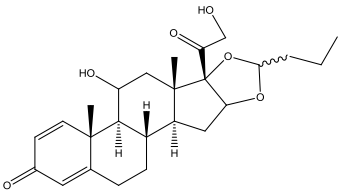
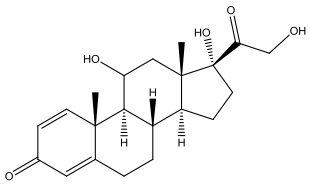
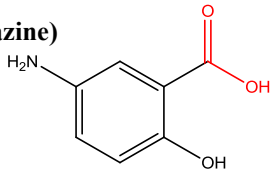
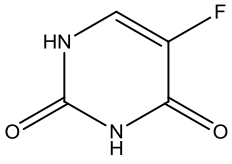
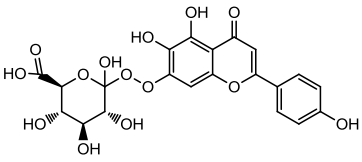
Several drugs have been combined with CDs to synthesize prodrugs enabling colonic specific release (Table 1.6). Cyclodextin conjugates of non-steroidal anti-inflammatory drugs include: byphenylacetic acid (Uekama, Minami et al., 1997a; Minami, Hirayama et al., 1998), ketoprofen (Kamada, Hirayama et al., 2002), etodolac (Rizzarelli, Vecchio et al., 2007), mefenamic acid (Dev, Mhaske et al., 2007), naproxen, flurbiprofen, sulindac (El-Kamel, A.M.Abdel-Aziz et al., 2008), ibuprofen, indomethacin (Namazi, Bahrami et al., 2005); short-chain fatty acids such as n- butyric acid (Hirayma, Ogata et al., 2000); steroidal drugs like prednisolone (Yano, Hirayama et al., 2001; Yano, Hirayama et al., 2002), budesonide (Prabhu, D.Satyanarayana et al., 2009); a drug for the treatment of inflammatory bowel disease, 5-aminosalicylic acid (Zou, Okamoto et al., 2005). There are also β-lactam antibiotics like ampicillin, amoxicillin, cephalexin (Namazi and Kanani, 2007); and recently anticancer drugs like 5-fluoracil (Udo, Hokonohara et al., 2010), and scuterallin, a flavonoid with anticancer activity (Yang, Zhao et al., 2013). All these examples have been designed

anticipating new candidates for colon-specific delivery prodrugs but, asfar as we know, there are no reports of cyclodextrin conjugate with diclofenac. In the case of prednisolone and budesonide a linker molecule, succinic acid, was used between drug and cyclodextrin, forming esters at both ends of the linker molecule (Yano, Hirayama et al., 2001; Prabhu, D.Satyanarayana et al., 2009). Modification of β-cyclodextrin with water soluble polymer poly(ethylene glycol) was used to produce conjugates of antibiotics (Namazi and Kanani, 2007) and conjugates of indomethacin and ibuprofen (Namazi, Bahrami et al., 2005).

In most of the drug-cyclodextrin conjugates, namely those with a ester linkage, the drug was released in the contents of large intestine, whereas only minor chemical hydrolysis occured in the upper GI tract (Uekama, 2004; Kurkov and Loftsson, 2013). These promising results highlight cyclodextrin-based prodrugs as an area for further research.

Table 1.6 Active molecules conjugated with cyclodextrins, including type of carrier, linkage and the presence/ absence of carrier.

Drug	Carrier cyclodextrins	Linkage Ester	Linkage amide	Spacer/ intermediate	Reference
biphenylacetic cid	α β ϒ	√ √ √	√ √ √		(Uekama, Minami et al., 1997b)
	Dimethyl-β	√			(Ventura, Paolino et al., 2003a)
etodolac	β	√			(Rizzarelli, Vecchio et al., 2007)
ketoprofen	α	√			(Kamada, Hirayama et al., 2002)
mefenamic acid	β	√			(Dev, Mhaske et al., 2007)
naproxen	α β	√ √			(El-Kamel, A.M.Abdel-Aziz et al., 2008)
flurbiprofen	α β	√ √			
sulindac	β α	√ √			
ibuprofen	β	√		Poly(ethylene glycol)	(Namazi, Bahrami et al., 2005)
indomethacin	β	√		Poly(ethylene glycol)	
butyric acid	β	√			(Hirayma, Ogata et al., 2000)
prednisolone	α β γ	√ √ √		succinic acid hemiester	(Yano, Hirayama et al., 2000; 2001)
budesonide	β	√		succinic acid hemiester	(Prabhu, D.Satyanarayana et al., 2009)
5-Aminosalicylic acid	α β ϒ	√ √ √			(Zou, Okamoto et al., 2005)
5-fluoracil	β	√	√	5-fluorouracil-1-acteic acid	(Udo, Hokonohara et al., 2010)
scutellarin	β		√	amino-cyclodextrin enamino- cyclodextrin dienamino-cyclodextrin	(Yang, Zhao et al., 2013)
ampicillin amoxicillin caphalexin	β β β	√ √ √		Poly(ethylene glycol)	(Namazi and Kanani, 2007)

[1]Biphenylacetic acid 	[2]Ketoprofen 	[3]Etodolac 
[4]Mefenamic acid 	[5]Naproxen 	[5]Fluribuprofen 
[5]Sulindac 	[6]Ibuprofen 	[6]Indomethacin 
[7]Butyric acid 	[8]Prednisolone 	[9]Budenoside 
[10] 5-Aminosalicylic acid (mesalazine) 	[11]Fluorouracil 	[12]Scuteralin 

[1](Hirayama, Minami et al., 1996; Uekama, Minami et al., 1997b; Ventura, Paolino et al., 2003a) , [2](Kamada, Hirayama et al., 2002) , [3](Rizzarelli, Vecchio et al., 2007), [4](Jilani, Pillai et al., 1997), [5](El-Kamel, A.M.Abdel-Aziz et al., 2008) [6] (Namazi, Bahrami et al., 2005)[7](Hirayama, Ogata et al., 2000) [8](Yano, Hirayama et al., 2000; Yano, Hirayama et al., 2002) [9](Prabhu, D.Satyanarayana et al., 2009), [10](Zou, Okamoto et al., 2005) [11](Udo, Hokonohara et al., 2010) [12](Yang, Zhao et al., 2013)

Figure 1.9 Structures of active molecules studied where cyclodextrins were used as a carrier to target the colon.

1.4 *In vivo* evaluation

1.4.1.1 Animal models

The ultimate investigation of any colon-specific drug delivery system must be performed *in vivo*, in humans. However, prior to the test in humans, it is common procedure to assess the performance of the delivery system in animal models. Different animal models such rabbit, rat and pig are used to evaluate the delivery of drugs to the colon because they resemble the conditions of the human GI tract (Figure 1.10). However, the choice of the animal model to perform *in vivo* studies is a very critical point, given the important anatomic, physiological, biochemical and metabolic differences that exist between human and the common laboratory animals (Kararli, 1995), which contributes to the noticeable differences on the behaviour of the drug delivery systems between species.

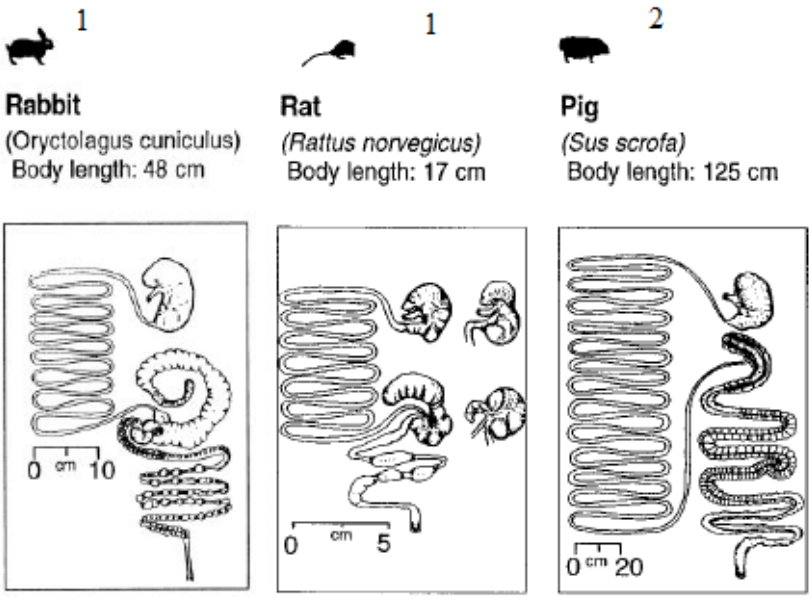


Figure 1.10 Gastrointestinal tracts of small hindgut-fermenting mammalian herbivore (rabbit) and gastrointestinal tracts of mammalian omnivores (rat, pig). Adapted from references [1](Stevens, 1977) and [2](Argenzio and Southworth, 1975).

In the case of colonic prodrugs different animal models have been used: rabbits, to evaluate aminoacids prodrugs (Nakamura, Kido et al., 1992; Nakamura, Tagami et al., 1992); pig, to evaluate dextran prodrugs (Harboe, Larsen et al., 1989); and rats, to study glucosides prodrugs (Simpkins, Smulkowski et al., 1988; Cui, Friend et al., 1994) and also cyclodextrin prodrugs (Kamada, Hirayama et al., 2002).

Below, it is described the main anatomic and physiological characteristics including differences on the pH (Table 1.7) and microbiota (Table 1.8) along the GI tract of each animal model considered thereafter in this dissertation.

1.4.1.2 Stomach

Like humans, pigs and rabbits have a glandular type stomach while the stomach of rats is divided in two main sections: glandular and non-glandular. The non-glandular section also called forestomach portion is an entry from esophagus and the site for storage and bacterial digestion (Kararli, 1995).

In rabbits, stomach is the major site of digestion (Yu, Chiou et al., 2000). Its stomach is normally never empty, herein the gastric emptying time is highly variable. After 24 hour of fasting, it has been shown that the stomach of an adult rabbit is still 50% full (Harboe, Larsen et al., 1989; Davies, Certzoomed et al., 2003).

The stomach of pigs is two to three times larger than the stomach of humans and the cardiac mucosa occupies a greater portion of the stomach compared to the human stomach (Kararli, 1995).

1.4.1.3 Small intestine, pancreas and gall bladder

The small intestine is the major site of absorption, due to its enormous surface and due to existence of villi and microvilli in all animals (Peetersg, Charlier et al., 1985; Kararli, 1995). In rabbits, the end of the ileum is expanded into a thick walled called *sacculus rotundus*, which seems to have an immunological function and is only found in *Lagomorphs* (Davies, Certzoomed et al., 2003; Merchant, Mcconnell et al., 2011). Like humans, rabbits are poor biliary excreters (Toutain, Ferran et al., 2010). Otherwise, rats are good biliary excreters, they do not have a gall bladder, and thus the bile enters the dudodenum continuously as it is made (Toutain, Ferran et al., 2010).

1.4.1.4 Caecum and colon

The large intestine provides a chamber for the final phase of digestion, for both herbivores (rabbits) and omnivorous (rats and pigs). The pig, rabbit and rat have a well-defined caecum whereas in man the caecum is very small and is continuous with the colon.

The colon of pig consists on, ascending, transverse and a portion of the descending colon coiled tightly into a series of centripetal and centrifugal coils. Pig has an extensive sacculation (haustra) and elongation of the large intestine (Swindle, Makin et al., 2011). The length and the capacity of the large intestine of pigs are about 1.5 - 3 times larger than that of humans (Christensen, Knudsen et al., 1999).

In rats, the caecum is the main site of fermentation and represents 26% of the length of the large intestine. The colon of rats is not sacculated nor long and it is mainly important for re-absorption of water and formation of faecal pellets (Desesso and Jacobson, 2001). Rats pratice coprofagy, i.e. they ingest part of their faeces, which allows the digestion of faecal bacteria and the intestinal absorption of vitamins and microbial protein (Hawksworth, Drasar et al., 1971).

In rabbits, the thin-walled caecum is a large organ (40–60% of the total volume of the gastrointestinal tract) that ends in an appendix which secretes bicarbonate ions into the caecal lumen, which are thought to act as a buffer for the volatile fatty acids produced by caecal fermentation (Davies, Certzoomed et al., 2003). The colon of rabbits is sacullated, and consists of a proximal and distal colon separated by a short transverse colon, and it ends with the *fusus coli*, which is responsible for the peristaltic waves on the colon (Davies, Certzoomed et al., 2003). Rabbits practice caecotrophy that is considered more sophisticated than coprophagy (Hörnicke, 1981). Rabbits produce two types of faeces: soft faeces, also named caecotrophs that are rich in vitamins, protein and minerals and regular hard faeces. The process of excretion of caecotrophs and immediate re-ingesting directly from the anus gives a second opportunity to benefit from valuable nutrient substances. Such physiological recycling usually occurs twice each day (Toutain, Ferran et al., 2010).

Table 1.7 Comparison of gastrointestinal pH between animal species under fed (*ad libitum*) state.

Species	Gastrointestinal tract region		Mean pH values
Rats [1]	stomach		3.2 ± 1.0
	small intestine	duodenum	5.0 ± 0.3
		jejunum	5.10 ± 0.3
		ileum	5.94 ± 0.4
	caecum		5.9 ± 0.4
	large intestine	proximal	5.51 ± 0.5
		distal	5.77 ± 0.5
Rabbit [2]	stomach	fundus	3.0
		antrus	1.6
	small intestine	proximal distal	6.4 -7.0
		medium	6.9 - 7.2
		distal	7.2 - 7.4
	ileo-caecal junction		6.6
	caecum		6.0 - 6.1
	appendix		6.4
	Colon	proximal	6.2 - 6.4
		distal	6.5
Pig [3]	stomach	fundus	4.6
		antrum	4.4
	small intestine	proximal	6.1 - 6.5
		medium	6.3 - 6.6
		distal	6.4 - 6.7
	caecum		6.1
	colon	proximal	6.1 - 6.4
		medium	6.3 - 6.5
		distal	6.5 - 6.6

Adapted from references [1](Mcconnell, Basit et al., 2008) [2](Marounek, Vovk et al., 1995; Merchant, Mcconnell et al., 2011).

1.4.1.5 Gastrointestinal microbiota

The large intestine has higher concentrations and larger numbers of bacteria than the stomach and small intestine in all species, including the human (Stella, Borchardt et al. 2007). Moreover, fermentation process occurs in the stomach of both rats and rabbits. In rats, it

occurs due to ingestion of coprophages and in rabbits due to ingestion of caecotrophes. In the stomach of rats bacteria are autochthonous, i.e. permanently attached to the epithelium, and the faeces ingested only provide the buffer necessary for fermentation process (Hörnicke, 1981). The presence of bacteria in stomach of rats is explained by the existence of a non-glandular compartment in the stomach which permits the bacteria to proliferate in this non-acid producing region. Consequently, rats have a large number of bacteria along the whole length of the gastrointestinal tract. The main organisms in stomach and small intestine of rats are Lactobacilli (Firmicutes Phylum), Bacteroides (Bacteroidetes Phylum) and Bifidobacteria (Actinobacteria Phylum) (Hawksworth, Drasar et al., 1971; Tannock, 1999) These organisms outnumber the enterobacteria (Proteobacteria phylum), mainly *Escherichia coli* by a factor of more than 10^2 (Hawksworth, Drasar et al., 1971).

In rabbits, caecothrophs are responsible for the existence of bacteria at the level of the stomach (Griffiths and Davies, 1963). The gastric pH in rabbits is generally maintained between 1 and 2 and increase to 3 in presence of caecotrophs (Davies, Certzoomed et al., 2003). This gastric pH avoids the occurrence of fermentation in the gastric luminal of rabbits. Fermentation takes place inside caecothrophs, which are unaffected by the luminal pH, given the fact that they are covered by a mucous membrane. This membrane remains morphologically intact once caecotrophs are swallowed without mastication (Griffiths and Davies, 1963; Davies, Certzoomed et al., 2003). The bacteria, maintained at high pH by phosphate buffer, produces amylase which, together with the salivary and food amylases, degrades food starch to maltose and glucose. Thus, in rabbits, the caecotrophes provide both the buffer and the active microbes in fermentation (Griffiths and Davies, 1963). Although caecotrophy, the flora of rabbits at the level of the upper gastrointestinal tract is very simple and similar to that in man and the number of bacteria is much lower than in rats (Table 1.8) (Hawksworth, Drasar et al., 1971). The prevalent organisms in the upper gastrointestinal tract of rabbits are Bacteroids (Bacteroidetes Phylum) and Bifidobacterias (Actinobacteria Phylum), like in human (Kararli, 1995).

Wkswortbh *et al.* reported the principal bacteria present in the large intestine of rats, rabbits and human. Microbiota present in this part of the gut of these animals belong to the Firmicutes Phylum (Enterococcus, Lactobacillus spp), Bacteroidetes Phylum (Bacteroids spp), Proteobacteria Phylum (Enterobacteriaceae spp, mainly *Escherichia coli*) and Actinobacteria Phylum (Bifidobacterium spp). The strictly anaerobic bacteria (Bacteroides

and Bifidobacteria) greatly outnumber the aerobes (enterobacteria and enterococci). Lactobacilli are present in very large number in the rat and in smaller numbers in the rabbit and man (Hawksworth, Drasar et al., 1971).

The main bacteria populations in the lower intestine of pig belong to Firmicutes Phylum (Lactobacillus spp, Eubacterium spp, Clostridium spp), Bacteroidetes Phylum (Bacteroids spp), Proteobacteria Phylum (Enterobacteriaceae spp/ *Echerichia coli*) and Actinobacteria Phylum (Bifidobacterium spp) (Mountzouris, Balaskas et al., 2006; Reilly, Sweeney et al., 2010).

Table 1.8 Microbiota in different regions of the GI tract of the most commonly used laboratory animal models.

Species	Stomach	Proximal small intestine	Distal small intestine	Large intestine	Rectum and feces
Rats[1]	10^7-10^9	10^6-10^8	10^7-10^8	10^8-10^9	10^9-10^{10}
Rabbits[1]	10^0-10^6	10^0-10^5	10^6-10^7	10^8-10^9	10^9-10^{10}
Pigs[2]	10^7-$10^{8.6}$	$10^{6.5}$ – $10^{7.0}$	$10^{7.6}$ – $10^{8.0}$	$10^{8.6}$ (caecum)	$10^{8.8}$

Adapted from references [1] (Kararli, 1995) and [2](Desesso and Jacobson, 2001)

From the viewpoint of bacteria distribution in the GI tract, rabbits may be more suitable than rodents as models to evaluate prodrug approach for colon-specific delivery (Stella, Borchardt et al., 2007).

Relative to microbiota in the lower intestine, a huge similarity in terms of bacteria Phylum between all animals and human exists. As described before, in human, the dominant bacterial Phyla are the Firmicutes (Clostridium, Roseburia, Faecalibacterium and Ruminococcus species), Bacteroidetes (Bacteroides and Prevotella) and Actinobacteria (Bifidobacterium), normally with lower abundances of Verrucomicrobia and Proteobacteria (Wu, Chen et al., 2011; Aziz, Doré et al., 2013; Chassard and Lacroix, 2013; Scott, Gratz et al., 2013).

1.5 Project aims

The research work presented in this thesis aims to synthesize a cyclodextrin-based prodrug of diclofenac and investigate its ability to target the colon.

Therefore, the research work was designed and organized to meet the following aims:

- Development of a method to prepare cyclodextrin conjugates of diclofenac in a short reaction time and physical-chemical characterization obtained molecules (Chapter II). This would provide a reliable method to produce efficiently and conveniently new cyclodextrin conjugates in adequate quantities to proceed for further studies.

- *In vitro* assessment of the stability of diclofenac-cyclodextrin conjugates in the upper gastrointestinal tract using simulated gastrointestinal fluids and animal fluids to predict *in vivo* behaviour. Investigate the ability of the conjugate to site-specific release of diclofenac on the colon and compare the performance of this type of prodrug with the classic prodrug sulfasalazine. This would allow accessing the inter-species variability on the metabolism of produgs and the establishment of the best animal model to perform the *in vivo* studies (Chapter III).

- Investigate the influence of different feeding regimens on the distribution and pH contents along the gut and on the metabolism of cyclodextrin conjugates (Chapter IV). This would provide information necessary to understand the importance of the feeding state on the drug disposition.

- Investigate the *in vivo* behaviour of the cyclodextrin conjugates *versus* sulfasalazine and determine the potential of cyclodextrin to be used as a carrier to colon specific delivery (Chapter V).

CHAPTER II

SYNTHESIS AND CHEMICAL CHARACTERIZATION OF DICLOFENAC-CYCLODEXTRIN CONJUGATES

2 SYNTHESIS AND CHEMICAL CHARACTERIZATION OF DICLOFENAC-CYCLODEXTRIN CONJUGATES

2.1 Introduction

Natural cyclodextrins (CDs), namely α-, β- and γ- have been studied as carriers for colonic drug delivery. Due to their size and hidrophilicity, they are not absorbed through the intestine. Cyclodextrins, α- and β- , contrarily to γ-cyclodextrin can pass intact through the upper gastrointestinal tract and only when reach the colon they are metabolized, as described in Chapter I. Thus, α- and β-cyclodextrins are the most interesting cyclodextrins to work as carriers.

To develop a stable colonic specific prodrug, it is important to produce a linkage between CDs and a drug. The linkage should be sufficiently stable to survive the transit through the stomach and small intestine, but also susceptible to be broken only at the colon level where the drug is released.

However, to generate this linkage between a cyclodextrin and a drug, several challenges related with the own CDs and the structure of the drug need to be addressed. Not all the drugs that are candidates to colon delivery can be modified to a prodrug. For a drug to become a colon-specific prodrug, it needs to have an appropriate functional group suitable for conjugation (Jung and Kim, 2010). Otherwise, synthesis of CDs conjugates is associated with a wide variety of physical-chemical processes that can succeed, owing to the presence of the hydrophobic cavity and a large number of hydroxyl groups that provides molecule nucleophilicity.

For this reason, what follows is a description of the main physical-chemical characteristics of cyclodextrins as a molecule, as well as of diclofenac, with the purpose of link both in order to get specific delivery of the anti-inflammatory drug in the colon.

2.1.1 *Cyclodextrin chemistry*

The α-, β-, and γ-cyclodextrin contain 18, 21 and 24 hydroxyl groups, respectively. Thus, each cyclodextrin moiety potentially has several sites of modification. The available modifications of cyclodextrin will occur at the hydroxyl groups, taking advantage of their

nucleophilicity, which directs regioselectivity and the extent of modification (mono-, di-, etc) (Khan, Forgo et al., 1998).

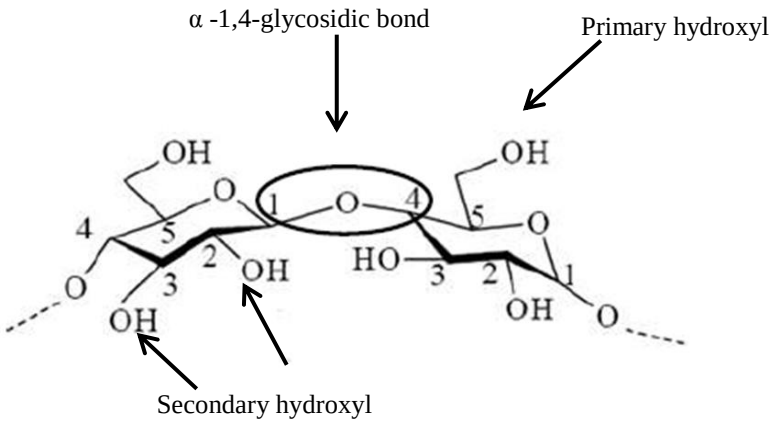


Figure 2.1 α-1,4 glycosidic bond and the three different types of hydroxyl groups of a cyclodextrin.

The primary and secondary hydroxyl groups have different reactivities, but also the two secondary hydroxyl groups between them. This last difference is due to the fact that the hydroxyl groups at C-2 are closer to the hemi-acetal than the hydroxyl groups at C-3. This makes the hydroxyl groups attached at C-2 slightly more acidic, hence slightly more reactive and consequently more susceptible to deprotonation than the hydroxyl groups at C-3. These hydroxyl groups at position 3 are the most inaccessible because they are sterically hindered. On the other side, the hydroxyl groups at the C-6-position are the most basic (and often most nucleophilic) (Hybl, Rundle et al., 1965; Saenger, Noltemeyer et al., 1976). Thus, an electrophilic reagent attacks at the most reactive C-6 hydroxyl groups. Then, under anhydrous conditions, the C-2 hydroxyl groups are the most vulnerable to deprotonation (Saenger, Noltemeyer et al., 1976). Bulky reagents tend to react preferentially at the primary C-6 hydroxyl groups of the macrocycle because these have easier access (Khan, Forgo et al., 1998). It is important to recognize that more reactive reagents will attack the hydroxyl groups less selectively (Khan, Forgo et al., 1998).

Synthesis of cyclodextrin conjugates at position 6 has been investigated by some authors (Uekama, Minami et al., 1997b; Udo K, Hokonohara K et al., 2010). This modification is easier since primary hydroxyl groups are more nucleophilic than their secondary counterparts.

However, is not only the nucleophilicity of the hydroxyl groups that dictates the site of reaction. Other factors determine the nature of the product. If the electrophilic reagent forms a

complex with cyclodextrin, then the orientation of the reagent within the complex introduces an additional factor in determining the nature of the product (Ueno and Breslow, 1982). If the complex formed is very strong, then the predominant product formed will be directed by the orientation of the reagent within the complex. On the other hand, if the complex is weak, then the product formation will be directed by the relative nucleophilicities of the hydroxyl groups (Ueno and Breslow, 1982).

It is also important to note that solvents play an important role in determining the strength and the orientation of the complex between the reagent and cyclodextrin. Thus, for example tosyl chloride reacts with α-cyclodextrin in pyridine to give the 6-tosylated product whereas, in aqueous base, it gives the 2-tosylated product (Fujita, Nagamura et al., 1984).

The size of the cyclodextrin cavity also affects the strength and the orientation of the complex and affects the product of the reaction. For example, in aqueous solutions, tosyl chloride reacts with α-cyclodextrin to give the 2-substituted product; whereas with β-cyclodextrin, it gives the 6-substituted product (Takahashi, Hattori et al., 1984b).

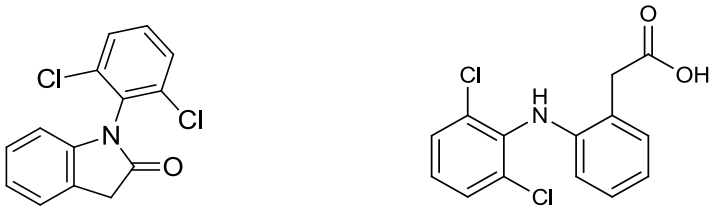
2.1.2 *Diclofenac chemistry*

Diclofenac, 2-[(2,6-dichlorophenyl)amino] benzeneacetic acid is a synthetic non-steroidal anti-inflammatory. It is a weak acid with a pKa of 4 and a partition coefficient (*n*-octanol:aqueous buffer, pH 7.4) of 13.4 (Palomo, Ballesteros et al., 1999).

It is manufactured via a stable intermediate, 1-(2,6-dichlorophenyl) indolin-2-one (Figure 2.2), which is commonly known as the indolinone derivative, by heating in the presence of sodium hydroxide (Yasumitsu Tamura, Jun-Ichi Uenish et al., 1984; Roy, Islam et al., 2001).

The molecule of diclofenac, from a point of view of its structure, is a hybrid between a fenamic and an acetic acid class derivative; the characteristic feature is the presence of a secondary amino group (N-H) bridging two aromatic rings and representing the source of a series of intramolecular H-bonds towards a chlorine atom of one and the carboxyl group of the other aromatic ring of the diclofenac molecule. Other H-bonds exists between the carboxyl groups of two different molecules of diclofenac, which face together in a dimer. The dimer form represents a structural unit of the solid state of diclofenac, like that of most carboxylic acids. All the H-bonds involve the hydrophilic groups inside the dimer inter- and intra-molecularly and therefore make the diclofenac molecule less available to intermolecular

interaction with the environment, such as the water molecules of the solvent (Castellari and Ottani, 1997; Fini, Cavallari et al., 2010).



I **II**

Figure 2.2 I- 1-(2,6-dichlorophenyl) indolin-2-one structure, precursor on the synthesis of diclofenac. II- Diclofenac: (2-(2,6-dichloranilino) phenylacetic acid) structure.

2.1.3 *Design of diclofenac-cyclodextrin ester conjugate*

Although an ester bond is not a suitable linkage for colon-specific drug delivery considering that esterases are abundant in the whole gastrointestinal tract without significant regional preference (Inoue, Morikawa et al., 1979), ester prodrugs of not only cyclodextrins but also dextrans have been developed with a good colon-targetibility (Jung and Kim, 2010).

2.1.3.1 Strategies to synthesize ester cyclodextrin conjugates

The literature describes the synthesis of ester conjugates of cyclodextrins using several methods, including: *i)* activation of drug carboxylic group using a carbodiimide derivative, following reaction with an hydroxyl group of the cyclodextrin (Yano, Hirayama et al., 2001; Namazi, Bahrami et al., 2005; Zou, Okamoto et al., 2005; Dev, Mhaske et al., 2007; Prabhu, D.Satyanarayana et al., 2009; Udo, Hokonohara et al., 2010) *ii)* formation of an acid chloride of the drug and posterior reaction with cyclodextrin (Ventura, Paolino et al., 2003b); *iii)* modification of CDs hydroxyl groups, by an electrophilic reagent, leading to the formation of one group more labile to nucleophilic substitution.

i) Activation of drug carboxylic group using a carbodiimide derivative, following reaction with a hydroxyl group of the cyclodextrin.

Carbodiimide derivatives are powerfull desidratant agents that react with the carboxylic group of the drug and allow the formation of an intermediate derivative sufficiently active to react with the hydroxyl groups of the cyclodextrin. The rate of this reaction also depends very

much on the nucleophilicity of their anions. Dicyclohexylcarbodiimide (DCC) has been the most commonly used carbodiimide and is regarded as a standard (Michael Smith, J.G. Moffatt et al., 1958). It is a non-aqueous carbodiimide that has been used to synthesize cyclodextrin conjugates of mefenamic acid (Dev, Mhaske et al., 2007), ibuprofen, indomethacin (Namazi, Bahrami et al., 2005) and β-lactam antibiotics (Namazi and Kanani, 2007). Carbonyldiimidazole (CDI) represents an alternative carbodiimide, but it is less frequently used, most probably due to cost and its sensitivity to moisture. This carbodiimide has been used to form cyclodextrin conjugates of 5-fluorouracil-1-acetic acid (Udo K, Hokonohara K et al., 2010), 5-aminosalicylic acid (Zou, Okamoto et al., 2005) and prednisolone (Yano, Hirayama et al., 2001).

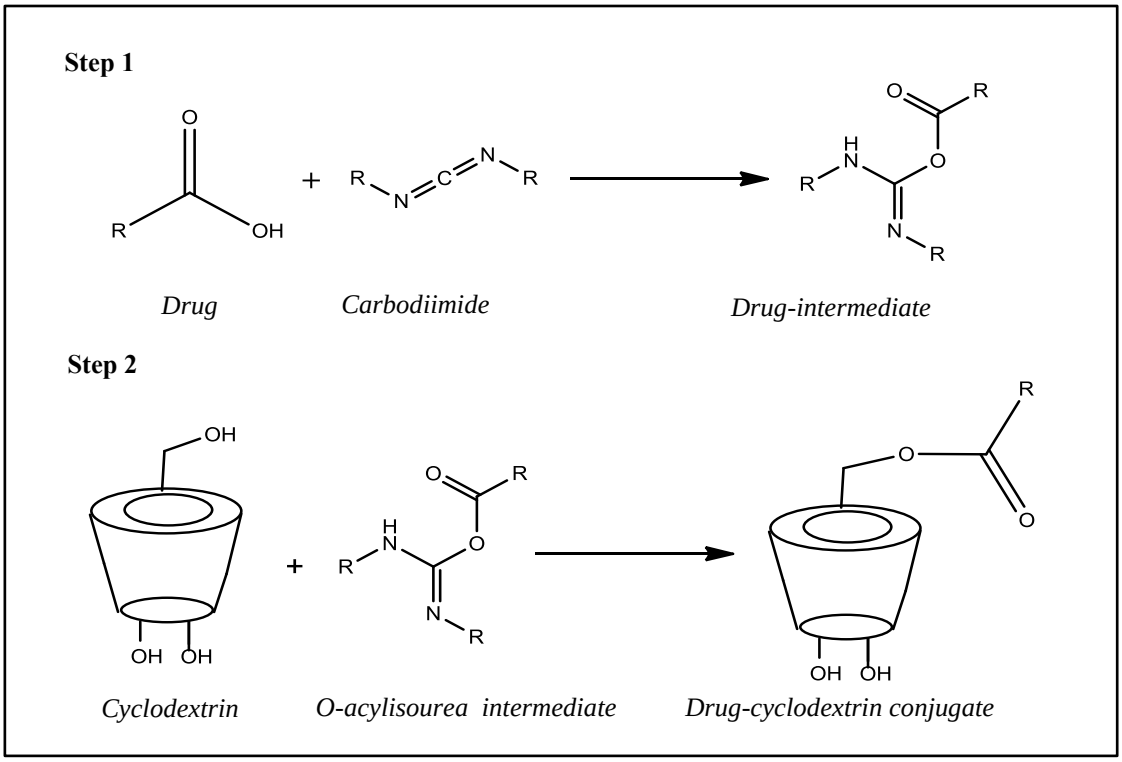


Figure 2.3 Synthetic scheme of synthesis of drug-cyclodextrin conjugate by a carboodimiide activation of the drug.

A water soluble carbodiimide such as dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) can be used to facilate the purification of the product (Albert and Hamilton, 2001).

Based on the literature, reactions between acids and carbodiimides in general occurs rapidly (Khorana, 1953). 4-dimethylaminopyridine (DMAP) (pKa=9.70) can assist on the

deprotonation of the nucleophile working as a nucleophilic acylation catalyst (Albert and Hamilton, 2001).

ii) Formation of an acid chloride of the drug and posterior reaction with cyclodextrin.

Formation of an acid chloride of anti-inflammatory drug and posterior reaction with cyclodextrin constitutes other method to synthetize cyclodextrin conjugates. This method was adopted to synthetize dimethyl-beta-cyclodextrin-4-biphenylylacetic acid conjugate (Ventura, Paolino et al., 2003a)

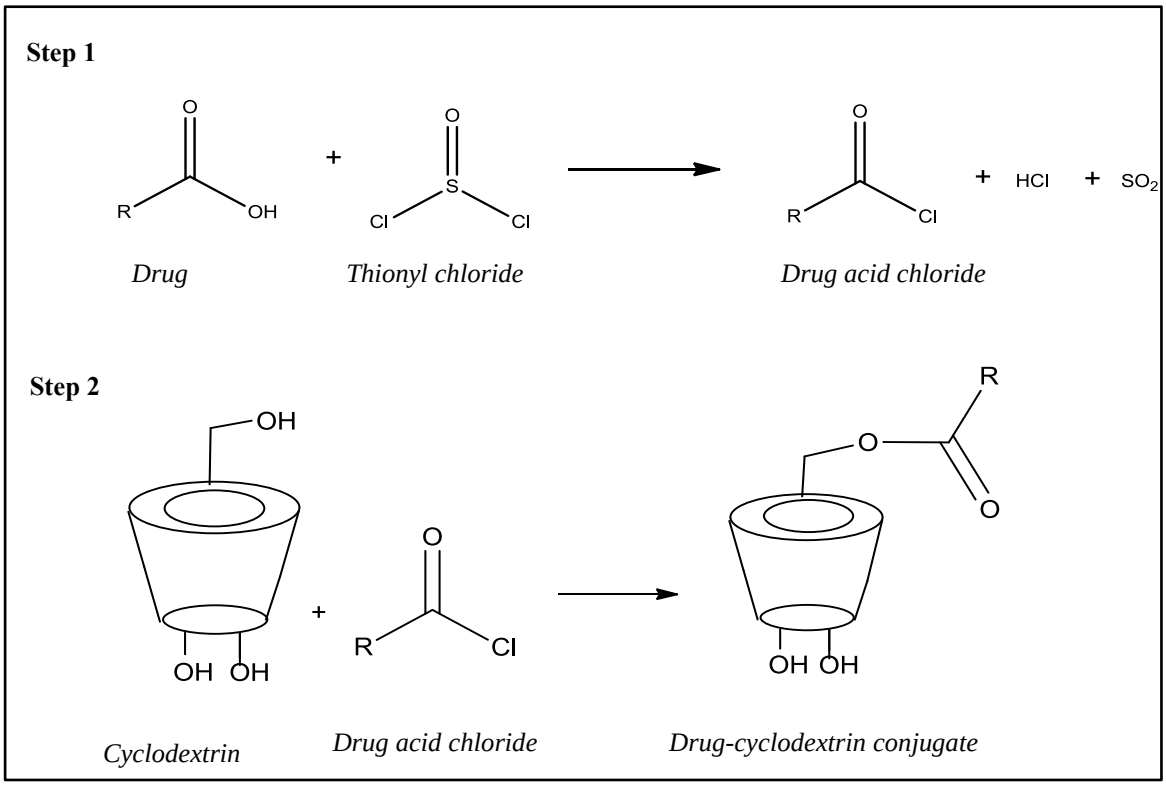


Figure 2.4 Synthetic scheme of synthesis of drug-cyclodextrin conjugate by formation of an acid chloride of the drug and posterior reaction with cyclodextrin.

iii) Modification of β-cyclodextrin hydroxyl groups, by an electrophilic reagent, leading to the formation of one group more labile to nucleophilic substitution.

Another possibility to achieve the required conjugation is through transformation of the hydroxyl groups of cyclodextrin by an electrophilic agent, followed by nucleophylic attack by the diclofenac sodium salt.

Transformation of the hydroxyl group at 6-position into a 6-toluenesulfonylcyclodextrin was adopted by many authors. Since the tosyl group is a good leaving group, it may undergo displacement with a wide variety of nucleophiles to produce a series of monosubstituted cyclodextrins.

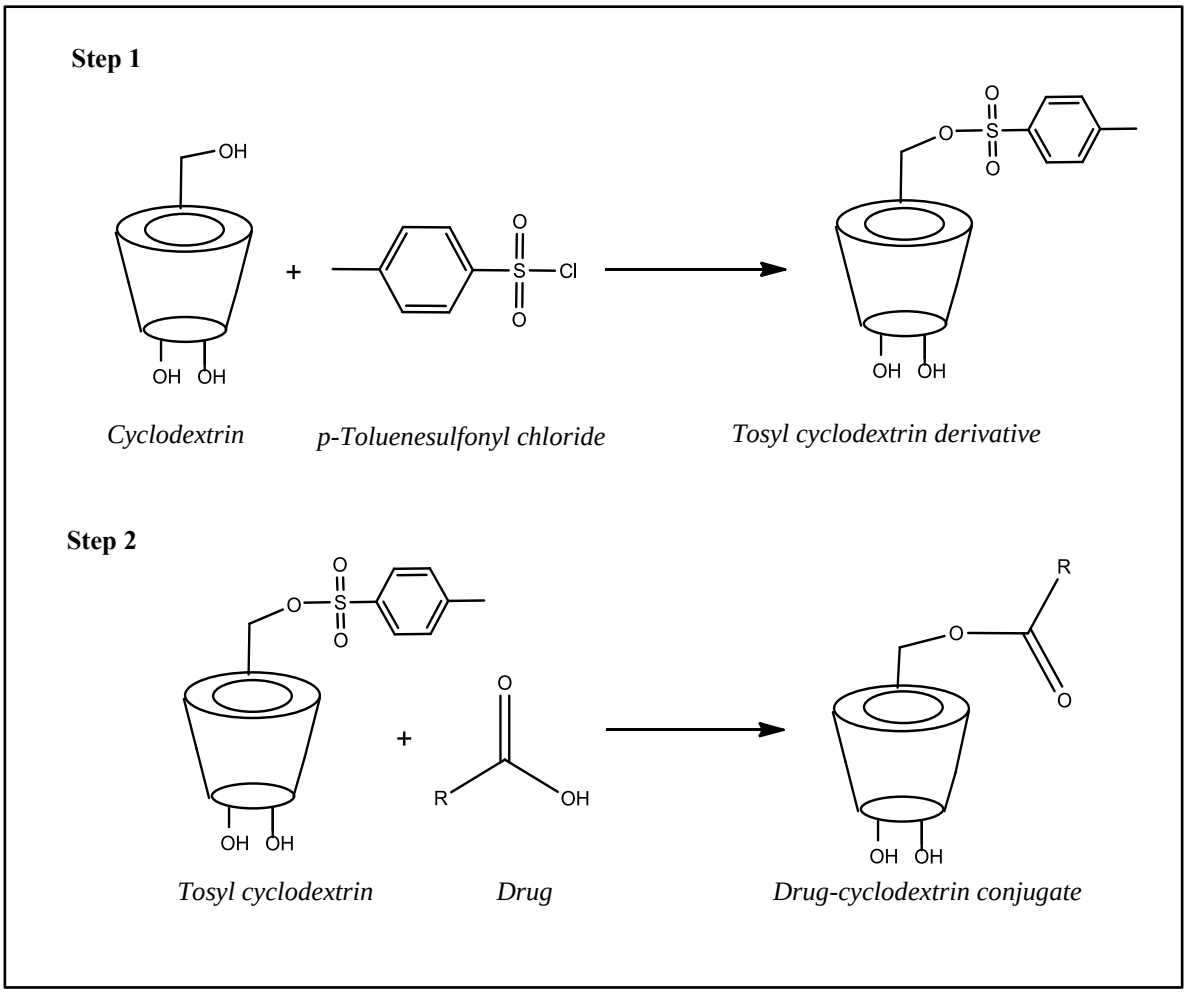


Figure 2.5 Synthetic scheme of synthesis of drug-cyclodextrin conjugate by nucleophilic substitution at the cyclodextrin structure.

This strategy was adopted particularly in the synthesis of the β-cyclodextrin ester of ibuprofen (Coates, Easton et al., 1991) fluribuprofen, 2-phenylpropanoic acid and *N*-acetylphenylalanine (Coates, Easton et al., 1994). Also for the synthesis of cyclodextrin conjugates with fenilacetic acid (Uekama, Minami et al., 1997b), etodolac (Rizzarelli, Vecchio et al., 2007), ketoprofen (Kamada, Hirayama et al., 2002), n-butyric acid (Hirayma, Ogata et al., 2000), naproxen, and flurbiprofen (El-Kamel, A.M.Abdel-Aziz et al., 2008).

2.2 Aims and Objectives

The aim of this Chapter is to investigate a method to synthesize diclofenac-cyclodextrin conjugates. In order to pursue this, the following objectives were defined:

- establishment of a reproducible method to synthesize diclofenac-β-cyclodextrin conjugate;

- chemical characterization of the obtained conjugate;

- evaluation of the ability of the chosen method used to synthesize diclofenac-β-cyclodextrin, and also to synthesize the other γ- and α- conjugates.

2.3 Materials

2.3.1 *Chemicals*

β-cyclodextrin was purchased from Roquette. β-cyclodextrin hydrated (M_W=1,134.98 g/mol), DCC, CDI EDAC, DMAP, *p*-toluenesulfonyl chloride, 2,4,6-triisopropylbenzenesulfonyl chloride, Diaion-HP-20 trifluroacetic acid and acetone were purchased from Sigma. Sodium diclofenac (M_W=318.13 g/mol) was kindly provided by Labesfal Genéricos (Portugal). Dimethylformamide (DMF), sodium hydroxide, ethyl acetate, isopropanol, ethanol, ammonium chloride, methanol, acetonitrile, thionyl chloride, tetrahydrofuran (THF), triethylamine (TEA) ethanol, acetonitrile and water for HPLC, were purchased from Fisher Scientific. Deuterated dimethyl sulfoxide (DMSO-d_6) was purchased from Euriso-top (Peypin, France). Ammonia, acetic acid, pyridine, sulfuric acid and *p*-anisaldehyde were purchase from Acros Organics. DC Silicagel 60 F254 was purchased from VWR.

Pyridine was kept dry over potassium hydroxide and DMF over molecular sieves 4Å prior to use.

Diclofenac free acid was obtained according to the method described by Fadda *et al*. Briefly, a saturated aqueous solution of diclofenac sodium salt was acidified with diluted hydrochloric acid until a white precipitate of diclofenac acid was observed. The precipitate was filtered, washed with bidistilled water and air dried (Lai, Sinico et al., 2009).

Other chemicals and solvents were of analytical reagent grade and deionized water was used.

2.4 Methods

2.4.1 *Synthesis of diclofenac-β-cyclodextrin conjugate.*

Several methods were investigated to synthesize ester diclofenac-β-cyclodextrin conjugate including:

2.4.1.1 Activation of diclofenac carboxylic group using a carbodiimide derivative, following reaction with a hydroxyl group of the cyclodextrin.

Briefly, sodium diclofenac (1.57 mmol) was dissolved in DMF. DCC (1.57 mmol) was then added to the solution, and the mixture stirred at 0 ^{o}C for 2 hours, adapting the method reported previously (Dev, Mhaske et al., 2007). After the addition of the cyclodextrin in DMF, the mixture was stirred at room temperature. Different conditions were tested following this procedure, including the addition of DMAP as a catalyst (Albert and Hamilton, 2001). Replacement of sodium diclofenac by diclofenac free acid was also performed (Manfredini, Pavan et al., 2002). Other carbodiimides derivatives were tested, namely CDI (Udo, Hokonohara et al., 2010) and EDAC (Bakar, Oelgemöller et al., 2009).

2.4.1.2 Formation of an acid chloride of the drug followed by reaction with cyclodextrin.

For the synthesis of diclofenac chloride, diclofenac free acid (3.37 mmol) was refluxed with thionyl chloride (10 mL) for 5 hours. After this step, β-cyclodextrin was added to diclofenac chloride and the reaction proceeded until formation of the conjugate. This method was performed adapting those described by Puglisi *et al.*(Ventura, Paolino et al., 2003a) and by Picci *et al.* (Cassano, Trombino et al., 2010).

2.4.1.3 Modification of β-cyclodextrin hydroxyl groups by an electrophilic reagent, leading to the formation of one group more labile to nucleophilic substitution.

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

2.4.1.3.1 Step 1: tosylation of β-cyclodextrin

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

2.4.1.3.2 Step 2: nucleophilic reaction

This step was performed following two different approaches - with and without the use of microwaves.

Non-microwave reaction:

In this method sodium diclofenac (0.7 mmol) was added to β-cyclodextrin tosylate (0.5 mmol) in anhydrous DMF (5 mL) and the mixture stirred at 100 ºC for 24 hours (Hirayma, Ogata et al., 2000).

Microwave reaction:

Reactions were carried out in an appropriate 10 mL thick walled glass vials under closed vessel conditions using CEM Discover S-class single mode microwave reactor with temperature, pressure and microwave power monitoring.

Sodium diclofenac (0.333 mmol) was dissolved in 3 mL of anhydrous DMF containing β-cyclodextrin tosylate (0.327 mmol) in an appropriate thick-walled glass vial. The reaction vessel was then sealed with a teflon® cap and the reaction mixture magnetically stirred and heated at 140 ^{o}C for 40 minutes under focused microwave irradiation with an initial power setting of 75 W. The product obtained was precipitated in acetone, filtered, washed several times with acetone and ethyl ether and dried. In order to obtain a purified sample, 300 mg of

the crude product was passed through DIAION HP-20 ion-exchange chromatography column eluting with water/methanol, and steadily increasing the methanol content. The conjugate was eluted with 80% methanol. Methanol in the eluate was removed under reduced pressure, the solution lyophilized in a freeze-dryer (Lyph-lock 6 apparatus, Labconco) for 72 hours and the diclofenac-β-cyclodextrin was obtained with a yield of 20%.

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 7.52 (d, H-5', H-3' of Diclofenac), 7.22 (d, H-4'' of Diclofenac), 7.20 (t, H-4 of Diclofenac), 7.05 (t, H-6'' of Diclofenac), 6.83 (t, H-5'' of Diclofenac), 6.25 (d, H-7'' of Diclofenac), 5.66–5.84 (m, OH-2 and OH-3 of β-cyclodextrin), 4.83 (d, H-1 of β-cyclodextrin), 4.21–4.55 (m, OH-6, H-6 of β- cyclodextrin), 3.50–3.90 (m, H-6, H-3 and H-5 of β-cyclodextrin), 3.25–3.43 (m, H-2, H-4 of β-cyclodextrin), MS (MALDI) m/z for $C_{56}H_{79}Cl_2NNaO_{36}$, found 1434.308 [conjugate+Na].

2.4.2 *Applicability of the nucleophilic microwave approach on the synthesis of diclofenac-γ-cyclodextrin and diclofenac-α-cyclodextrin*

2.4.2.1 Diclofenac-γ-cyclodextrin

2.4.2.1.1 Step 1: tosylation of γ-cyclodextrin

Synthesis of 6-monotosyl-γ-cyclodextrin

Tosylated γ-cyclodextrin was synthesized adapting the procedure described in the literature to produce γ-cyclodextrin ditosylated (Kahee Fujita, Hatsuo Yamamura et al., 1988). In this case, *p*-toluenesulfonyl chloride (2.02 g, 10.5 mmol) was added to a solution of γ-cyclodextrin (2.02 g, 1.55 mmol) in pyridine (20 mL) and stirred for 2 hours at room temperature under nitrogen atmosphere followed by addition of ethanol (20 mL). After addition of acetone, a solid precipitated was collected by filtration, washed with acetone, and dried under vacuum and at 100 ºC to afford 1-2 g of tosylate. This material can be re-crystallized by dissolving it in isopropanol at boiling temperature and then addition of water followed by cooling to room temperature, and storage in a refrigerator overnight.

Synthesis of Mono-6-(O-2,4,6-triisopropylbenzenesulfonyl)-γ-cyclodextrin

The synthesis was perfomed adapting the method described by Ronald Palin *et al.* (Palin, Grove et al., 2001). To a dry round-bottomed flask pyridine (10 mL) was added, followed by γ-cyclodextrin (1.0 g, 0.77 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (0.7 g, 2.3

mmol) at room temperature under nitrogen. After 24 hours, 100 mL of acetone was added to the mixture.

2.4.2.1.2 Step 2: Nucleophilic reaction - microwave approach

Sodium diclofenac (79.5 mg, 0.25 mmol) was dissolved in 3 mL anhydrous DMF containing γ-cyclodextrin tosylated (376 mg) in an appropriate thick-walled glass vial. The reaction vessel was then sealed with a teflon® cap and the reaction mixture was magnetically stirred and heated at temperature varying between 130-160 ºC for 20-40 minutes under focused microwave irradiation with an initial power setting of 75 W.

After cooling to room temperature, the product was precipitated in acetone. The precipitate was filtered and washed several times with acetone and diethyl ether, filtered off and then dried. Then the product was purified through by DIAION HP-20 ion-exchange chromatography eluting with water/methanol with increasing methanol content. The conjugate appeared with a composition of 80% methanol. Methanol in the eluate was removed under reduced pressure and the solution was lyophilized. Yield of isolated, pure product: 10-30%.

2.4.2.2 Diclofenac-α-cyclodextrin

2.4.2.2.1 Step 1: tosylation of α-cyclodextrin

Tosylated α-cyclodextrin was synthesized adapting the procedures described in the literature (Melton and Slessor, 1971; Weihua Tang and Ng, 2008). Briefly, 1 g (1 mmol) of γ-cyclodextrin was added to pyridine (10 mL) under stirring at room temperature under nitrogen. *p*-Toluenesulfonyl chloride was dissolved in 5 mL of pyridine and then added slowly during 30 minutes to the mixture of α-cyclodextrin in pyridine for 3 hours. After that, the reaction was stopped by the addition of acetone. The crude product was washed with methanol in order to eliminate pyridine.

2.4.2.2.2 Step 2: nucleophilic reaction - microwave approach

The protocol followed was the same used to prepare diclofenac-γ-cycldoextrin conjugate.

2.4.3 *Characterization*

The control of reactions was perfomed using thin layer chromatography (TLC). TLC was also used to monitorize elutes from the chromatography and the purification of the end products.

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

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

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

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

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

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

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

For analysis of the products of reactions, a gradient system of 0.4% TFA in water (A) and acetonitrile (B) was followed: 0-15 minutes 10-90% B; 15-22 minutes 90-10% B. The sample injection volume was 20 μl, the flow rate is 1.2 mL/min and detection wavelength was 254 nm at 30 ºC. For the analysis of the products of the chemical stability assays, the gradient

system was: 0-15 minutes 25-60% B; 15-22 minutes 60-25% B, with a flow rate of 1.0 mL/min.

2.4.4 *Chemical stability of diclofenac-β-cyclodextrin in aqueous buffer solutions*

Hydrolyses of conjugate was assessed in different buffer solutions namely: pH 1.2, acetate buffer 4.5, phosphate buffer pH 6.8, phosphate buffer 7.4. Preparation of the buffers is described in Appendix A.

Solutions of conjugate were prepared by dissolving an accurately weighed amount of conjugate (4 mg) in 5 mL of each buffer containing 1% of DMF (Table 2.1).

The samples were incubated at 37 ºC and shaken at 100 rpm. At fixed intervals, 100 µL were withdrawn, mixed with 400 µL of methanol and analyzed by HPLC to determine the percentage of conjugate under the aforementioned conditions. Each experiment was performed in triplicate.

Table 2.1 Schematic representation of the stability tests of diclofenac-β-cyclodextrin in different buffers.

Buffer solution (1% DMF)	[Conjugate] buffer solution	Sampling volume	Final volume (addition methanol)	Final [conjugate]
pH 1.2	800 µg/mL	100 µL	500 µL	160 µg/mL
pH 4.5				
pH 6.8				
pH 7.4				

2.5 Results and discussion

2.5.1 *Synthesis and characterization of diclofenac-β-cyclodextrin conjugate*

2.5.1.1 Activation of diclofenac carboxylic group using a carbodiimide derivative, following reaction with a hydroxyl group of the cyclodextrin

The first approach was the carboxylic activation by dicyclohexylcarbodiimide derivatives following a general procedure (Dev, Mhaske et al., 2007) with (DMAP) (Manfredini, Pavan et al., 2002; Li, Rossoni et al., 2007; Nemmani, Mali et al., 2009). This attempt failed to generate the required product. The substitution of DCC by other water insoluble carbodiimide derivative, CDI, or by EDAC, a water soluble carbodiimide also failed. Analysis of the NMR spectra did not show the expected product.

Results suggest that the nucleophylic attack of the hydroxyl group of cyclodextrin cannot compete with the more favorable intramolecular acylation by the nearby amino group (Palomo, Ballesteros et al., 1999).

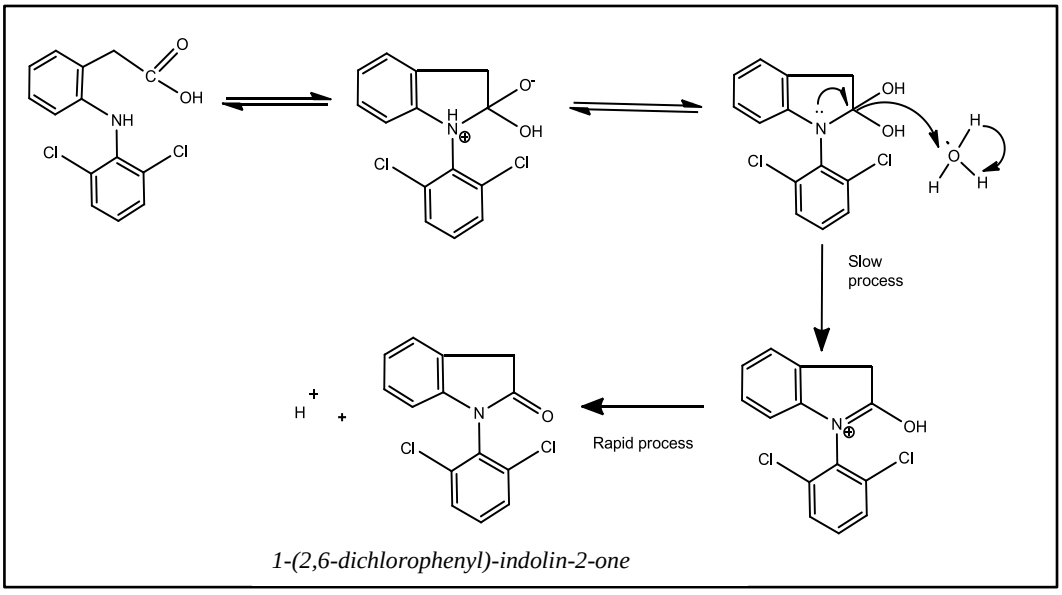


Figure 2.6 Intramolecular cyclization reaction of diclofenac (Palomo, Ballesteros et al., 1999).

Additionally, in order to clarify the reactivity of the two diiferent nucleophilic centers of diclofenac (NH, COOH) the reaction of diclofenac with benzyl bromide was performed in a mixture of DMF and acetonitrile. An ester of diclofenac was obtained in the form of crystals with considerable dimensions (see Appendix B). This result allows concluding that the carboxylic acid is more reactive than the amine and that carbodiimides are not reacting at the level of the amine group.

2.5.1.2 Formation of an acid chloride of the drug followed by reaction with cyclodextrin

Following the procedure described for the formation of an acid chloride of the drug, diclofenac was dissolved in thionyl chloride, but immediately the reaction solution became intensely red. The isolated product did not correspond to chloride acid of diclofenac. It is possible, therefore, that an intramolecular acylation of the diclofenac amino group had occured (Mcmaster and Ahmann, 1928).

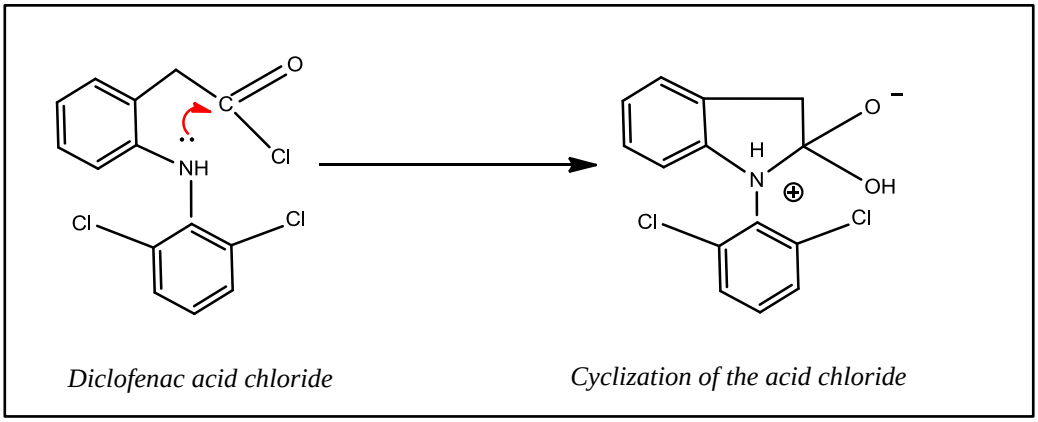


Figure 2.7 Preferential cyclization of the diclofenac molecule suggested by the lack of success on the attempt of synthesize an acid chloride.

2.5.1.3 Modification of β-cyclodextrin hydroxyl groups, by an electrophilic reagent, leading to the formation of one group more labile to nucleophilic substitution

This method involved two main steps: the transformation of the hydroxyl group at 6-position into a 6-*p*-(toluenesulfonyl) cyclodextrin (Step 1) and nucleophylic substitution of tosylated cyclodextrin by sodium diclofenac (Step 2), as described previously.

2.5.1.3.1 Step 1: tosylation of β-cyclodextrin

The major challenge of the first step was to achieve monotosylation of cyclodextrin specifically to the 6-position without considerable amounts of primary and secondary side multi-tosylated by-products. (Khan, Forgo et al., 1998; Brady, Lynam et al., 2000) This selective sulfonylation generally occurs in dry pyridine (Weihua Tang and Ng, 2008), or in water at alkaline pH (Brady, Lynam et al., 2000).

Firstly, it was thought that the reaction between tolylsulfonyl chloride and β-cyclodextrin in water resulted in the introduction of the tosyl group on the secondary alcohol side of cyclodextrin (Onozuka, Kojima et al., 1980). This was later corrected (Takahashi, Hattori et al., 1984a), proving the formation of monotosylated cyclodextrin on the primary hydroxyl of cyclodextrin. This process is accompanied by host-guest complex formation between cyclodextrin and tosyl chloride in aqueous solution (Onozuka, Kojima et al., 1980; Takahashi, Hattori et al., 1984a). Both solvents, pyridine and alkaline solution allow substitution of primary hydroxyl of β-cyclodextrin (Keiko Takahashi, Kenjiro Hattori et al., 1984). However, pyridine is a non-user-friendly solvent and forms a pyridinium complex with the cyclodextrin cavity, complicating the process of purification (Khan, Forgo et al., 1998). Due to these facts, the method using water as solvent was the one selected to prepare the 6-*p*-toluenosulfonyl-β-cyclodextrin derivative using *p*-tolylsulfonyl chloride (Brady, Lynam et al., 2000).

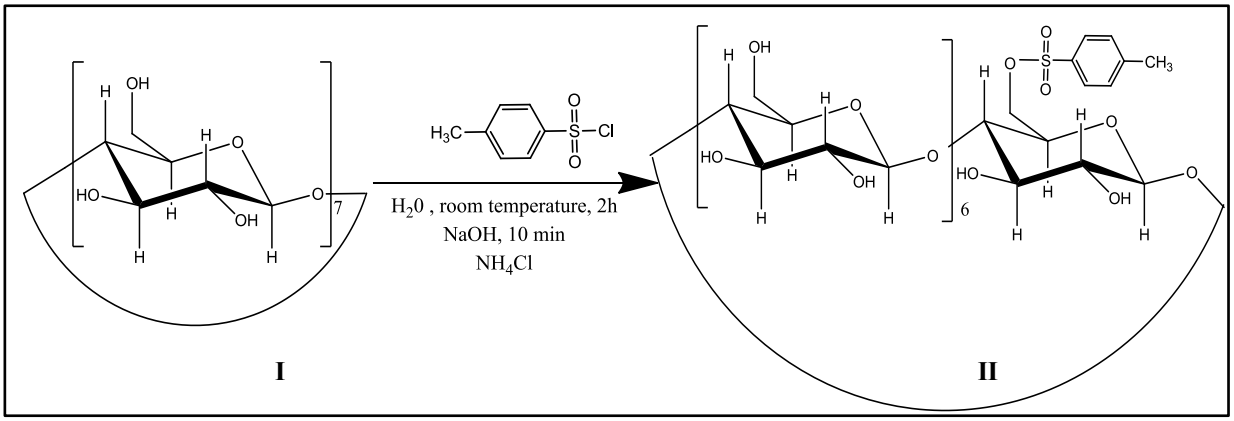


Figure 2.8 Synthetic scheme of preparation of mono-6-tosyl-β-cyclodextrin. I- β-cyclodextrin; II- Tosyl β-cyclodextrin.

Following the procedure using *p*-toluenesulfonyl chloride in water (Mcnaughton, Engman et al., 2004), gram amounts of the 6-*p*-toluenosulfonyl-β-cyclodextrin were obtained being then

characterized by NMR spectroscopy and MALDI-TOF spectrometry. For the substitution reaction, this product was used without purification, despite the presence of small amounts of di- and tri-tosilated derivatives as verified by MALDI analysis.

Mono-6-sulfonylciclodextrins can also be prepared in alkaline solution using other sulfonyl derivatives namely *p*-toluenesulfonic anhydride (Zhong, Byun et al., 1998) or 1-(*p*-toluenesulfonyl) imidazole (Brady, Lynam et al., 2000) accompanied by higher yields (61% and 40% respectively), rather than that obtained using of *p*-toluenesulfonyl chloride also in water: 34% (Jicsinszky and Iványi, 2001), 11% (Petter, Salek et al., 1990), 6.3% (Dragos Vizitiu, Caroline S. Walkinshaw et al., 1997) 25% (Brady, Lynam et al., 2000) and 32% (Mcnaughton, Engman et al., 2004). However, difficulties in preparing tosic acid free *p*-tolunesulfonic anhydride frequently results in lower yield, 1-(*p*-toluenesulfonyl) imidazole also needs to be prepared previously, which implies a higher procedure to obtain sulfonated cyclodextrin (Brady, Lynam et al., 2000).

2.5.1.3.2 Step 2: nucleophilic reaction

The required following step was the nucleophylic substitution of the tosyl group by the diclofenac.

i) Non-microwaves reaction

Following the conventional strategies the reaction between tosylcyclodextrin and diclofenac in DMF does not result in an ester formation. The work of Jiben Roy *et al.* and also of Petar Tudja *et al.*, showed that under heating conditions diclofenac can suffer dehydratation and an intramolecular cyclization (Roy, Islam et al., 2001; Tudja, Khan et al., 2001).

Our efforts led us to the conclusion that with this strategy some different approach should be taken.

ii) Microwaves reaction

Our efforts led us to conclude that an alternative strategic approach should be taken.

Microwave irradiation has long been viewed as an interesting alternative to the use of classical heating systems, and particularly in the field of organic synthesis, largely due to the

increases in reaction rates, improved yields and selectivities associated with this technique (Richel, Laurent et al., 2011).

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

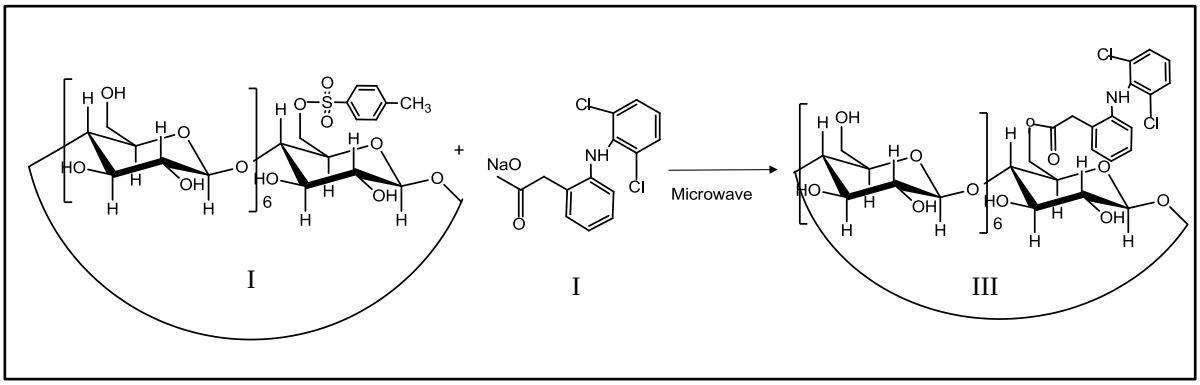


Figure 2.9 Synthetic scheme for the preparation of diclofenac-β-cyclodextrin conjugate. I - tosyl β-cyclodextrin, II - sodium diclofenac, III - diclofenac-β-cyclodextrin.

An HPLC study of different samples prepared under different reaction temperatures showed that reactions undertaken at 140 ^{o}C produced the best yield. As temperature increases, it is also possible to observe a decrease in diclofenac-β-cyclodextrin yields. This is likely due to the degradation that diclofenac suffers at high temperatures (Tudja, Khan et al., 2001; Galmier, Bouchon et al., 2005).

The use of microwave radiation additionally led to the conjugate formation, with a short reaction time and less solvent, comparatively to the conventional processes required for the preparation of cyclodextrin conjugates (Hirayama, Ogata et al., 2000).

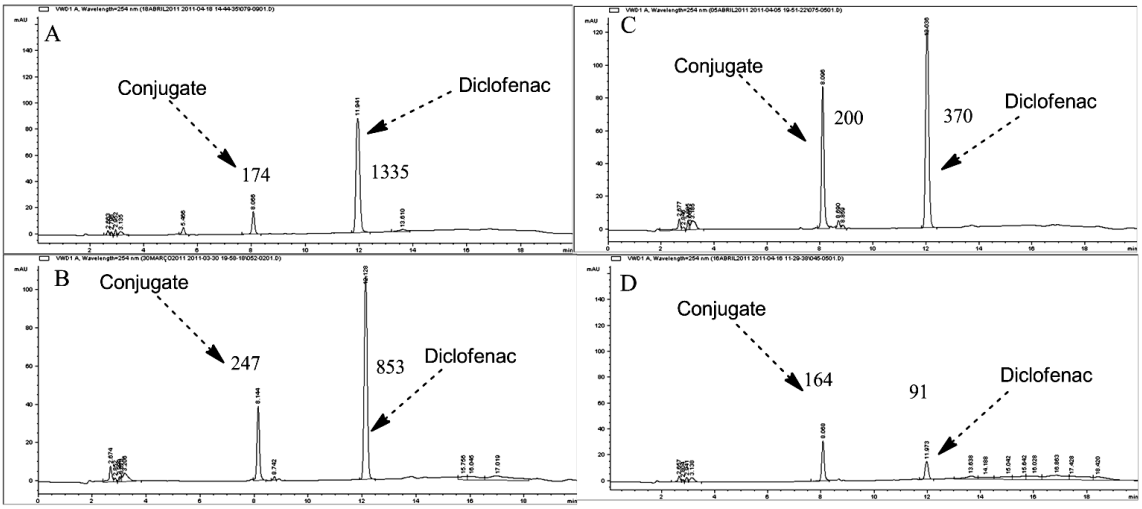


Figure 2.10 HPLC chromatograms of crude product of conjugates synthesized under microwave irradiation conditions for 40 minutes under different temperatures: A: 100 ºC; B: 140 ºC; C: 160 ºC; D: 200 ºC. The numbers represent the area of the corresponding peak. Conjugate retention time: 8.0 minutes; diclofenac retention time 11.9 minutes. Elution conditions are described in point 2.4.3.

After chromatographic purification, about 20% yield of product was achieved, and the microwave approach to the synthesis of this conjugate proved to be consistently reproducible.

It is reported that solvolysis of the tosylated cyclodextrins compete with the desired nucleophilic substitution when the reaction is carried out in water, producing cyclodextrins (Melton and Slessor 1971), which can contribute to the low yield of the reaction. Thus, it is important the use of dry DMF as well as the use of very well dry intermediate tosylated cyclodextrin.

Moreover, it was observed that when diclofenac is dissolved in DMF in the absence of cyclodextrin tosylated reagent and submitted to the same conditions than that used to perform the nucleophilic reaction in microwaves, the solution presented a green dark colour. This proves that simultaneous decomposition of sodium diclofenac into other compounds occurs when nucleophilic reaction is carried out. Previously, it were found some changes of the colour of diclofenac, when heated. The same study reported that the colour of the cyclic derivative, 1-(2,6-dichlorophenyl)-indolin-2-one is white but black when heated (Tudja, Khan et al., 2001).

2.5.1.4 Characterization of 6-*p*-toluenosulfonyl-β-cyclodextrin

2.5.1.4.1 Matrix-assisted laser desorption/ionization (MALDI) spectroscopy

Mass spectrometry, demonstrated that the product of the tosylation reaction is mainly the mono-tosylated-cyclodextrin (1311.605 [M+Na] adduct) contaminated with some di-tosylcyclodextrins (1465.667 [M+Na] adduct) (Figure 2.11).

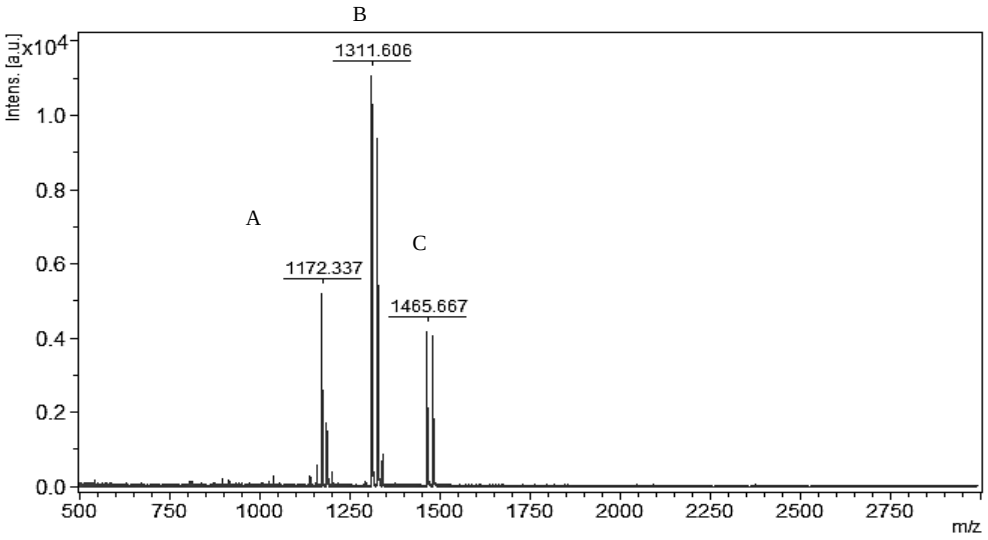


Figure 2.11 MALDI mass spectrum of tosylated β-cyclodextrin compound. A- β-cyclodextrin, B- monotosylated β-cyclodextrin, C- ditosylated β-cyclodextrin

2.5.1.4.2 Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

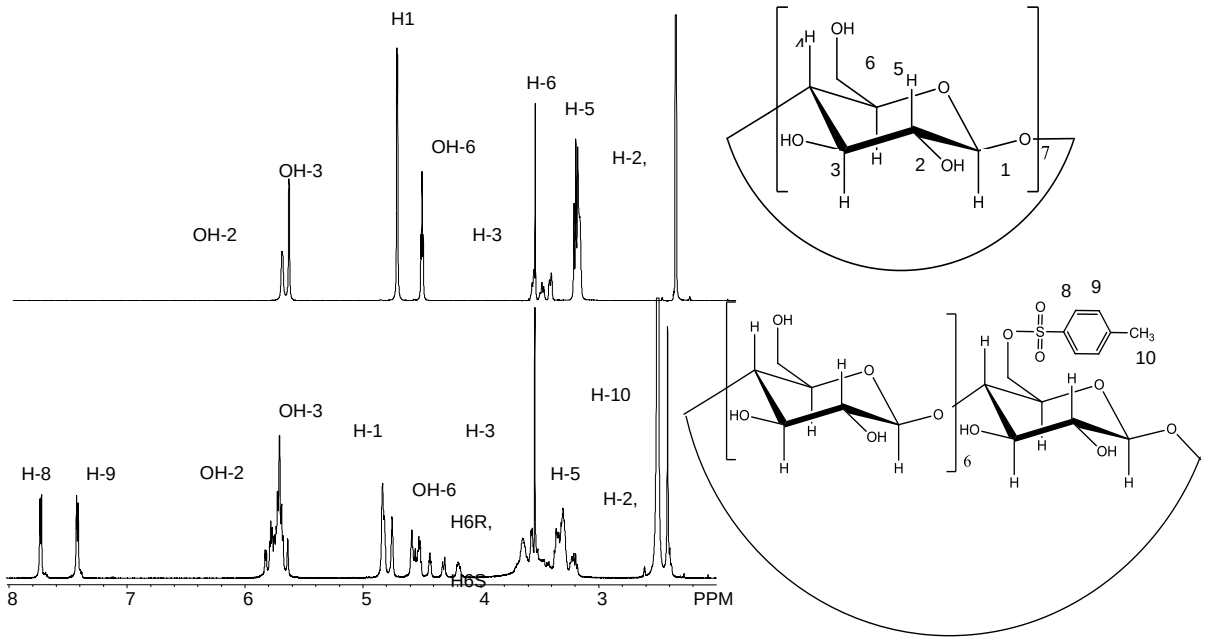


Figure 2.12 ^{1}H NMR spectrum of β-cyclodextrin (A); ^{1}H NMR spectrum of β-cyclodextrin tosylated (B); Structure of β-cyclodextrin (I); structure of β-cyclodextrin tosylated (II).

NMR confirmed the substitution at the 6-position due to an upfield shift of protons of primary hydroxyl origin, and the existence of an AB system due to protons linked to the tosyl group (Figure 2.12).

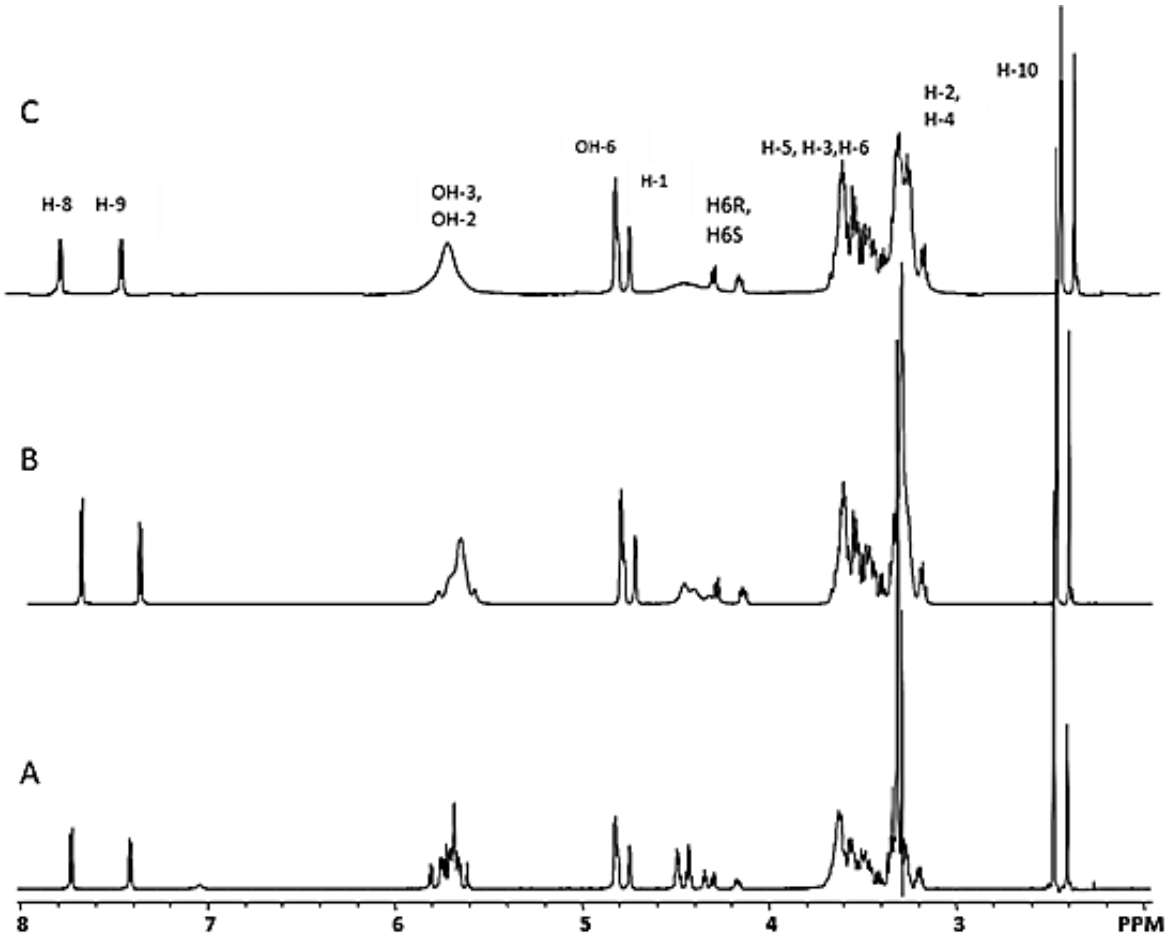


Figure 2.13 A- ^{1}H NMR spectrum of β-cyclodextrin tosylated without recristalization. B- ^{1}H NMR spectrum of β-cyclodextrin tosylated with one recristalization. C- ^{1}H NMR spectrum of β-cycodextrin tosylated after two purifications.

Re-crystallization of the tosylated product, as shown in Figure 2.13, only changes the kinetics of the intermolecular proton exchange, without modifying the product.

2.5.1.5 Characterization of diclofenac-β-cyclodextrin conjugate

2.5.1.5.1 High Performance Liquid Chromatography (HPLC)

After purification by column chromatography the diclofenac-β-cyclodextrin conjugate was fully characterized and the purity by HPLC analysis was high. The retention time of the conjugate (8.1 minutes) is distinct from the retention time of the tosylated β-cyclodextrin (5.5 minutes) as shown in Figure 2.14**.**

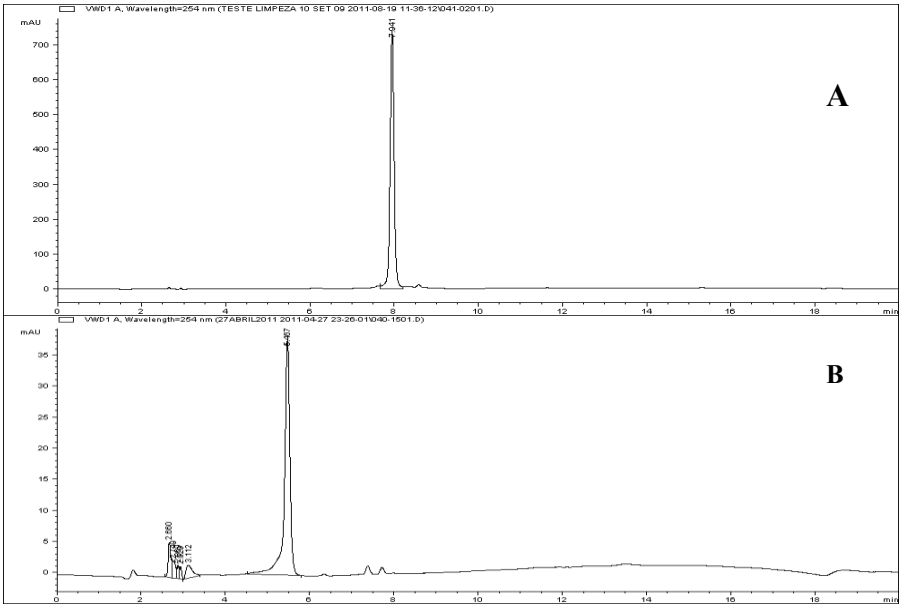


Figure 2.14 HPLC chromatogram of diclofenac-β-cyclodextrin conjugate (retention time of 7.9 minutes) after purification (A), and of tosylated β-cyclodextrin (retention time of 5.5 minutes) (B). Matrix-assisted laser desorption/ionization (MALDI) spectroscopy.

MALDI-TOF of the product shows only a signal m/z of 1434.308 corresponding to the [M+Na] adduct. No other relevant mass signal was observed as shown in Figure 2.15.

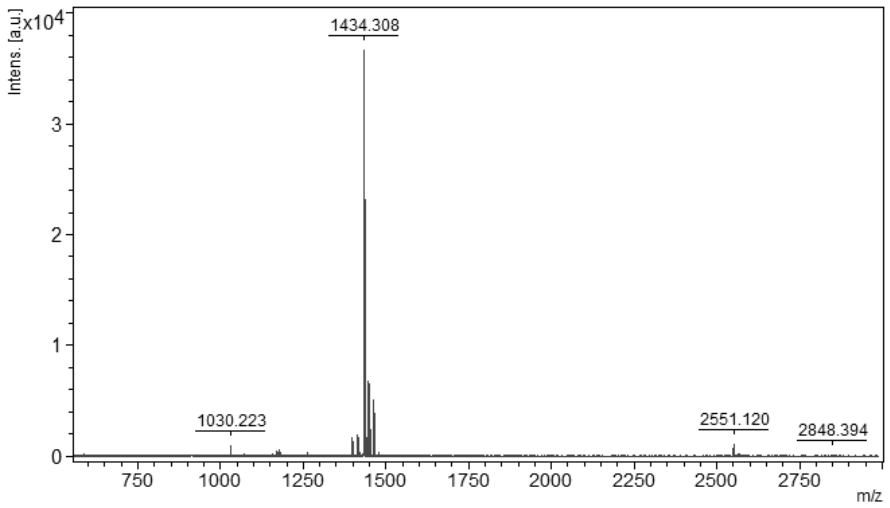


Figure 2.15 MALDI mass spectrum of diclofenac-β-cyclodextrin conjugate.

2.5.1.5.2 Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

The formation of the conjugate was proved by comparison of the ^{1}H NMR spectrum of the free β-cyclodextrin (Figure 2.16 II-A) and of the sodium diclofenac (Figure 2.16 II-B) with the spectrum of the diclofenac-β-cyclodextrin conjugate (Figure 2.16 II-C).

On the spectrum of sodium diclofenac, the H-2 protons appear at 3.38 ppm. Protons from the phenyl acetate ring protons are affected by the proximity of two chemical groups, the carboxylic group and the 2,6 – diclorophenyl ring. H-5'' (6.83 ppm) and H-6'' (7.05 ppm) appear as a triplet, and H-4'' (7.07 ppm) as a duplet. H-7''is the most shielded due to its proximity to the amine group. The signals of the 2, 6 diclorophenyl ring protons appear downfield. H-5' and H-3' are chemically and magnetically equivalent (7.43 ppm) and appear as a duplet, while H-4' (7.06 ppm) appears as a triplet.

In the diclofenac conjugate, all the signals related to diclofenac suffer downfield shifts, except for the H-2 protons. These modifications in the chemical shifts suggest that the diclofenac is not included in the cyclodextrin cavity when in the conjugate form. The upfield shift observed for H-2 protons of diclofenac is otherwise typical for the ones observed for an ester bond.

On the ^{1}H NMR analysis of the unmodified β-cyclodextrin (Figure 2.16 II-A), however, the secondary hydroxyl groups appear downfield (OH-2, singlet 5.76; OH-3, singlet 5.71), where these protons form intramolecular hydrogen bonds that contribute to a deshielding effect. The proton from the primary hydroxyl is a free-rotating proton and thus appears much more shielded at 4.61 ppm, as a triplet. A duplet due to H-1 protons appears at 4.81 ppm. The H-3 (triplet, 3.61 ppm) and H-5 (duplet of triplets, 3.54 ppm) protons are located within the cavity, while H-4 (triplet, 3.33 ppm) and H-2 (duplet of duplets, 3.30 ppm) are on the exterior of the cavity. This AB system corresponds to 1/7th of the area of the β-cyclodextrin H-1 protons, indicating once again a 1:1 stoichiometric relationship.

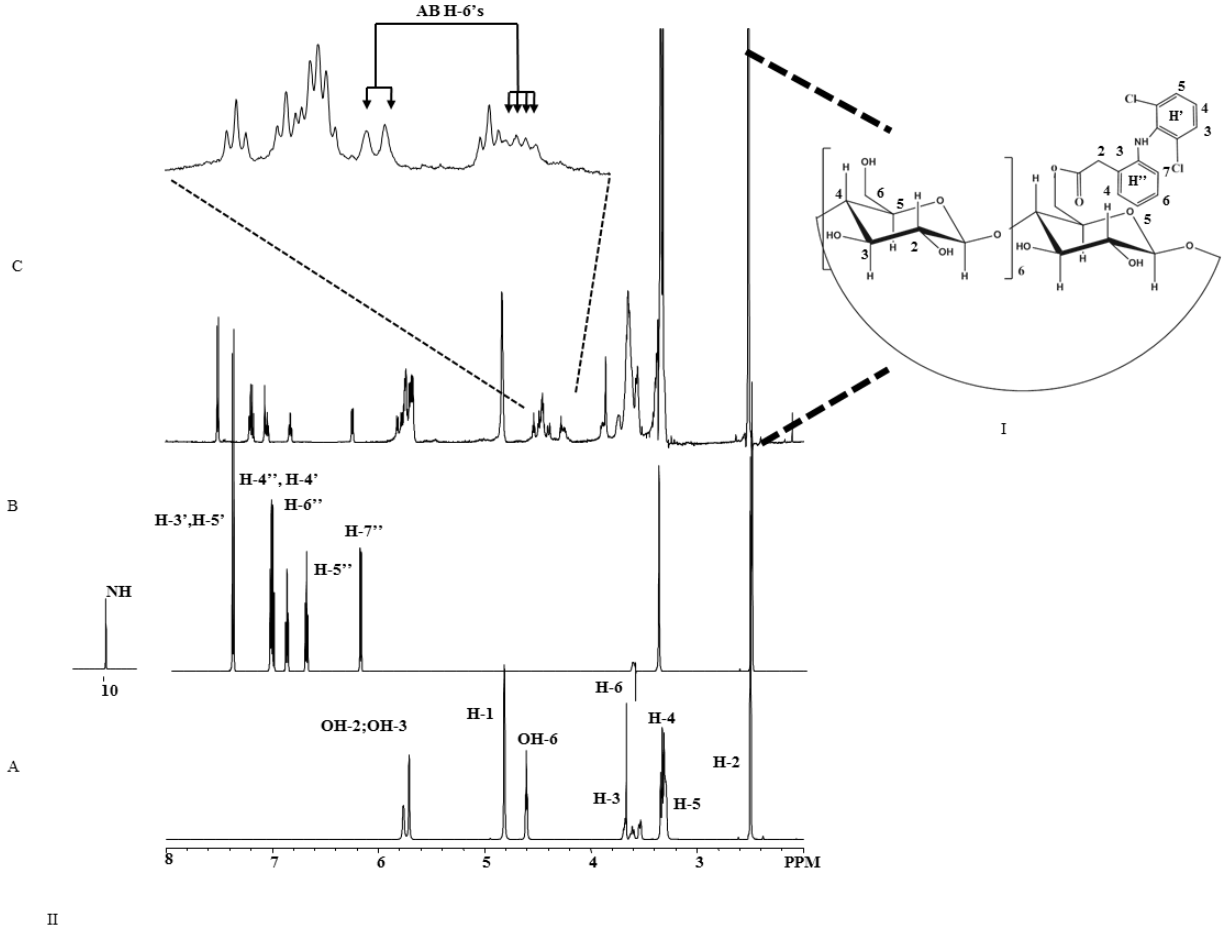
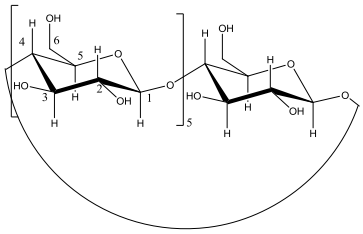
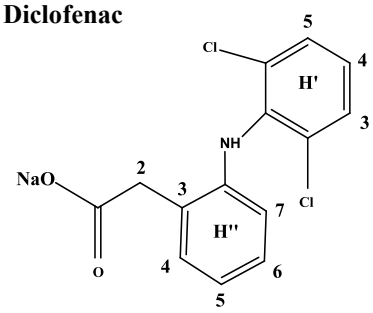


Figure 2.16 I-Structure of the diclofenac-β-cyclodextrin; II-A-^{1}H NMR spectrum of hydrated β-cyclodextrin hydrated. II-B-^{1}H NMR spectrum of sodium diclofenac. III-C-^{1}H NMR spectrum of diclofenac-β-cyclodextrin conjugate.

Tabela 1 ^{1}H Chemical Shifts (ppm) for the protons of diclofenac and of cyclodextrin in the free state and in the conjugated form in DMSO-d_6.

Molecule	Proton type	δ (ppm)	
		Free	Conjugate
B-cyclodextrin 	OH-2 (singlet)	5.76	Multiplet (OH-2;OH-3) (5.66-5.84)
	OH-3 (singlet)	5.71	
	OH-6 (triplet)	4.60	Multiplet (OH-6, H6 (AB system)) 4.21-4.55
	H6 (singlet)	3.66	
	H3 (triplet)	3.61	Multiplet (H6, H3,H5) 3.5-3.9
	H5 (duplet)	3.50	
	H2 (singlet)	3.38	Multiplet (H2,H3) 3.25-3.43
	H4 (triplet)	3.33	
	H1 (douplet)	4.81	4.83
Diclofenac 	H5', H3'(duplet)	7.43	7.52
	H4''(duplet)	7.07	7.22
	H4'(triplet)	7.06	7.20
	H6''(triplet)	6.92	7.05
	H5''(triplet)	6.74	6.83
	H7''(duplet)	6.22	6.25
	H-2 (singlet)	3.38	3.30

2.5.1.5.3 ROESY experiments

ROESY experiments shown in Figure 2.17 indicate that the only intermolecular correlations observed were between the H4'' and H6'' protons of the phenyl acetate ring and the H-6 protons of the β-cyclodextrin, corroborating the hypothesis of non-penetration of diclofenac into the hydrophobic core of cyclodextrin.

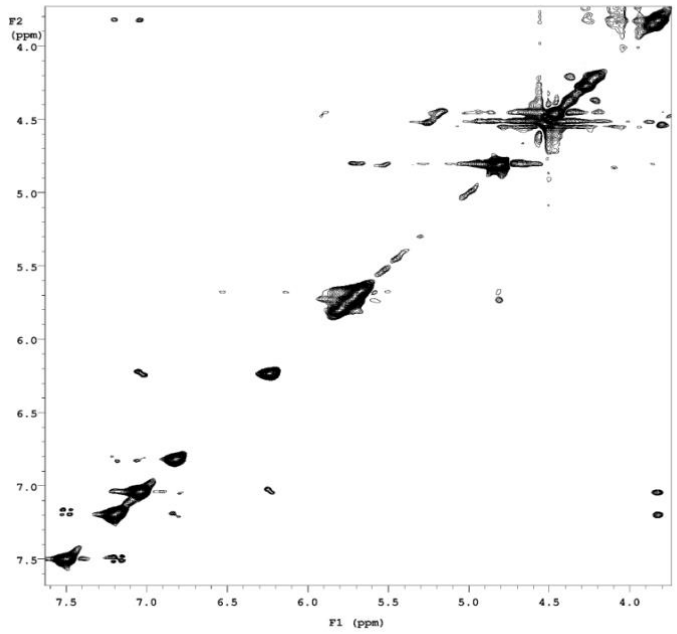


Figure 2.17 ^{1}H NMR ROESY spectra of the diclofenac-β-conjugate.

2.5.1.5.4 Infrared (IR) spectroscopy

IR spectroscopy also aided the identification of the diclofenac-β-cyclodextrin conjugate (see Figure 2.18) (Özkan, Atay et al., 2000; Wang, Han et al., 2006; Hamdi, Abderrahim et al., 2010; Jing, Yanping et al., 2011). The spectrum of the conjugate (Figure 2.18-C) shows a pattern distinct from the spectrum of β-cyclodextrin (Figure 2.18-B) and of the diclofenac (Figure 2.18-A). The broad band at 3300-3500 cm^{-1}, from the O-H stretching, is narrower and more closely resembles the corresponding β-cyclodextrin. Equally, the region at 1000-1200 cm^{-1}, from the C-O-C stretching, is similar in spectrum (Figure 2.18-B), but with a new prominent band located at 1028 cm^{-1}. Near the H-O-H bending band of β-cyclodextrin at 1650 cm^{-1}, a new band appears at 1729 cm^{-1} due to the created ester bond.

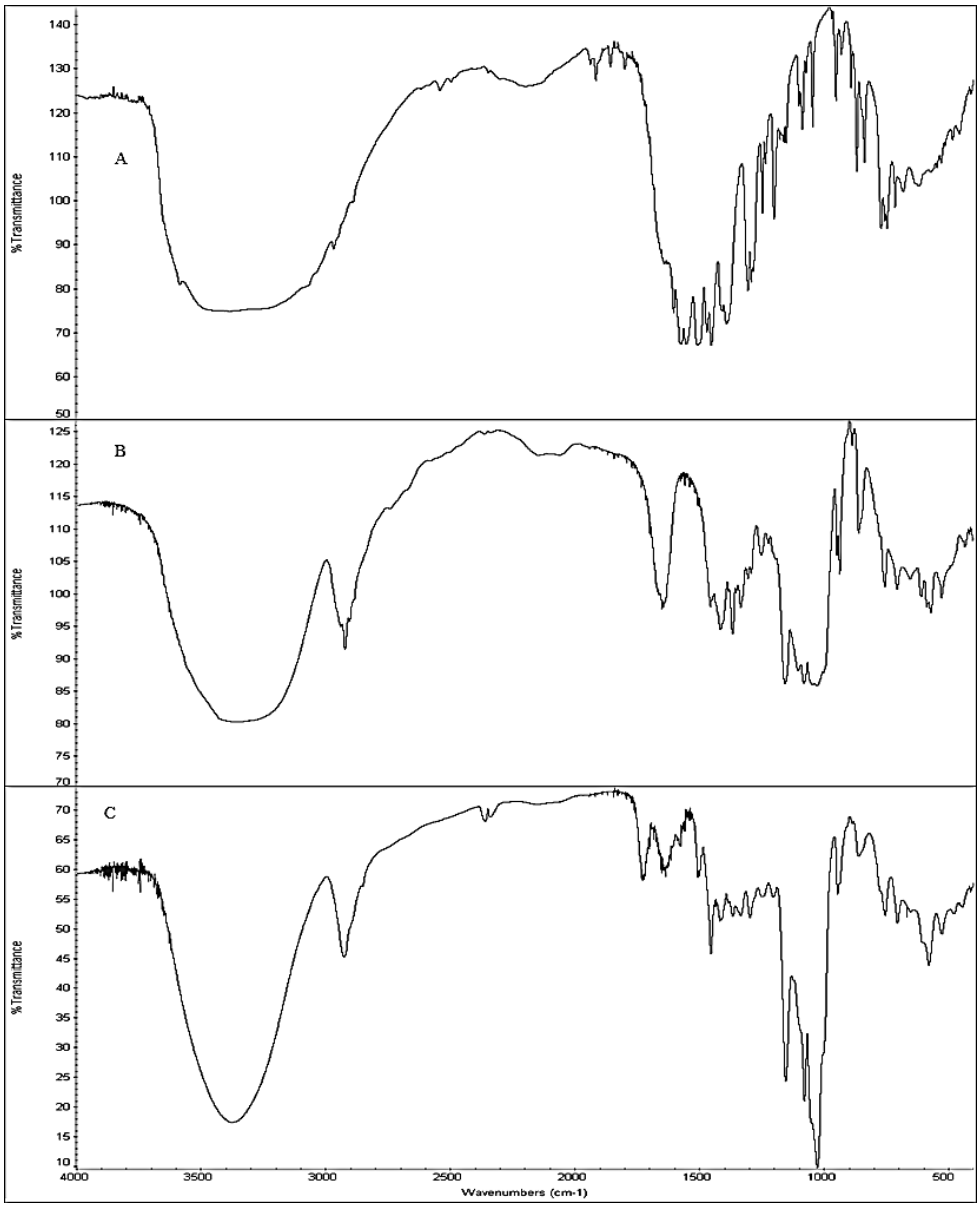


Figure 2.18 IR spectra of diclofenac (A), β-cyclodextrin (B) and diclofenac-β-diclofenac conjugate (C).

2.5.1.5.5 Chemical stability of diclofenac-β-cyclodextrin conjugate in aqueous buffer solutions

One of the physiological factors affecting drug release from prodrugs is the pH of the medium. Therefore in a first approach our objective was to investigate the effect of pH on diclofenac liberation from the obtained conjugates.

Results of *in vitro* hydrolysis studies, followed by HPLC, has shown in Figure 2.19, revealed that the conjugate is markedly stable to all selected pH values during the first 4 h.

After 24 hours, only for the pH of 1.2, the conjugate suffered degradation of about 25%. These results are very significant and showed that this conjugate can be chemically stable in the pH environment of the gastrointestinal tract.

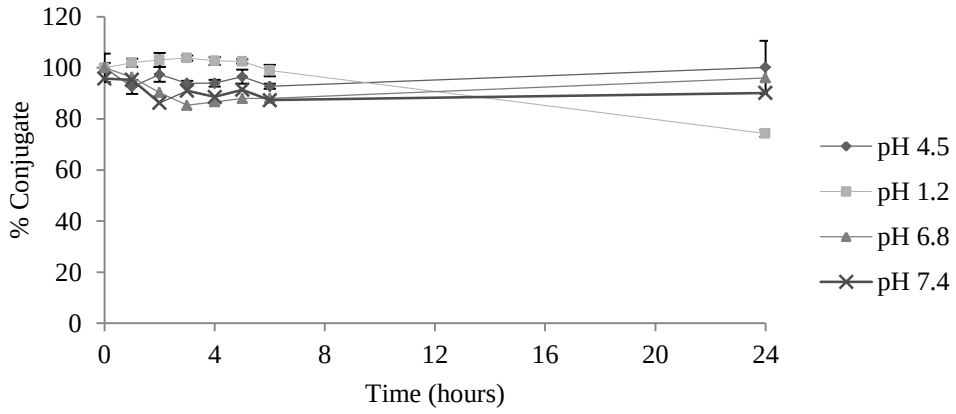


Figure 2.19 Stability studies of diclofenac-β-cyclodextrin in differents aqueous buffer solutions. Each value represents mean ± S.D (n=3).

During these experiments, it was found out that in some samples rather than conjugate and diclofenac peaks, in HPLC chromatograms other peaks with a retention time closer to the retention time of diclofenac were observed, as it is shown in Figure 2.20-A.

In order to understand the origin of these new peaks, sodium diclofenac and diclofenac free acid were incubated in pH 1.2 and the results were analyse by HPLC. Extra peaks were also observed in the samples of diclofenac free acid (Figure 2.20-B) and sodium diclofenac (Figure 2.20-C) incubated at pH 1.2, which allowed to conclude that the extra peaks were originated from the contact of the diclofenac with the acid solution. Further studies need to be done in order to investigate the precise structure of the derivative from diclofenac molecule.

It has already been reported that diclofenac suffers an intramolecular cyclization under the acidic conditions, which can cause its inactivation. As a consequence of the intramolecular cyclization, Na^+ is lost hence the solubility of the compound decreases (Palomo, Ballesteros et al., 1999). It is reported the formation of 1-(2,6-dichlorophenyl)-indolin- 2-one, which is an indol-cyclic amide and an intermediate product of the synthesis of diclofenac sodium. (Yasumitsu Tamura, Jun-Ichi Uenish et al., 1984).

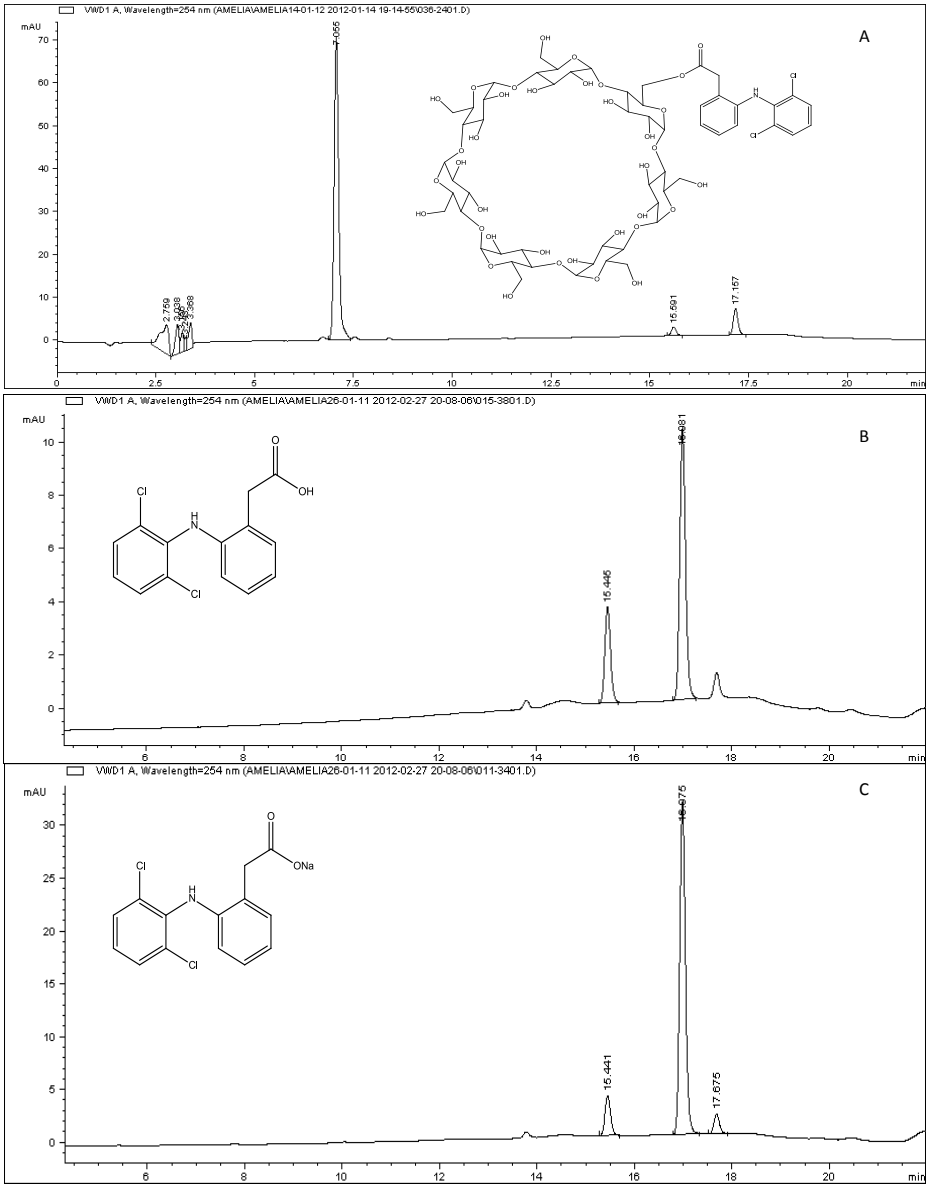


Figure 2.20 HPLC chromatograms of diclofenac-β-cyclodextrin (A), diclofenac free acid (B) and sodium diclofenac (C) after incubation in buffer at pH 1.2 for 24 h in case of A and B and 5 h in case of C. Diclofenac-β-cyclodextrin conjugate retention time is 7.0 and retention time of diclofenac is 16.9 minutes.

2.5.2 *Applicability of the nucleophilic microwave approach on the synthesis of diclofenac-γ-cyclodextrin and diclofenac-α-cyclodextrin*

The synthesis of other diclofenac conjugates derived from the natural α- and γ- cyclodextrins was attempted by the method that successfully allowed the synthesis of diclofenac-β-cyclodextrin.

The main goal is to assess if the reaction is possible using other cyclodextrins, once physical differences exist between them. Both, α- and β-cyclodextrins are much more soluble in water than β-cyclodextrin, and the molecular size is also different with consequently different reactivities. Thus, the ability of the nucleophilic reaction under microwaves was tested.

Like observed for β-cyclodextrin, modification of the hydroxyl group by electrophilic reagent on α- and γ-cyclodextrins had to be produced first (Step 1) followed by the nucleophilic reaction (Step 2).

Different problems were encountered on the synthesis of α- and γ-cyclodextrin conjugates as described below.

2.5.2.1 Synthesis and characterization of diclofenac-γ-cyclodextrin conjugate

2.5.2.1.1 Step 1: tosylation of γ-cyclodextrin

Tosylation of γ-cyclodextrin was carry out according to the method described to prepare ditosylated cyclodextrin (Kahee Fujita, Hatsuo Yamamura et al., 1988). The synthesis of monotosilated γ-cyclodextrin is very difficult since γ-cyclodextrin does not form an inclusion complex with tosyl derivatives as described by Bruno Perly *et al.*. Consequently, its big cavity causes polysubstitution when it reacts with *p*-toluenesulfonyl chloride (Djedaini-Pilard, Azaroual-Bellanger et al., 1995). Tosylation occurs preferentially at hydroxyl groups at C-6. However, 8 primary hydroxyl groups are available and therefore the reaction needs to be controlled.

As it is shown in Figure 2.21 (Section 2.5.2.1.3), the product of this reaction is a mixture of different tosylated products with multiple degrees of substitution even after recrystallization process. In order to get the monotosylated product, some changes were made in the reaction conditions namely the decrease of the ratio tosyl chloride/cyclodextrin, time of reaction, and temperature; however no significant differences in product distribution were observed.

Moreover, besides its toxicity, working with pyridine led to the formation of an unstable stick mass during isolation, which rendered stirring impossible during the course of the reactions and during the isolation of the product.

The attempt of tosylation γ-cyclodextrin adopting the method in aqueous solution as used described to synthesized β-cyclodextrin tosylated (Brady, Lynam et al., 2000) failed. As described by Florence Djeda'ini-Pilard *et al.* the reaction of γ-cyclodextrin with 4-toluenesulfonylchloride in aqueous solution results in polysubstitution at C-6 probably because of the larger γ-cyclodextrin annulus (Djedaini-Pilard, Azaroual-Bellanger et al., 1995). Moreover, once γ-cyclodextrin presents higher water solubility when compared with β-cyclodextrin, the final product in water is very difficult to isolate, once it did not precipitate by the addition of common organic solvents, such as acetone.

In order to get mono-6-(O-2,4,6-triisopropylbenzenesulfonyl)-cyclodextrin, the method described by Palin *et al.* was adopted. This procedure resulted in less polisubstitution than that obtained using the 4-toluenosulphonyl chloride. In our case the mono but also dissubstituted product were obtained, as confirmed by the mass spectrum (Figure 2.22**)** (Palin, Grove et al., 2001).

Beyond, 2,4,6-triisopropylbenzenesulfonyl, other larger arenesulfonyl chlorides, such as 2-naphthalenesulfonyl chloride (Djedaini-Pilard, Azaroual-Bellanger et al., 1995), have been explored by other authors in order to control the production of monosubstituted γ-cyclodextrins (Djedaini-Pilard, Azaroual-Bellanger et al., 1995). For instance, the synthesis of mono-6-(2-naphthalenesulfonyl)-γ-cyclodextrin (Ueno, Tomito et al., 1983) was adopted by Kaneto Uekama *et al.* 1998 (Uekama, Minami et al., 1997b) to produce BPAA-γ-cyclodextrin conjugate. However, Palin *et al.* obtained di- and tri-adducts when preparing mono-6-(2-naphthalenesulfonyl)-γ-cyclodextrin according to literature procedures, reporting the difficulty inherent to purification of the monosubstitued cyclodextrin. Additionally, the use of this alternative arenesulfonyl provides low yields with a more expensive (Palin, Grove et al., 2001).

2.5.2.1.2 Step 2: nucleophilic reaction

This step is performed using the crude product poli-substitued derivative obtained on the former step.

Using the microwave approach to synthesize diclofenac-γ-cyclodextrin conjugate, di-diclofenac-γ-cyclodextrin resulted as a contaminant. Purification of the product allowed isolation of diclofenac-γ-cyclodextrin as it can be observed in Figure 2.24**.** After purification, the monosubstituted product was obtained with 10% yield and characterized, according as described in point 1.4.2.1.2.

However, the reaction revelead not to be reproducible and the yield depended on the batch of the tosylated γ-cyclodextrin that was formerly obtained. Although, all the tosylated cyclodextrins presented the same degrees of substitution, different results were obtained when the nucleophilic attack was attempted. The small amount of crude product obtained in some of the nucleophilic reactions seemed to be related with the amount of pyridine present in the tosylated product. Recrystallization revealed not to be efficient in the elimination of this solvent. Therefore, it is suggested that the success of this step depends on the purity of the tosylated derivative.

Thus, the polisubstitution resulted from the reaction with *p*-toluenosulphonyl chloride and the lack of reproductibility of the nucleophilic reaction using the *p*-toluenosulfonyl cyclodextrin, led us to use the product resultant from the reaction between 2,4,6-triisopropylbenzenesulfonyl and γ-cyclodextrin, as an alternative. The nucleophile reaction in microwaves resulted only in trace amounts of crude product, which contained γ-cyclodextrin and diclofenac-γ-cyclodextrin conjugate. It is possible that the steric hindrance caused by the substitution pattern of this derivative is not favourable for the occurrence of nucleophilic attack by diclofenac. Results suggested that the prevalent reaction is the hydrolysis of the tosylated cyclodextrin.

Thus, further studies involving the synthesis and purification of monotosylaled γ-cyclodextrin need to be performed in order to improve the quality and yield of this intermediate, that is the key step to get reproducibility of the nucleophilic reaction (Step 2).

2.5.2.1.3 Characterization of 6-*p*-toluenosulfonyl-γ-cyclodextrin

Mass spectrometry demonstrated that the product of the tosylation reaction of γ-cyclodextrin with *p*-toluenosulphonyl chloride is mainly the monotosylated γ-cyclodextrin (1512.4 [M+Na+K] adduct) and ditosylated γ-cyclodextrin (1666.4 [M+Na+K] adduct) and

tritosylated γ-cyclodextrin (1820 [M+Na+K].adduct) contaminated with some γ-cyclodextrin (1372 [M+K+Na+H2O] adduct).

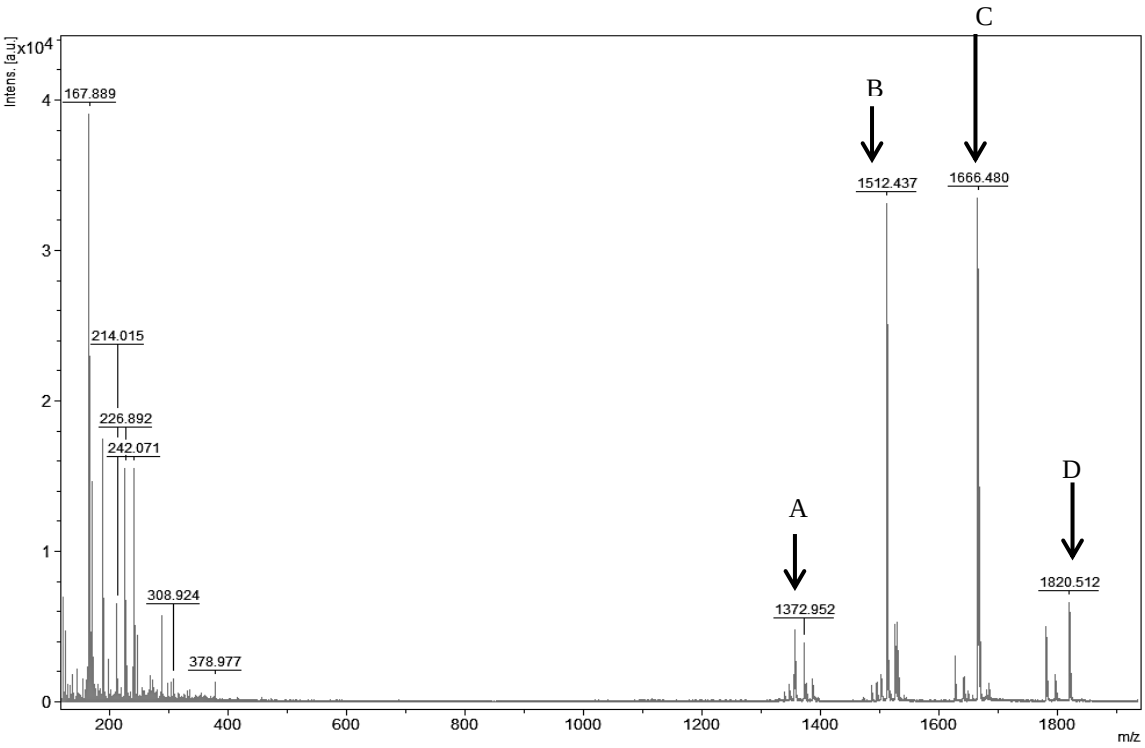


Figure 2.21 MALDI mass spectrum of the product from tosylation of γ-cyclodextrin with *p*-toluenosulphonyl chloride. (A) γ-cyclodextrin, (B) monotosylated γ-cyclodextrin, (C) ditosylated cyclodextrin, (D) tritosylated γ-cyclodextrin.

2.5.2.1.4 Characterization of Mono-6-(O-2,4,6-triisopropylbenzenesulfonyl)-γ-cyclodextrin

Mass spectrometry demonstrated that the product from tosylation reaction with triisopropylbenzenesulphonyl chloride is mainly the mono-γ-6-(*O*-2,4,6-triisopropylbenzenesulfonyl)-γ-cyclodextrin (1624 [M+2Na] adduct) and di-6-(*O*-2,4,6-triisopropylbenzenesulfonyl)-γ-cyclodextrin (1890 [K+2H_2O] adduct).

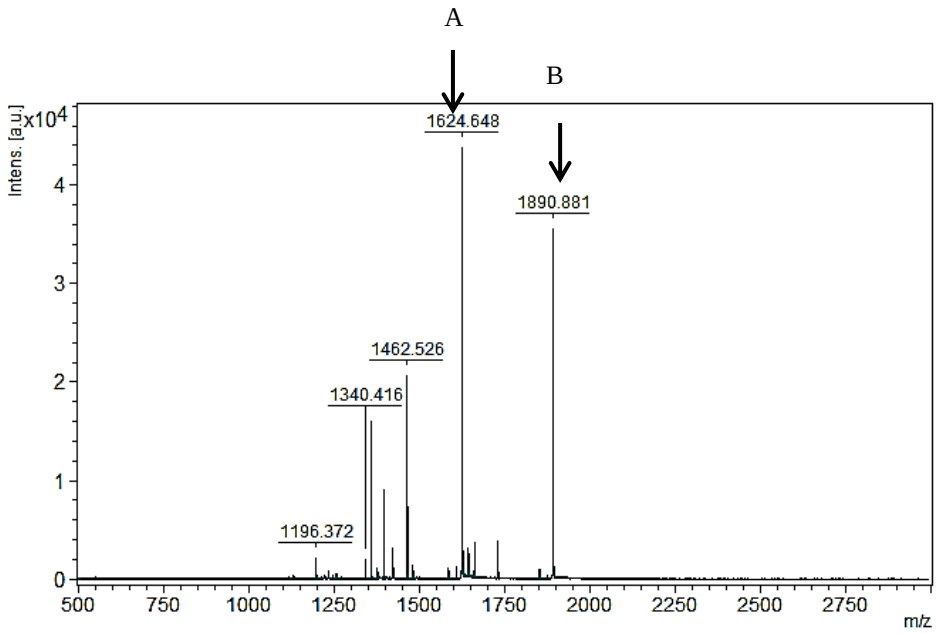


Figure 2.22 MALDI mass spectrum of the product from tosylation of γ-cyclodextrin with *triisopropylbenzenesulfonyl* chloride. (A) mono-γ-6-(*O*-2,4,6-triisopropylbenzenesulfonyl)-γ-cyclodextrin; (B) di-6-(*O*-2,4,6-triisopropylbenzenesulfonyl)-γ tolsylcyclodextrin.

2.5.2.1.5 Characterization of the diclofenac-γ-cyclodextrin conjugate

Matrix-assisted laser desorption/ionization (MALDI) spectroscopy

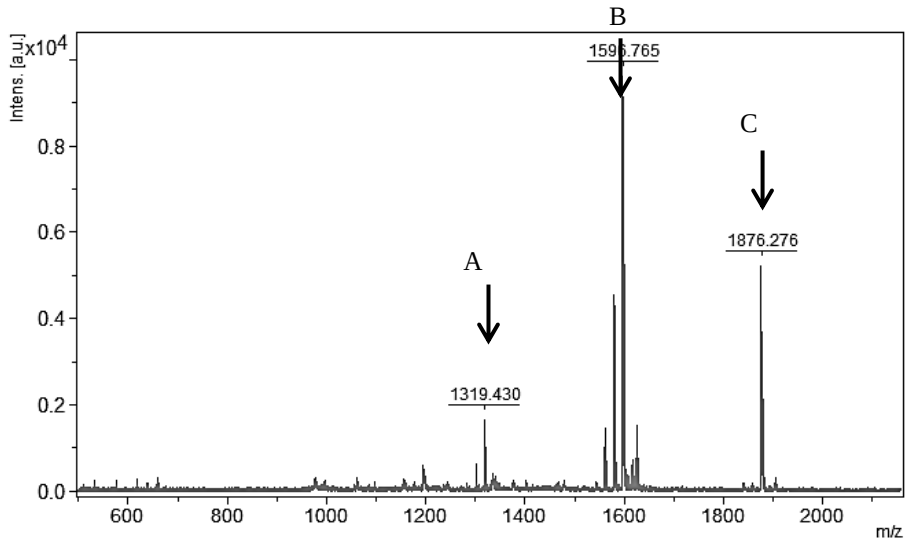


Figure 2.23 MALDI mass spectrum of crude product obtained after microwave reaction between γ-cyclodextrin tosylated and sodium diclofenac.(A) γ-cyclodextrin; (B) diclofenac-γ-cyclodextrin, (C) di-diclofenac-γ-cyclodextrin.

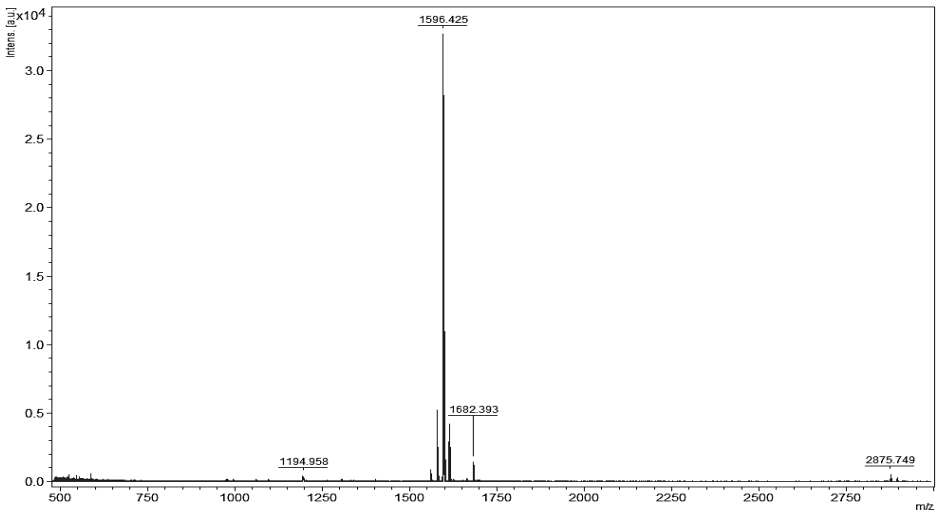


Figure 2.24 MALDI mass spectrum of compound diclofenac-γ-cyclodextrin conjugate after purification using DIAION HP.

Figure 2.23presents the mass spectra of the diclofenac-γ-cyclodextrin-conjugate obtained from microwave conditions before purification. Otherwise, in Figure 2.24 we have the mass spectrum after chromatographic isolation using DIAION HP resin. The value of m/z of 1596.308 correspond to the [M+Na] adduct.

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

The NMR and mass spectrometric analysis indicated that the carboxyl group of diclofenac is covalently bound to one of the primary hydroxyl groups of γ-cyclodextrin through an ester bound.

In the spectrum of the diclofenac conjugate, integration of the signals allows concluding that ratio between cyclodextrin and diclofenac is exactly 1:1. In this conjugate, similarly to β-cyclodextrin conjugate the ester bond occurs at the hydroxyl of 6-position. The H-6 protons linked to diclofenac resonate downfield relative to the unsubstituted H-6 protons, and present a distinct multiplicity. In Figure 2.24, we can observe an AB system (centered at 4.308) due to such protons.

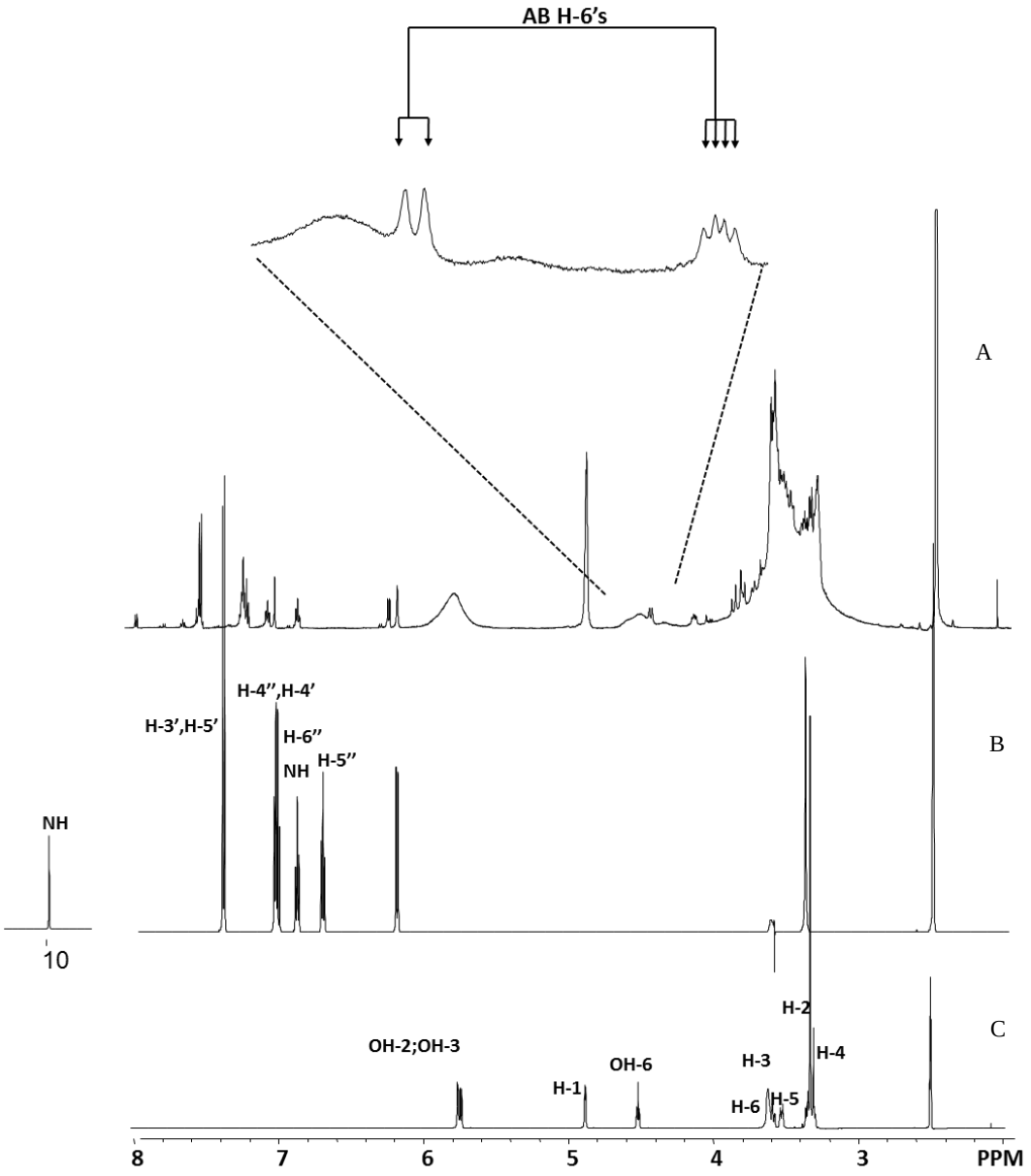


Figure 2.25 (A) ^{1}H NMR spectrum of γ-cyclodextrin. (B) ^{1}H NMR spectrum of sodium diclofenac. (C) ^{1}H NMR spectrum of diclofenac-γ-cyclodextrin conjugate.

2.5.2.2 Synthesis and characterization of diclofenac-α-cyclodextrin conjugate

2.5.2.2.1 Step 1: tosylation of α-cyclodextrin

Tosylation of α-cyclodextrin in water was not attempted, once it was reported that tosylation in alkaline solution results in substitution in the C-2 hydroxyl (Takahashi, Hattori et al., 1984b; Fujita, Nagamura et al., 1985).

Tosylation of α-cyclodextrin was performed combining two procedures, one described by Keiko Takahashi *et al,* and the other Tang *et al.* after several attempts to reproduce each method individually (Keiko Takahashi, Kenjiro Hattori et al., 1984; Weihua Tang and Ng, 2008).

The reaction was performed at room temperature. It were studied different times of reaction as well as the proportions of reagents in order to get the monosubstituted α-cyclodextrin.

The best results were obtained using 1/2.5 (cyclodextrin/6-*p*-toluenosulfonyl chloride) ratio, which led to obtain minimum degree of polysubstitution. However, as the mass spectra shows, part of the α-cyclodextrin did not react. The increase of the time of reaction did not give better results.

Attempts to purify this product, by crystallization or solvent washings in order to eliminate the non-reacted cyclodextrin, were accompanied with loss of the major part of the product. Additionally, like experienced with tosylation of γ-cyclodextrin, pyridine was very difficult to eliminate, which was confirm by the ^{1}H NMR spectra. Results suggest that pyridine can remain within the cyclodextrin, which is a very difficult task to remove from the mixture.

Chromatographic purification of the product of tosylation of α-cyclodextrin with DIAION resin was attempted and it was successful in eliminating pyridine. However, after chromatography a mixture of mono and ditosilated α-cyclodextrin was obtained (6% of the initial amount of crude product).

The use of an alternative solvent, like colidine, was made revealing to allow the formation of the same substituted cyclodextrins. However, the problem to eliminate this solvent after the reaction still remains.

2.5.2.2.2 Step 2: nucleophilic reaction: microwave approach

Due to the low quantities of tosylated cyclodextrin obtained after recrystallization, there had been few attempts to produce diclofenac-α-cyclodextrin conjugate. Furthermore, when the isolated was analysed by mass spectra it did not show any signal of diclofenac-α-cyclodextrin conjugate. Once again, the lack of product resulted from the absence of a clean tosylated derivative, free of pyridine. Further studies are needed to improve the process of tosylation of this cyclodextrin.

2.5.2.2.3 Characterization of 6-*p*-toluenosulfonyl-α-cyclodextrin

Mass spectrometry demonstrated that the product from the tosylation reaction of α-cyclodextrin with *p*-toluenosulphonyl chloride is mainly the monotosylated α-cyclodextrin (1188.382 [M+Na+K] adduct) and ditosylated α-cyclodextrin (1342.402 [M+Na+K] adduct) contaminated with some α-cyclodextrin (1034 [M+K+Na] adduct).

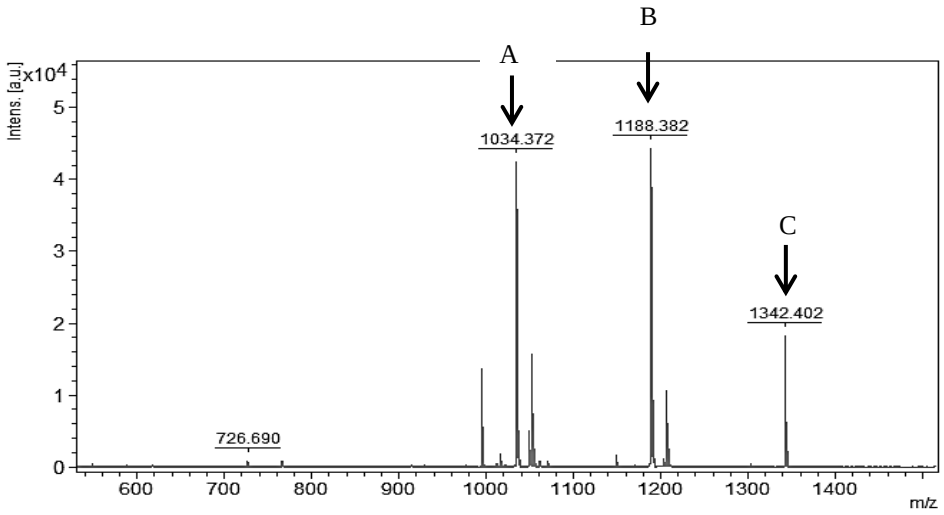


Figure 2.26 MALDI mass spectrum of the product of tosylation of α-cyclodextrin with *p*-toluenosulphonyl chloride. (A) α-cyclodextrin; (B) monotosylated α-cyclodextrin; (C) ditosylated α-cyclodextrin.

2.6 Conclusions

An efficient chemical method to synthesize diclofenac-β-cyclodextrin conjugate was developed. Additionally, the possibility to apply the same method to synthesize γ-cyclodextrin and α-cyclodextrin derivatives was explored.

Initially, various approaches were investigated to synthesize β-cyclodextrin conjugates. These included: nucleophylic substitution with conventional heating, activation of diclofenac using carbodiimides, and formation of an acid chloride. All three approaches were unsuccessful in the synthesis of the conjugate. We came to the conclusion that these approaches did not work due to the particular structure of diclofenac since they were successfull in other cyclodextrin conjugates.

Microwave radiation is being explored in organic synthesis in order to accelerate reactions. Some authors have explored the use of this technique to promote reactions of cyclodextrin. However, this type of non-conventional conditions was not applied in reactions that allow the formation of cyclodextrin conjugates.

In this work, using the nucleophylic approach under microwave irradiation, diclofenac-β-cyclodextrin conjugate was synthesized in efficient conditions and conveniently short reaction time. The product was then purified through a polyaromatic ionic adsorbent resin. ^{1}H NMR studies confirmed the covalent link of the carboxylic group of diclofenac with the hydroxyl of β-cyclodextrin, and bidimensional ^{1}H NMR ROESY allowed to conclude that diclofenac is not included inside the cavity of cyclodextrin.

Preliminary chemical hydrolysis assays were performed in order to discern about the stability of the conjugate. The drug release behavior of these conjugates was studied in different buffer solutions (pH 1.2, 4.5, 6.8 and 7.4) at 37 ºC. Results showed that the conjugate was stable in pH 4.5 and pH 6.8 and 7.4. Moreover, hydrolysis in pH 1.2 is negligible.

Preliminary results of β-cyclodextrin conjugate in different physiological conditions allows predicting that this conjugate resists to different gastrointestinal tract pHs.

The synthesis of γ-cyclodextrin conjugate was made using the same approach and the end product was characterized. However, this reaction showed not to be reproducible. On the other hand, the production of α-cyclodextrin conjugate was not successful. Synthesis of diclofenac α-cyclodextrin and γ-cyclodextrin conjugates was hindered by difficulties in the synthesis and purification of the intermediary tosylated cyclodextrins. Further work needs to be done to improve the process to prepare these tosylated intermediaries, since this seems to be the key step to produce these conjugates.

The first step of this thesis was very challenging and demonstrated that the chemical differences between cyclodextrins have to be taken in account when the aim is to synthesize prodrugs. Additionally, diclofenac is a very instable molecule, which prevents its conjugation in some chemical conditions. Overall, diclofenac-β-cyclodextrin revealed to be a molecule with a strong potential for colon delivery of diclofenac. Therefore, the next Chapters focus on the investigation of the potential of diclofenac-β-cyclodextrin to be used as a colonic prodrug.

CHAPTER III

STABILITY OF DICLOFENAC-β-CYCLODEXTRIN IN THE GASTROINTESTINAL TRACT. *IN VITRO* AND *EX VIVO* STUDIES USING SIMULATED FLUIDS, GASTROINTESTINAL FLUIDS FROM ANIMALS.

3 STABILITY OF DICLOFENAC-BETA-CYCLODEXTRIN IN THE GASTROINTESTINAL TRACT. *IN VITRO* AND *EX VIVO* STUDIES USING SIMULATED FLUIDS AND GASTROINTESTINAL FLUIDS FROM ANIMALS

3.1 Overview

An ideal prodrug for colon specific drug delivery should be stable in the stomach and small intestine and reach the colon intact. Once the prodrug reaches the colon, the enzymes should catalyse the drug-carrier bond breakdown, hence releasing the drug. Moreover, this process should be rapid and complete.

In Chapter II, stability studies were carried out by incubation of diclofenac-β-cyclodextrin conjugate in different buffer solutions in order to establish its ability to resist chemical hydrolysis. According to those results, the conjugate revealed to be chemically stable. The next aim is to evaluate the pre-systemic behaviour of the conjugate, namely its stability in the GI tract by performance of *in vitro* and *ex vivo* studies using appropriate media able to represent the physiological conditions of the GI tract.

This Chapter firstly pursues the investigation the ability of this based cyclodextrin prodrug to release diclofenac in the lower intestine, using human faecal slurry to simulate colonic fluid. These stability studies in the lower gastrointestinal tract are performed using a well-established marketed available model of colonic delivery, sulfasalazine, as a positive control. When sulfasalazine is orally administered, approximately 90 percent of it reaches the large intestine as an intact molecule, where bacterial azoreductase cleaves the azo bond thereby releasing mesalazine and sulfapyridine (Zoetendal, Akkermans et al., 1998).

Moreover, the stability is performed in the presence of enzymes, namely ester hydrolysing enzymes and sugar-degrading enzymes in order to find an enzymatic media able to mimic the metabolism of cyclodextrin-β-cyclodextrin in the colonic environment. Concomitantly, these studies with enzymes are worth to understand the process involved on the metabolism of this conjugate.

Further, this Chapter investigates the ability of the conjugate to be intact in the upper gastrointestinal fluids, namely at gastric and intestinal level, using simulated gastric and intestinal fluids, described in the Pharmacopeia.

The Chapter ends with stability studies conducted in fluids from different animals (pig, rabbit and rat) in order evaluate the validity of each animal model to perform *in vivo* experiments.

3.2 Introduction

Any new molecule destined to oral delivery should be tested *in vitro* at an early stage of development regarding its stability in the different environments of the GI tract, namely, stomach, small intestine and colon. This allows an understanding of the pre-systemic behavior of any molecule. Moreover, in the case of prodrugs, the stability tests will make it possible to determine the site and rate of prodrug-to-drug conversion.

Different approaches are attempted in order to study and establish cyclodextrin-β-cyclodextrin stability in the complex environment that constitutes the luminal content of the gastrointestinal tract.

According to FDA Guidelines (Fda, 2000):

"Stability in the gastrointestinal tract may be documented using gastric and intestinal fluids obtained from human subjects. Drug solutions in these fluids should be incubated at 37 ^{o}C for a period that is representative of *in vivo* drug contact with these fluids; for example, 1 hour in gastric fluid and 3 hours in intestinal fluid. Drug concentrations should then be determined using a validated stability-indicating assay method. Significant degradation (>5%) of a drug in this protocol could suggest potential instability. Obtaining gastrointestinal fluids from human subjects requires intubation and may be difficult in some cases. Use of gastrointestinal fluids from suitable animal models and/or simulated fluids such as Gastric and Intestinal Fluids USP can be substituted when properly justified".

This guidance for the determination of drugs stability in the gastrointestinal tract can be adopted to guide the stability of prodrugs to site colonic delivery, namely of diclofenac-β-cyclodextrin prodrug.

3.2.1 *Simulated gastrointestinal fluids*

3.2.1.1 Simulated colonic fluid

The primary region of the GI tract of interest to evaluate the effectiveness of a colonic delivery system is the colon. The lack of a Pharmacopeia simulated colonic fluid for *in vitro* studies warrants the use of other methodology to mimic the environment of the human colon. Studies in fresh human fluids constitute an alternative that closely reproduces the environment *in vivo.* In case of colonic release systems, the ascending colon is the primary region of interest not only because of the substantial residence time in this region of the lower intestine (Metcalf, Phillips et al., 1987; Kilian and Clive, 2005), but also due to the limited free water volume in the transverse colon (Schiller, Fröhlich et al., 2005). Consequently, the potential of colonic prodrugs should be evaluated in fluids from this region of the colon. However, human fluids are not easily available due to the difficulty in sampling. An alternative is the use of faeces to study metabolism at the level of the lower intestine. The advantage is that the microbiota is essentially confined to the distal intestinal tract and epithelium associated bacteria are apparently absent. The use of fresh human feces within an appropriate medium has been identified as a suitable alternative, the well-known faecal slurry (Sousa, Paterson et al., 2008). These faecal slurries from humans provide accurate information concerning not only the intestinal microbiota, but also the enzymatic and electrolytes composition (Tannock, 1999; Basit and Lacey, 2001; Basit, Newton et al., 2002). Additionally, it is known that microbiota is a major component and constitutes 40-45% of faecal solids in people consuming Western-type diets (Cummings and Macfarlane, 1991).

3.2.1.2 Enzymatic colonic media containing enzymes

Given all the drawbacks associated with the use of biological fluids, namely the odour, complexity of its composition, the lack of homogeneity, the thickness of the faeces matrix and further the difficulties in the analysis of the drug release into this complex medium, alternative media able to simulate the colon have been investigated.

Maria Vertzoni *et al*. developed two media (fasted state and fed state simulated media) able to mimic the ascending colonic fluids useful for the determination of solubility of drugs at this level (Vertzoni, Diakidou et al., 2010). These media do not contain enzymes and therefore are not reliable to determine the drug release from colonic delivery systems activated by

microbiota. Other authors have incorporated specific enzymes in a buffer medium able to simulate the colonic fluids. For instance, galactomannanase was included in the medium for the dissolution of guar gum based system (Wong, Larrabee et al., 1997), a pectinolytic enzyme was added to a phosphate buffer (pH 6.8) to assess the *in vitro* drug-release behaviour of pectin cross-linked with glutaraldehyde nanogel particles, a matrix for colonic drug delivery (Chang, Wang et al., 2007) and dextranase was included in buffer media to simulate the release of dextran-conjugates in the lower intestine (Mcleod, Friend et al., 1994).

The selection of a medium to simulate the colon fluids is dependent on the rationale design of the delivery system. This Chapter discusses the development of a simpler enzymatic simulated medium able to reproduce the digestion profile of diclofenac-β-cyclodextrin in the large intestine. Thus, ester-hydrolysing and sugar-degrading enzymes were tested as potential enzymes involved on the drug release from this cyclodextrin based prodrug at colonic level.

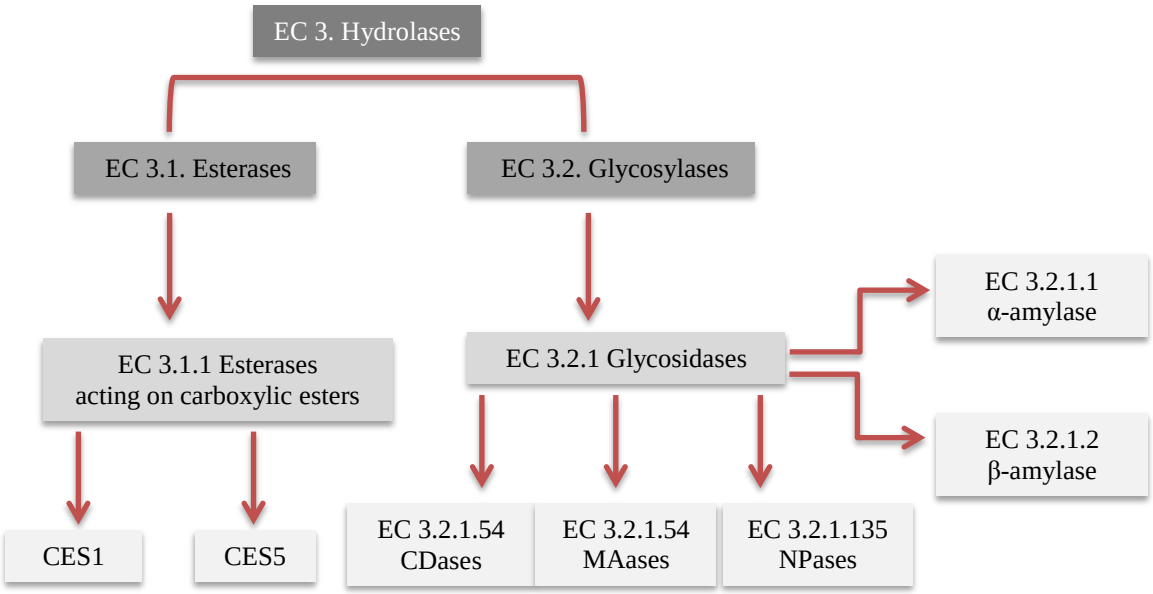


Figure 3.1 Schematic representation of enzymes involved in the hydrolysis of various bonds (Hydrolases EC 3.), including those acting on ester bonds (esterases EC 3.1.) and those that hydrolyse glycosyl compound (glycosylases EC 3.2.). Glycosidases hydrolyse O- and S-glycosyl compounds (EC 3.2.1). This subgroup includes cyclomaltodextrinases (CDases; EC 3.2.1.54), maltogenic amylases (MAases; EC 3.2.1.133) and neopullulanases (NPases; EC 3.2.1.135). This classification is according to Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB).

The stability of diclofenac-β-cyclodextrin is tested against hydrolases (EC 3) used to mimic enzymatically the behavior in the large intestine. Once the carrier is an oligosaccharide, its susceptibility to be hydrolysed by glycosylases (EC 3.2), namely by glycosidases (EC 3.2.1),

i.e. enzymes hydrolysing *O*- and *S*-glycosyl compounds needs to be examined. Additionally, given the fact that diclofenac-β-cyclodextrin possesses an ester linkage between the drug and the carrier, studies are conducted in the presence of hydrolases able to act on the ester bonds, esterases (EC 3.1.), namely those acting on carboxylic ester (EC 3.1.1) (see Figure 3.1).

Below is described briefly the main characteristics and occurrence of esterase and glycolytic enzymes in the gastrointestinal tract capable of acting against diclofenac-β-cyclodextrin conjugate.

- ***Carboxylesterases (CES) (EC 3.1.1.)***

The mammalian CES comprise a multigene family and the isozymes are classified into five main CES groups (CES1-CES5) and several subgroups. The majority has been segregated into CES1 and CES2 families. These are involved in both drug metabolism and activation of ester, amide and carbamate bonds. Typically the expression of CES is maximal in the epithelia of most organs (Imai, 2006; Landowski, Lorenzi et al., 2006). CES1 is present predominantly in the liver; its expression is low along the gastrointestinal tract. CES2 is predominantly present in the small intestine and colon (Imai, Imoto et al., 2005). CES1 hydrolyses a substrate with a small alcohol group and a large acyl group, but its wide active pocket sometimes allows it to act on structurally distinct compounds of either a large or small alcohol moiety (Hosokawa, 2008; Wheelock and Nakagawa, 2010). In contrast, the CES2 isozyme recognizes a substrate with a large alcohol group and small acyl group, and its substrate specificity may be restricted by the capability of acyl-enzyme conjugate formation due to the presence of conformational interference in the active pocket (Hosokawa, 2008; Ohura, Nozawa et al., 2011).

- ***Glycosidases (E.3.2.1.)***

CDs are relatively stable compared to the corresponding linear malto-oligosaccharides, but are readily degraded to glucose and maltose by specific hydrolases including cyclomaltodextrinases (CDases; EC 3.2.1.54), maltogenic amylases (MAases; EC 3.2.1.133) and neopullulanases (NPases; EC 3.2.1.135). (Park, Kim et al., 2000; Lee, Kim et al., 2002). Due to the fact that these enzymes have rather similar Xray structures and amino acid sequence identities (above 40%) it has been proposed to name them all cyclomaltodextrinases

(Lee, Kim et al., 2002). They hydrolyse cyclic dextrins and also linear maltodextrins, but have little or no activity for polysaccharides, such as starch and glycogen (Saha and Zeikus, 1992).

Generally, cyclodextrins are not hydrolyzable by exo type amylases, such as β-amylases (EC 3.2.1.2), which remove successive maltose units from the non-reducing ends of chains of polysaccharides. Cyclodextrin works as an inhibitor of β-amylases (Suetsugu, Koyama et al., 1974). In an other hand, α-Amylase (EC. 3.2.1.1) is an endo-type carbohydrase that hydrolyzes α-1,4-glycosidic bonds, mainly in starch, producing α-limit dextrins and oligosaccharides (Alkazaz, Desseaux et al., 2001; Maarel, Veen et al., 2002). Some α-amylases, such as human salivary α-amylase, human and porcine pancreatic α-amylase have been reported to hydrolyse CDs, although the rate is much slower than that for starch. Moreover, some bacterial and fungal α-amylases revealed to be able to hydrolise cyclodextrins, such as that from *Aspergillus oryzae* (Suetsugu, Koyama et al., 1974; Jodal, Kandra et al., 1984). Interestingly, cyclomaltodextrinases are intracellular enzymes, whereas most enzymes of the α-amylase family are extracellular enzymes (Depinto and Campbell, 1964; Saha and Zeikus, 1990; Hashimoto, Yamamoto et al., 2001; Lee, Kim et al., 2002).

3.2.1.3 Simulated gastric and intestinal fluids

Beyond the investigation of the ability of a colonic prodrug to release a drug in the colon, the stability of it in the upper GI tract needs also needs to be evaluated. Pharmacopeia establishes the composition of simulated gastric and intestinal fluids, which are well-accepted by the worldwide scientific community. A suspension of pepsin (E.C. 3.4.23.1), a gastric endopeptidase, at pH 1.2 is well-known to simulate gastric fluid (SIG). To replicate the intestinal environment, Pharmacopeia describes a suspension of 1% (w/v) of pancreatin at pH 6.8, the called simulated intestinal fluid (SIF). It is worthwhile pointing out that these simulated fluids used for *in vitro* testing do not faithfully reproduce the *in vivo* condition of the human gastrointestinal environment. These are simple acid and phosphate buffer solutions that contrast with the complex, dynamic and fluctuating gastrointestinal fluids (Lindahl, Ungell et al., 1997; Moreno, Oth et al., 2006). Some elements such as electrolytes, bile acids and a wide range of other lipids and important enzymatic co-factors are not present in the composition of these fluids (Lindahl, Ungell et al., 1997; Mcconnell, Fadda et al., 2008; Sarti, Barthelmes et al., 2011). Otherwise, other elements are available in excess as in the case of

pancreatic proteins, the amount of which is 5-fold higher in comparison with that in the intestinal lumen of humans (Lindahl, Ungell et al., 1997; Moreno, Oth et al., 2006).

3.2.2 *Animal gastrointestinal fluids*

The simulated fluids do not represent all aspects of the physiological conditions of the oral administration, such as pH, volume, contraction patterns, buffer capacity, bacteria, enzymes, components of food and others already mentioned. For this reason, and although there is variability between human and animals, namely in terms of bacterial activity, the fluids collected from animals can be used for human predictions of drug metabolism as reviewed by Sousa *et al.* (Sousa, Paterson et al., 2008). These GI fluids from animals can represent better the *in vivo* metabolic fate than simulated fluids (Sarti, Barthelmes et al., 2011). The challenge is to establish the best animal model to be further used in the *in vivo* experiments. The main anatomic and physiological differences between some animal models are described on Chapter I. In the case of colonic prodrugs, when the prodrug is converted to the active drug by the enzymatic reaction, the conversion can be highly species-dependent (Stella, Borchardt et al., 2007). For instance, Beagle dogs (*Carnivora* order) are not suitable to perform pharmacokinetics studies of ester prodrugs, due to the absence of carboxylesterase isozyme in the small intestine of these animals (Imai, 2006). Dogs are not suitable models to test non-steroid anti-inflammatory drugs once they are very sensitive to ulcer-inducing properties of these drugs (Swindle, Makin et al., 2011). As a non-rodent species, pig (*Artiodactyla* order) is considered an alternative to the dog or monkey (Swindle, Makin et al., 2011). Although pig is physiologically similar to humans, some anatomic differences are significant namely: the existence of a well-defined caecum contrarily to that in human, and also a bigger colon (Kararli, 1995). Pig is considered a superior model for colonic delivery when compared with smaller laboratory animals, namely for studies of oral intake of intact dosage forms intended for man, due to the large size (Sarfraz, Sarfraz et al., 2011). It has been used to study colonic ester prodrugs, namely of colonic prodrugs dextran-based (Harboe, Larsen et al., 1989). As for an non-rodent, rabbit (*Lagomorpha* order) is other alternative and it has been use to test colonic formulations (Freire, Podczeck et al., 2010; Mustafin, Kabanova et al., 2011). However, rats (*Rodentia* order), due namely to the small size and low cost, are used extensively in pre-clinical tests of drugs, including prodrugs to colon delivery (Mcleod, Friend et al., 1994; Kamada, Hirayama et al., 2002). Additionally rats have been used to perform the *in vitro* stability test of colonic prodrugs, as described previously. In this Chapter,

it is explored the behaviour of diclofenac-β-cyclodextrin in gastrointestinal fluids from 3 different animal models: pig, rat and rabbit.

3.3 Aims and Objectives

The aim of this Chapter is firstly to investigate the ability of the diclofenac-β-cyclodextrin conjugate to release diclofenac in the colon and its capacity to resist to the environment in the upper GIT tract.
To pursue this, the following objectives were defined (Table 3.1):

- Determination of the stability of the conjugate in simulated colon fluids (faecal slurries) and in an enzymatic media able to reproduce the environment of the colon;
- Determination of the stability in the upper GI tract, namely in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF).

Secondly, this Chapter aims to investigate the best animal model to be further used on the *in vivo* studies. For this, the following objective was defined:

- Conduct stability experiments in gastrointestinal fluids from animals (pig, rabbit and rats).

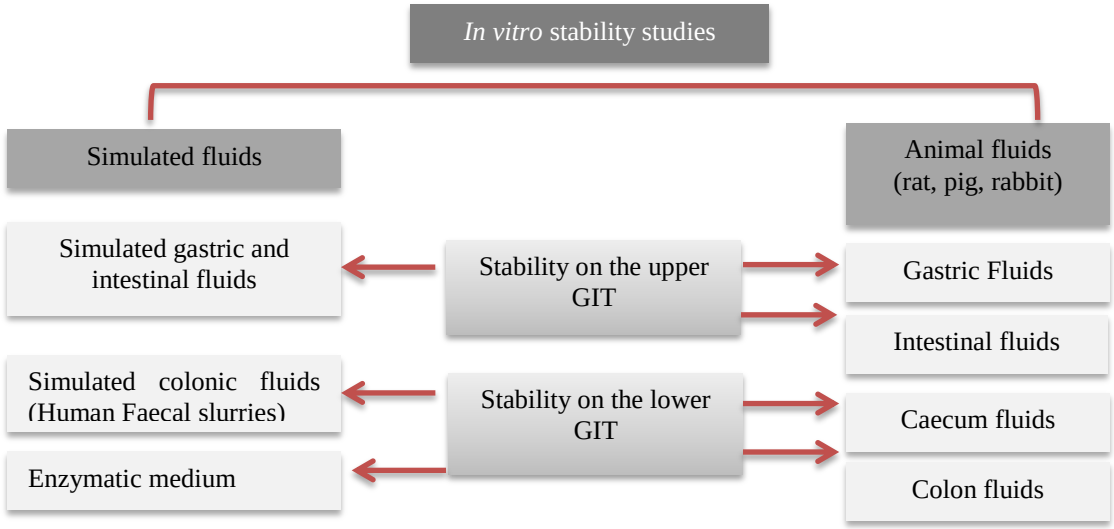


Table 3.1 Schematic representation of the main objectives of this Chapter. Determination of stability of diclofenac-β-cyclodextrin in upper and lower GI tract using simulated fluids and animal fluids.

3.4 Materials

3.4.1 *Reagents and chemicals*

Sodium diclofenac (M_W = 318.14 g/mol) and sulfasalazine (M_W = 398.394 g/mol) were purchased from Sigma Aldrich, diclofenac-β-cyclodextrin (M_W = 1411 g/mol) was synthetized according to the method reported in Chapter II.

Sodium chloride (NaCl), potassium hydroxide (KOH), sodium hydroxide (NaOH), dipotassium hydrogen orthophosphate (K_2HPO_4) potassium dihydrogen orthophosphate (KH_2PO_2), hydrochloric acid (HCl), HPLC grade acetonitrile, HPLC grade methanol and HPLC grade water were purchased from Fisher Scientifics UK Limited.

Trifluoroacetic acid (TFA) and dimethylformamide (DMF), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), L-cysteine HCl, bile salts, vitamin K, sodium bicarbonate ($NaHCO_3$), haemin, resazurin, and calcium chloride dehydrate $CaCl_2.2H_2O$ were acquired from Sigma Aldrich (Dorset, UK).

Calcium chloride hexahydrate ($CaCl_2.6H_2O$), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) and sodium acetate trihydrate ($NaCH_3COONa.3H_2O$) were obtained from VWR (Leicestershire, UK).

Peptone water and yeast extract were purchased from Oxoid Limited (Hampshire, UK).

3.4.2 *Enzymes*

All the enzymes were purchased from Sigma-Aldrich.
Pepsin derived from porcine gastric mucosa (Reference: P700) (EC. 3.4.23.1);
Pancreatin from porcine pancreas (≥3 x USP specification) (Reference: P1625) (EINECS 232-468-9);
α-Amylase from *Aspergillus oryzae* (Reference: 10065) (EC. 3.2.1.1);
α-Amylase from *Bacillus lechiniformes* (Reference: A3403) (EC. 3.2.1.1);
α-Amylase from porcine pancreas (Reference: A 3176) (EC. 3.1.1.1);
Esterase form porcine liver (PLE) (Reference: 46058) (EC. 3.1.1.1).

3.4.3 *Animals*

All procedures with animals were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act 1986.

The GI tract of pigs was obtained from freshly killed animals (cross-breed of large white and landrace, 95–110 kg, 6 months) at Cheale Meats Ltd. (Essex, UK).

The GI tract of rabbits was obtained from Adult New Zealand white rabbits (2.1-2.3 kg, 9-10 weeks) at UCL Institute of Ophthalmology.

The GI tract of Wistar rats (8 weeks) were obtained from Harlan Olac Ltd at UCL.

All animals were healthy and fed *ad libitum* prior to the experiments.

The animals were killed humanely and the gastrointestinal tracts from pig, rabbit and rats were immediately sectioned into the stomach, small intestine, caecum, and colon, with the small intestine and colon being further subdivided. The small intestine was divided in 3 parts: duodenum, jejunum and ileum. The lower intestine was divided into caecum and colon. Further, the pig colon was divided in two parts, anterior (colon A) and posterior (colon B).

After division, the gastrointestinal fluids were collected from each section of the GI tract and kept in flasks in the freezer at - 80 ^{0}C.

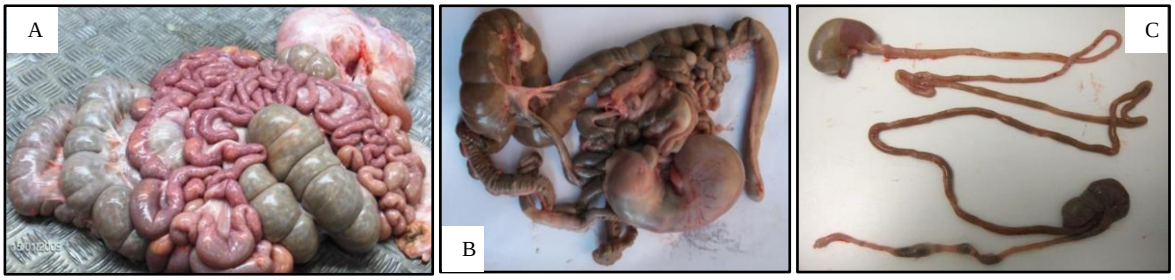


Figure 3.2 Gastrointestinal tract of pig (A), rabbit (B) and rat (C) before collection of the respective fluids.

3.4.4 *Instrumentation and chromatographic conditions*

An HPLC system (model HP1100 series, Hewlett Packard, Germany) equipped with an autosampler (Agilent 1100 series, 119 Germany) was used. A reversed-phase X-Terra C-18 column, 5 µm, 4.6 mm × 250 mm (Waters, USA), with a pre-column, was employed. Mobile phase consisted of 0.1% TFA in water (A) and acetonitrile (B). The injection volume

was 20 µL, the flow rate 1.0 mL/min and the detection wavelength was 254 nm. All the assays were performed at 30 ^{0}C. The gradient system was: 0-15 minutes 25-60% B; 15-22 minutes 60-25% B, with a flow rate of 1.0 mL/min.

3.5 Methods

3.5.1 *Stability of the diclofenac-β-cyclodextrin conjugate in simulated fluids*

3.5.1.1 Stability studies in the lower GI tract

The stability was carried out in simulated colonic fluid prepared as human faecal slurry and the use of an enzymatic media able to mimic the release in the colonic medium was investigated.

3.5.1.2 Stability studies in human faecal slurries

Faecal slurries were utilized to simulate the conditions of the colon (Basit and Lacey, 2001; Basit, Newton et al., 2002). Fresh faeces from healthy adults were collected in pre-weighed sterile plastic containers. The volunteers were on no medication and had not taken antibiotics for at least the previous six months. The plastic receptacle containing freshly voided human faeces was transferred into an anaerobic workstation (Electroteck 500TG workstation, Electrotek, UK) (37 ^{o}C and relative air humidity of 70%) as soon as possible after defecation in order to preserve viability of the microbial population.

Faeces were diluted with PBS pH 6.8 (Appendix A) in order to obtain 40% (w/w) slurry. The mixture was then homogenized and sieved through an open mesh fabric (Sefar Nitex^{TM} Heiden, Switzerland, pore size 350 µm) to remove any unhomogenised fibrous material. Afterwards, the sieved homogenized faecal slurry was diluted 50% (w/w) with basal medium, which was prepared as described below.

- **Preparation of basal medium:**

The quantity of each ingredient is described in Table 3.2. Peptone water and yeast extract were weighed into a glass flask containing 1.3 L of distilled water and autoclaved at 125 ^{o}C for 20 minutes. In a 200 mL volumetric flask containing about 150 mL of distilled water, NaCl, K_2HPO_4, $MgSO_4.7H_2O$ and $CaCl_2.6H_2O$ were weighed and dissolved under

stirring. After that, tween 80 was added and stirred until completely dissolved. Bile salts followed by L-cysteine were added and stirred until completely dissolved. Vitamin K, haemin (dissolved in 2 drops of NaOH 1M) and the resazurin solution were added. Sodium bicarbonate and distilled water were added to make final volume of 200 mL. The addition of $NaHCO_3$ to the basal medium was just added before filtering and the flask was stopped during stirring to avoid dissolution of oxygen. The attained solution was filtered through 0.45 μm filters (Millex GP syringe-driven filter units, Millipore, Ireland) into the autoclaved 1.3 L solution containing the peptone water and yeast extract. This step was performed aseptically in a Laminar Flow Cabinet. The bottle was kept tightly closed and at room temperature until be used.

Basal media was used to support the activity of microbiota in the faecal slurry for accurate prediction of the degradation of the compounds.

Table 3.2 Composition of basal medium.

Ingredient	Quantity per Litre
Peptone water	2 g
yeast extract	2 g
NaCl	0.1 g
K_2HPO_4	0.04 g
$MgSO_4.7H_2O$	0.01 g
$CaCl_2.6H_2O$	0.01 g
$NaHCO_3$	2 g
Haemin	0.005 g
L-cysteine HCl	0.5 g
Bile salts	0.5 g
Tween 80	2 mL
Vitamin K	10 μL
Resazurin olution 0.25%	4 mL

Solution of diclofenac-β-cyclodextrin (2.4 mg/mL) was prepared in PBS 6.8 with DMF to guarantee complete dissolution of the conjugate in the medium, as described by Koichi Udo *et al*.(Udo, Hokonohara et al., 2010), and 300 μL of this solution was mixed with 900 μL of faecal slurries in the anaerobic workstation (at 37 ^oC and relative air humidity of 70 %). A control experiment was also run in parallel in faecal slurry that was subjected to autoclaving

at 130 ◦C for 20 minutes. This was conducted to inactivate the bacterial enzymes in the slurry.

Thereafter, these solutions were incubated and shaken at 100 rpm (VXRbasic Vibrax®, Leicestershire, UK). The final concentrations of the conjugate and DMF were 600 µg/mL and 1.0%, respectively. At fixed intervals, 100 µL of fluid from each solution was withdrawn, mixed with 300 µL of methanol and centrifuged at 10 000 rpm for 10 minutes at room temperature. The supernatant was then removed and analyzed by HPLC (see Table 3.3). Each experiment was performed in triplicate.

In order to compare the inter-individual degradation profile of conjugate stability tests were performed in faecal slurries from 3 different male subjects processed in separate. Sulfasalazine was used as a positive control. Solutions of diclofenac-β-cyclodextrin (2.4 mg/mL) and sulfasalazine (0.4 mg/mL) were prepared in PBS 6.8 with DMF and the experiment was performed following the steps described anteriorly and according to the described in Table 3.4.

Table 3.3 Details for stability experiments of diclofenac-β-cyclodextrin in faecal slurries, using autoclaved faecal slurries as a control.

Media	Conjugate concentration	Incubation	Conjugate concentration (incubation)	Sampling volume	Final volume (addition methanol)	Final conjugate concentration
Faecal slurry	2.4 mg/mL	300 µL of conjugate + 900 µL of media	0.6 mg/mL	100 µL	400 µL	0.15 mg/mL
Autoclaved faecal slurry						

Table 3.4 Details for stability experiments of diclofenac-β-cyclodextrin *versus* sulfasalazine in faecal slurries from 3 different subjects processed separately.

Prodrugs concentration	Incubation (faecal slurry)	Prodrugs concentration (incubation)	Sampling volume	Final volume (addition methanol)	Final prodrugs concentration
2.4 mg/mL of conjugate and 0.4 mg/mL of sulfasalazine	300 µL of prodrug + 900 µL of media	0.6 mg/mL of conjugate and 0.1 mg/mL of sulfasalazine	100 µL	400 µL	0.15 mg/mL of conjugate and 0.025 mg/mL of sulfasalazine

3.5.1.3 Stability studies in simulated colonic media containing enzymes

In presence of esterase from porcine liver solution (39 units/mL).

Solutions of conjugate (0.8 mg/mL) were prepared in HEPES/NaOH buffer (pH 7.4) (see Appendix C) with 1% of DMF. After that, esterase from porcine liver (39 units/mL) was added to the previous solutions. This procedure was adapted by that described by F. Hirayama *et al.* (Hirayma, Ogata et al., 2000).

In presence of α-amylases solutions: Bacillus lechiniformes (250 units/mL) porcine pancreas (250 units/mL) and Aspergillus oryzae (20-250 units/mL).

Solutions of conjugate (0.8 mg/mL) were prepared in 0.01M $CaCl_2$/0.2 M acetate buffer (pH 5.5) (see Appendix C) with 1% of DMF. After that, the respective α-amylase was added to the previous solutions. Stability tests with α-amylase from *Aspergillus oryzae* were conducted with different concentrations of enzyme 20, 40 or 250 units/mL while with α-amylase from *Bacillus lechiniformes* and with α-amylase from porcine pancreas were conducted at 250 units/mL.

In presence of α-Amylase from Aspergillus oryzae (250 units/mL) and esterase from porcine liver (39 units/mL) simultaneously.

Solutions of conjugate (0.8 mg/mL) were prepared in 0.01 M $CaCl_2$/0.2 M acetate buffer pH 5.5 or in HEPES/NaOH buffer (pH 7.4) with 1% of DMF. After that, α-amylase and esterase were added to the previous solution.

In presence of α-Amylase from Aspergillus oryzae (250 units/mL) followed by the presence of esterase (39 units/mL).

Solutions of conjugate (0.8mg/mL) were prepared in 0.01 M $CaCl_2$/0.2 M acetate buffer (pH 5.5) with 1% of DMF. After that, α-amylase (250 units/mL) was added and the solution incubates at 37 ºC under 100 rpm. After 12 hours of incubation, the pH of the solution was changed to 7.4 by the addition of NaOH 0.1 N and the esterase was added to the solution.

All the previous prepared samples were incubated at 37 ºC and shaken at 100 rpm. 100 µL of sample was withdrawn immediately after enzyme addition and at different time points hereafter, mixed with 400 µL of methanol and centrifuged at 10 000 rpm for 10 minutes at

room temperature (Centrifuge eppendorf 5415). The supernatant was then removed and analysed by HPLC to determine the concentration of conjugate. Each experiment was performed in triplicate. This procedure was adapted by that described by F. Hirayama (Hirayma, Ogata et al., 2000).

Table 3.5 Details for stability experiments of diclofenac-β-cyclodextrin conducted in the presence of different enzymes.

Conjugate	Enzyme	Enzyme concentration (units/mL)	Media	Final volume (addition methanol) (µL)	Final conjugate concentration (mg/mL)
0.8 mg/mL (1% DMF)	Esterase from porcine liver	39	HEPES/NaOH (pH 7.4)	400	0.15
	α-amylase from *Aspergillus oryzae*	20 40 250	0.01M $CaCl_2$/0.2M acetate buffer (pH 5.5)	400	0.15
	α-amylase from porcine liver	250	0.01M $CaCl_2$/0.2M acetate buffer (pH 5.5)	400	0.15
	α-amylase from *Bacillus lechiniformes*	250	0.01M $CaCl_2$/0.2M acetate buffer (pH 5.5)	400	0.15
	α-Amylase and esterase from porcine liver simultaneously	250 39	0.01M $CaCl_2$/0.2M acetate buffer (pH 5.5)	400	0.15
	α-Amylase and after 12 h addition of esterase from porcine liver	250 39	0.01M $CaCl_2$/0.2M acetate buffer (pH 5.5) for 12h. Ajust of pH to 7.4 before addition of esterase	400	0.15

3.5.1.4 Stability in gastric and intestinal simulated fluids

3.5.1.4.1 Stability in simulated gastric fluids (SGF) and in simulated intestinal fluids (SIF)

Solutions of diclofenac-β-cyclodextrin conjugate 0.8 mg/mL were prepared in SGF and in SIF with 1% of DMF. The simulated gastric and intestinal fluids were prepared according to USP

specifications (Test solutions, United States Pharmacopeia 30, NF 25, 2007), (Appendix C). These solutions were incubated at 37 ^{o}C and shaken at 100 rpm. At fixed intervals, 100 µL of fluid was withdrawn, mixed with 400 µL of methanol and centrifuged at 10 000 rpm for 10 minutes at room temperature (Centrifuge eppendorf 5415). The supernatant was then removed and analyzed by HPLC to determine the concentration of conjugate. Each experiment was performed in triplicate.

Table 3.6 Details for stability experiments of diclofenac-β-cyclodextrin in SGF and SIF.

Media	Conjugate concentration	Sampling volume	Final volume (addition methanol)	Final conjugate Concentration
Simulated gastric fluid (SGF) (0.1 N HCl, pH 1.2 with pepsin)	0.8 mg/mL	100 µL	500 µL	0.16 mg/mL
Simulated intestinal fluid (SIF) (phosphate buffer pH 6.8 with pancreatin)	0.8 mg/mL	100 µL	500 µL	0.16 mg/mL

3.5.2 *Stability of the diclofenac-β-cyclodextrin conjugate in animal fluids*

3.5.2.1 Stability in gastric and intestinal fluids

To performe the stability studies, gastric and intestinal fluids that had been collected and kept in the freezer were left at room temperature until completely thawed. After that, fluids were centrifuged at 10 000 rpm during 10 minutes. The supernatants obtained were separated and used to conduct the stability experiments. Solutions of diclofenac-β-cyclodextrin (2.4 mg/mL) were prepared in SGF without pepsin and in PBS pH 6.8 with DMF (Table 3.7). To conduct the stability in gastric fluids from animals, 100 µL of the conjugate solution prepared in SGF without pepsin was mixed with 900 µL of stomach fluids from pig, rabbit or rats. In the case of intestinal fluids, 100 µL of the conjugate solution prepared in PBS 6.8 was mixed with 900 µL of duodenum, jejunum or ileum fluids from pig, rabbit or rats. The final concentrations of the conjugate and DMF were 0.240 mg/mL and less than 1% respectively. Thereafter, these solutions were incubated at 37 ^{o}C and shaken at 100 rpm. At fixed intervals, 100 µL of fluid was withdrawn, mixed with 200 µL of methanol and centrifuged at 10 000 rpm for 10 minutes at room temperature. The supernatant was then removed and analysed by HPLC. Each experiment was performed in triplicate.

Table 3.7 Details for stability tests of conjugate in gastric and intestinal fluids from pig, rabbit and rats.

Conjugate concentration	Animal fluid	Incubation	Conjugate concentration Incubation	Sampling volume	Final volume (addition methanol)	Final Conjugate concentration
2.4 mg/mL in SGF without pepsin	Stomach	100 µL of conjugate + 900 µL of animal fluid	0.24 mg/mL	100 µL	300 µL	0.08 mg/mL
2.4 mg/mL in PBS 6.8	Duodenum Jejunum Ileum					

3.5.2.2 Stability studies in caecum and colonic fluids

The stability of conjugate was carry out in fluids collected form the lower intestine of animals, namely caecum and colonic fluids. Sulfasalazine was used as a control and was incubated concomitant with the conjugate. Caecum and colonic frozen fluids were left at room temperature until completely thaw. Thereafter, the caecum fluids (rat, rabbit and pig) and also the fluids from the anterior colon of pig (colon A) were centrifuged at 10 000 rpm during 10 minutes and the supernatant was used to perform the stability studies. The colon fluids (rats, rabbit and fluids from the posterior colon of pig (colon B)) were prepared according to the method described before for preparation of human faecal slurries (see 3.5.1.2) due to the absence of liquid fluid. The stability studies were performed as described above and according to the scheme presented in Table 3.8.

Table 3.8 Details for stability tests of conjugate *versus* sulfasalazine in caecum and colonic fluids from pig, rabbit and rat.

Animal fluid	Prodrugs concentration in PBS 6.8	Incubation	Prodrugs concentrations (Incubation)	Sampling volume	Final volume (addition methanol)	Final prodrug concentration
Caecum	2.4 mg/mL of conjugate and 0.4 mg/mL of sulfasalazine	100 µL of prodrugs solution + 900 µL of animal fluid	0.24 mg/mL of conjugate and 0.04 mg/mL of sulfasalazine	100 µL	300 µL	0.08 mg/mL of conjugate and 0.0133 mg/mL of sulfasalazine
Colon						

3.6 Results and discussion

3.6.1 *HPLC method development*

The HPLC method for quantification of conjugate, diclofenac and sulfasalazine is described in section 3.4.4 was developed taking in account the previous experiments. The choice of the solvent of precipitation was tested in one of the most complex mediums, faecal slurries. The aim was to confirm which solvent could provide better recovery of drugs from the incubated samples. The experiments showed that methanol allows the extraction of the drugs with better recovery than acetonitrile, which can be explained by the best solubility of the tested molecules in methanol. Additionally, samples precipitated with methanol providing cleaner chromatograms than those with acetonitrile.

A typical chromatogram produced after injection of one sample prepared by precipitation of a human faecal slurry sample containing diclofenac-β-cyclodextrin and sulfasalazine is represented in Figure 3.3. The retention times for the conjugate, sulfasalazine and diclofenac were 6.9, 13.0 and 18.6 minutes, respectively.

The same method was adopted to perform all the stability studies. All the experiments, blank samples were run previously to be certain of no existence of interferences between the componentes of each matrix and the analytes.

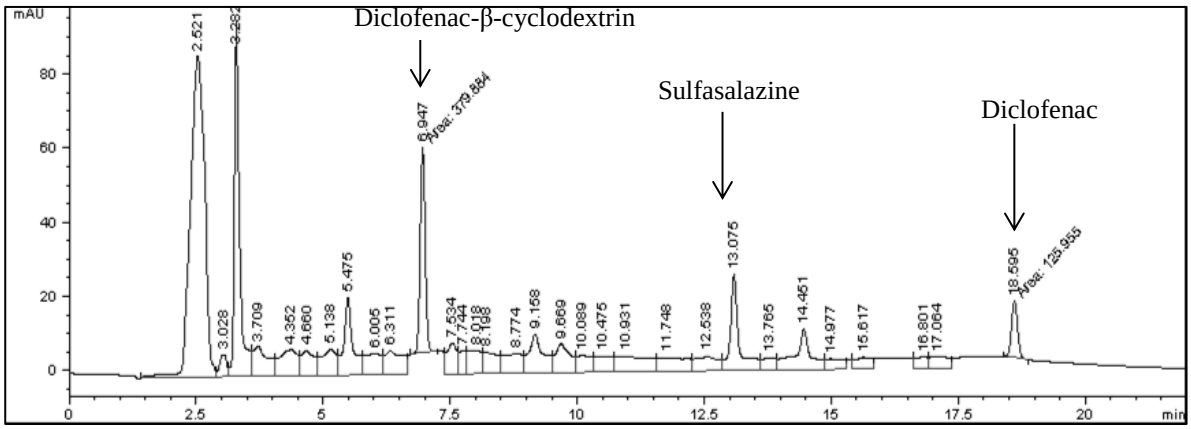


Figure 3.3 HPLC chromatogram of diclofenac-β-cyclodextrin (r.t. = 6.9 minutes), sulfasalazine (r.t = 13.0 minutes) and diclofenac (r.t. = 18.6 minutes) in human faecal slurry.

3.6.2 *Stability of diclofenac-β-cyclodextrin conjugate in simulated fluids*

3.6.2.1 Stability in the lower gastrointestinal tract

While in the upper gastrointestinal tract, the digestive enzymes play the most important role in the mechanism of hydrolysis, in the lower intestine, fermentation is the main process of digestion of subtracts.

3.6.2.1.1 Stability studies of diclofenac-β-cyclodextrin in human faecal slurries

Conjugate was added to the human faecal slurry and results demonstrated that the conjugate was readily hydrolysed in the human faecal slurry, and within 2 hours the conjugate was completely degraded, observed by the rapid liberation of free diclofenac, as shown in Figure 3.4. By contrast, the conjugate was stable in the autoclaved faecal slurry (control). These results confirmed that cleavage of the conjugate is due to bacterial enzymatic activity in the slurry. Bacteroides species account for ca. 20% of the human faecal and ferment a wide variety of polysaccharides (Reddy, Palmer et al., 1984).

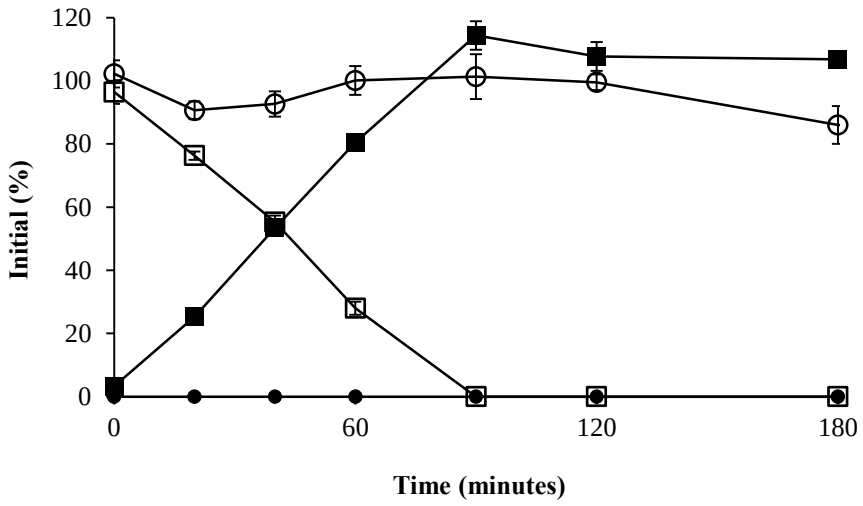


Figure 3.4 Mean levels of diclofenac-β-cyclodextrin (□) and diclofenac (▪) in human faecal slurry (test). Mean levels of diclofenac-β-cyclodextrin (○) and diclofenac (●) in autoclaved faecal slurry (control). Each point represents mean ± S.D. (n=3).

Zoetendaal *et al* reported the existence of considerable variation in human faecal microbiota composition between individuals. These authors concluded that, in humans, there is a strong genetic link between an individual and its microbiota, so that each individual has a unique

microbial community (Zoetendal, Akkermans et al., 1998). For this reason, it was recommend by McBurney and Thompson to use at least three donors when human faeces were used as inocula (Mcburney and Thompson, 1989). Taking in account this, the metabolism of the conjugate was studied in faecal slurry from 3 male humans in order to assess the variability inter-subjects. Sulfasalazine was used as positive control. The stability profile of conjugate and sulfasalazine is shown in Figure 3.5 A and B, respectively.

These results indicate that metabolism of both prodrugs depends on the source of faecal slurry, which can be associated with different diets.

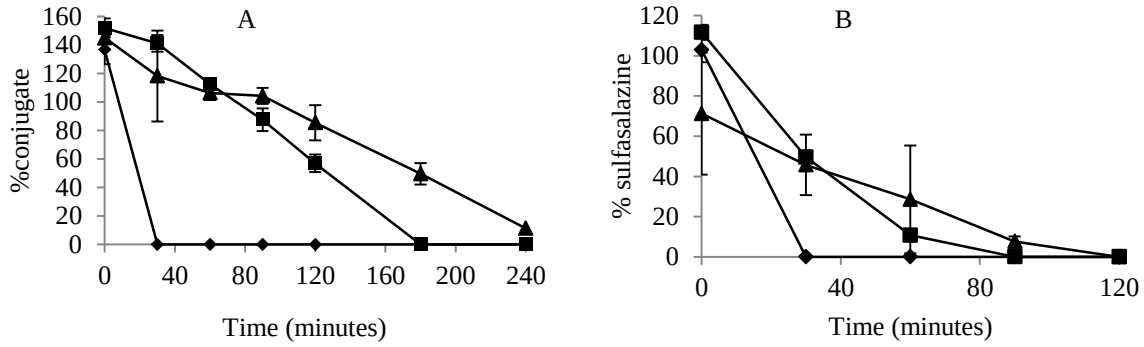


Figure 3.5 Mean levels of diclofenac-β-cyclodextrin conjugate (A) and mean levels of sulfasalazine (B) in three different samples of faecal slurry from male individuals from 3 different male individuals (▲, ■, ♦). Each point represents mean ± S.D. (n = 3).

It has been reported that diet may have a profound influence on microbiota. Hence, a diet rich in protein and animal fat is associated to a domine of *Bacteroides* genus (*Bacteroidetes* Phylum) while a diet rich in carbohydrates is associated to Prevotella genus (Bacteroidetes Phylum). Moreover, the same authors revealed that the European microbiome is dominated by Bacteroides enterotype, whereas the African microbiome was dominated by the Prevotella enterotype (Wu, Chen et al., 2011). It would be necessary to increase the number of donors to have evidence how the metabolism of prodrugs varies according to the source of matrix.

Further, these results show that in all the faecal slurries the degradation of sulfasalazine is faster than the degradation of the conjugate. This suggests that the mechanism involved in the degradation of sulfasalazine is faster than that responsible for the degradation of conjugate. As described in Chapter I, azoreductase (EC 1.7.1.6) is the enzyme responsible for the activation of sulfasalazine. This is a flavin mononucleotide (FMN)-dependent enzyme, that is

produced by numerous intestinal bacteria, namely *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis*, *Rhodobacter sphaeroides*, and *Pseudomonas aeruginosa* (Ryan, Laurieri et al., 2010). There is some evidence that azoreductase activity occurs inside the cell, on certain bacterial species (Peppercorn and Goldman, 1972; Song, Zhou et al., 2003). However, azoreduction can be accomplished outside the bacterial cells with the assistance of electron transport agents such as nicotinamide adenine dinucleotide phosphate (NADPH), which is a more general reaction (Scheline, 1973). Otherwise, in the case of diclofenac-β-cyclodextrin, as described before, esterases and/or glycosylases will be involved in its metabolism. Also, it was reported that *Bacteroides* are the main species responsible for degradation of cyclodextrins (Antenucci and Palmer, 1984). Therefore, the metabolism rate of both prodrugs is different not only due to the different mechanism of degradation but also due to different microbiota species involved on the enzymes production.

3.6.2.1.2 Stability studies in simulated colonic media containing enzymes

It is difficult to specify which enzymes are responsible for the release of diclofenac from the conjugate in the faecal slurry. There are a huge variety of microorganisms that are able to produce a variety of enzymes. The hydrolysis studies of the conjugate, using ester hydrolysing enzymes and sugar-degrading enzymes were conducted in order to attempt the development of an *in vitro* enzymatic media able to mimic the biological fluids.

Stability in presence of esterases

Results from stability studies in the presence of esterases showed that diclofenac is not released during the 24 hours of incubation. This allows concluding that this ester bond is not susceptible to esterase hydrolysis, as observed in Figure 3.6.

Pig liver esterase (PLE) is a carboxylic esterase (E.C 3.1.1.1.) and it was chosen since it has been used as a widely accepted model enzyme for ester-hydrolysing molecules. Stability, low costs, and the ability of hydrolysing a wide range of substrates with high stereoselectivity represent additional advantages of this enzyme, which operates without the need of co-enzymes (Tamm, 1992; Huang, Shiotsuki et al., 1996).

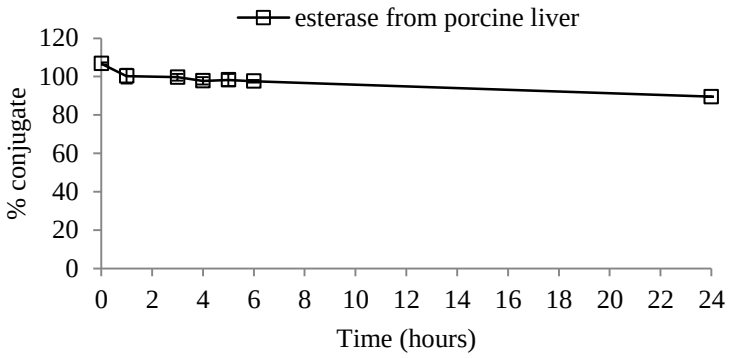


Figure 3.6 Stability of diclofenac-β-cyclodextrin in the presence of esterase from porcine liver (39 Units/mL) in HEPES NaOH buffer (pH 7.4). Each point represents mean ± S.D. (n = 3).

This study was performed according to previous studies that investigated the stability of cyclodextrins conjugates, namely of dimethyl-β-cyclodextrin-4-biphenylylacetic acid conjugate (Ventura, Paolino et al., 2003a), β-cyclodextrin 5-fluorouracil acetic acid conjugate (Udo, Hokonohara et al., 2010) and of β-cyclodextrin-butyric acid(Hirayma, Ogata et al., 2000). Basically, the conjugate was incubated with 39 units/mL of esterase in HEPES/NaOH buffer pH 7.4 (Hirayma, Ogata et al., 2000). As observed with diclofenac-β-cyclodextrin, esterase had no effect on the ester linkage of β-cyclodextrin-butyric acid conjugate (Hirayma, Ogata et al., 2000). However, in the case of dimethyl-β-cyclodextrin biphenylacetic acid conjugate, the drug was released in presence of this esterase with a $t_{1/2}$ value of 4 hours (Ventura, Paolino et al., 2003a). This result obtained with diclofenac-β-cyclodextrin conjugate reinforces the idea that the conjugation with β-cyclodextrin is less susceptible to hydrolysis by esterase than the conjugation with a derivative of β-cyclodextrin. This is probably due to the higher hydrophilicity of the methylated carrier when compared with the natural β-cyclodextrin and consequently less intramolecular association between drug and carrier. Moreover, it was found that esterase activity is higher towards more water soluble substrates (Fojan, Jonson et al., 2000).

A previous study showed that complexation of diloxanide furoate with β-cyclodextrin, caused a stabilization of this drug against esterase. This effect was not only due to protection of the drug through formation of cyclodextrin complexes. The authors suggested that the inhibitory effect against esterase can occur due to the complexation of cyclodextrin with amino acids of the lateral chains that participate in the active site of the enzyme (Monteiro, Chiaradia et al.,

2003). Therefore, interactions between cyclodextrins and enzymes should be taking into account when stability studies are performed in the presence of enzymes.

Stability in presence of amylases

The assessment of efficacy of 3 different types of amylases (amylase from *Bacillus lechiniformes*, amylase from porcine pancreas and amylase from *Aspergillus Oryzae)* to digest the conjugate was carried out. Figure 3.7 shows the quantity of conjugate (%) after incubation in presence of three different amylases plotted *versus* time.

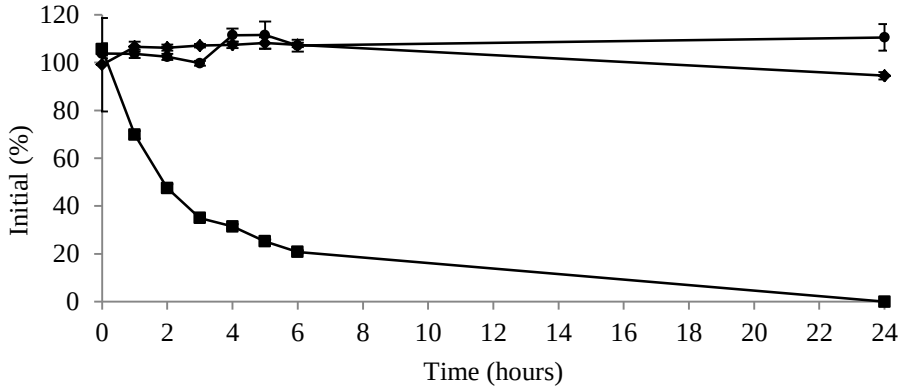


Figure 3.7 Mean levels of diclofenac-β-cyclodextrin in presence of different α-amylases (250 units/mL): α-amylase from *Bacillus lechiniformes* (♦), α-amylase from *Aspergillus oryzae* (■) and α-amylase from porcine pancreas (●) in 0.2 M acetate buffer (pH 5.5) containing 0.01 M $CaCl_2$ at 37 °C. Each point represents mean ± S.D. (n = 3).

Amylase from porcine pancreas is part of pancreatin, which is commonly used to represent the digestion within the small intestine, as described before. The intention here is to study its activity against cyclodextrins isolated, since in the studies with pancreatin amylases are mixed with a variety of other enzymes, including lipases and proteases. Results observed in the presence of this amylase are in agreement with previous results with films of cyclodextrins, which had shown that this enzyme does not degrade β-cyclodextrin (Fetznera, Bo¨Hmb et al., 2004). This emphasizes the idea that α-amylase from porcine pancreas is not active against this conjugate.

α-Amylase from *Bacillus lechiniformes*, a highly thermostable enzyme, was selected as it resembles the properties of colonic amylases. It has been used as a model enzyme to resemble the colonic digestion of amylase (Siew, Man et al., 2004). When the conjugate was incubated with this enzyme, it was not observed its degradation.

The amylase from fungus *Aspergillus oryzae* was selected since it was able to degrade cyclodextrin (Jodal, Kandra et al., 1984). As observed in Figure 3.7, only this enzyme was able to digest the conjugate.

Analysis of HPLC chromatograms of the hydrolysates obtained after incubation of the conjugate with α-amylase from *Aspergillus Oryzae* after 0, 6 and 24 hours revealed that during incubation new peaks have apperead, however non of them correspond to the conjugate or the diclofenac. However, after 24 hours of incubation, although the conjugate had completely disappeared, the digestion of conjugate with this enzyme only allowed to release around 9% of diclofenac.

In order to understand the progress of the enzymatic reaction, and how the concentration of α-amylase from *Aspergillus Oryzae* can affect the rate of degradation and the formation of the intermediate compounds, the conjugate was incubated with three different concentrations of enzyme. The results obtained are present in Figure 3.8, and reveal that the conjugate was hydrolysed by the enzyme according to first-order kinetics, with $t_{1/2}$ values of 13.3, 7.4 and 2.7 hours at enzyme concentration of 20, 40 and 250 units/mL, respectively.

In Figure 3.9 is possible to observe the evolution of the reaction between the conjugate and different concentrations of amylase from *Aspergillus oryzae*. Chromatograms showed that after 24 hours of incubation, in samples incubated with 20 and 40 units/mL of enzyme, part of the conjugate was still intact, while in samples incubated with 250 units/mL, all the conjugate was digested. Otherwise, after 24 hours of incubation, in the case of low concentrations of enzyme, 20 and 40 units/mL, it was not observed any release of diclofenac from the conjugate. In the course of the hydrolysis, it was registered the formation of 2 new peaks that were not identified. These should be linear maltooligomers (Jodal, Kandra et al., 1984) coupled to diclofenac, since only the drug was responsible for the ultraviolet detector response at the wavelength used ($\lambda = 254$nm).

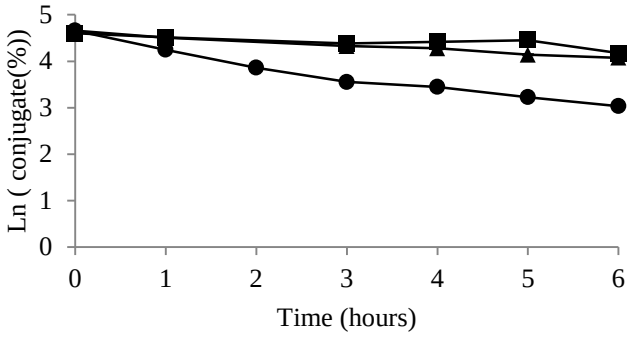


Figure 3.8 First-order plots for hydrolysis of the conjugate in the presence of *Aspergillus oryzae* α-amylase in acetate buffer (pH 5.5) at 37 °C at different concentrations of α-amylase (20 (■), 40 (▲) and 250 (●) units/mL).

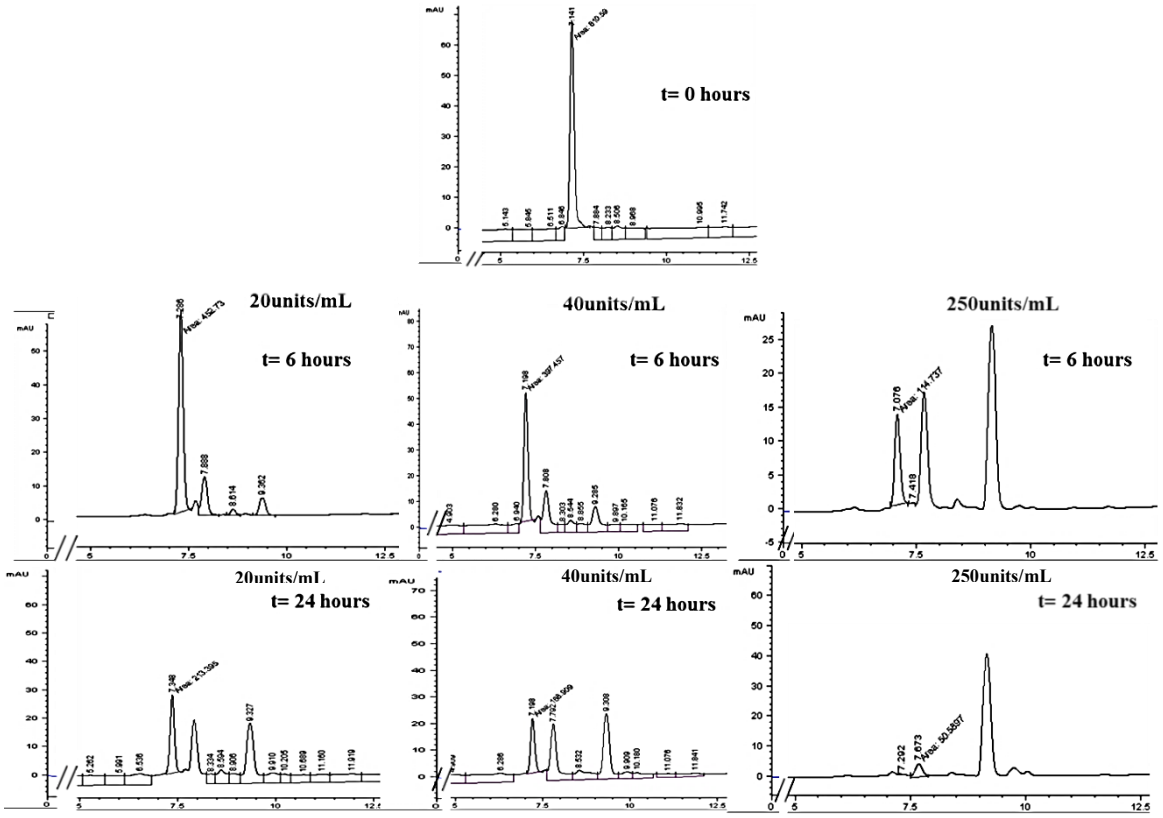


Figure 3.9 HPLC chromatograms of the enzymatic reaction of conjugate im presence of different concentrations of α- amylase from *Aspergillus Oryzae* (20, 40 and 250 units/mL) at 0, 6, and 24 hours.

α-amylase from *Aspergillus Oryzae* can digest the conjugate, however new intermediate compounds are formed prior to release of diclofenac. These unknown compounds, that are fragments from the digestion of cyclodextrin, can be identified using LC-MS.

It is important to note that in case of incubation with faecal slurry, no formation of intermediate peaks in HPLC chromatograms was observed. In the case of stability studies in human faecal slurry the rate of disappearance of diclofenac-β-cyclodextrin is inversely proportional to the rate of appearance (release) of diclofenac. These results suggest that this enzyme *per se* is not able to simulate the mechanism involved in the release of diclofenac, at the level of the lower intestine.

It is reported that the new compounds formed in the first period of the reaction between cyclodextrin and this enzyme, called higher-membered maltooligomers (maltohexaose, maltopentaose, maltotetraose), are also substrates of the enzyme and consequently these can be competitive inhibitors of the enzymatic reaction (Suetsugu, Koyama et al., 1974; Jodal, Kandra et al., 1984). Moreover, it is reported that lower-membered maltooligomers (glucose, maltose and maltotriose) formed during the reaction are not substrates of the enzyme, but by linking to the enzyme-protein they may become "non-competitive" inhibitors (Jodal, Kandra et al., 1984).

This inhibition effects caused by the degradation products can explain the slow digestion and consequently the poor release of drug. Otherwise, results suggest that amylase alone is not able to lead to complete release of drug, at least in 24 hours of experiment.

The combination of amylase and esterase was made in order to access the ability of both in the release of diclofenac from the conjugate. It was expected that, even if interaction occured between esterase and cyclodextrin, once the last one is degraded, esterase would be free to act against the ester linkage.

Stability of diclofenac-β-cyclodextrin in the presence of amylase and esterase

The concomitant incubation of amylase and esterase with the conjugate has the same effect than the incubation with amylase alone (Figure 3.10). This suggests that amylase was degrading the cyclodextrin carrier, while esterase did not act against cyclodextrin, independently on the structure of the carrier obtained after digestion by amylase.

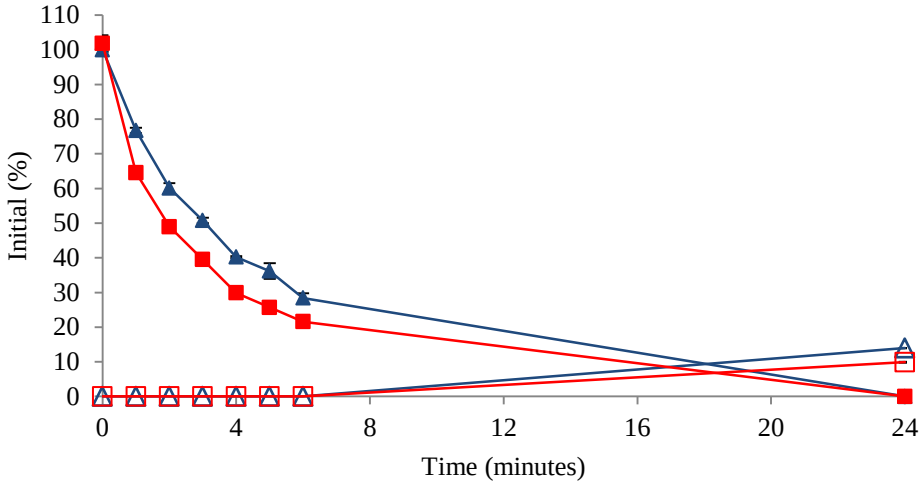


Figure 3.10 Mean levels of diclofenac-β-cyclodextrin (▲) and diclofenac (Δ) in the presence of α-amylases from *Aspergillus oryzae* (250 units/mL) and carboxylic esterase (39 units/mL) in acetate buffer (pH 5.5) at 37°C. Mean levels of diclofenac-β-cyclodextrin (■) and diclofenac (Δ) in the presence of α-amylases from *Aspergillus oryzae* (250 units/mL) in acetate buffer (pH 5.5) at 37°C.

This could be due to the fact that esterase enzyme was not incubated in its optimum medium (HEPES/NaOH pH 7.4). In order to clarify this presupposition, the same experiment was conducted in HEPES/NaOH pH 7.4 and results showed a decrease of the rate of degradation of the conjugate when compared to results observed in Figure 3.10. Moreover, it was not observed release of diclofenac during the first 6 hours of experiment (results not presented). The change on pH leads to alteration on the activity of α-amylase proved by the slower disappearance of the conjugate at pH 7.4. However, no alterations were observed on the quantity of diclofenac released.

Therefore, other experiment was performed in the presence of amylase at pH 5.5, and after 12 hours of incubation the pH was changed to 7.4 before incubation of esterase according to studies reported by Koichi Udo *et al.* (Udo, Hokonohara et al., 2010). Thus, the study was conducted using the optimum pH to guarantee the bioactivity of the enzymes.

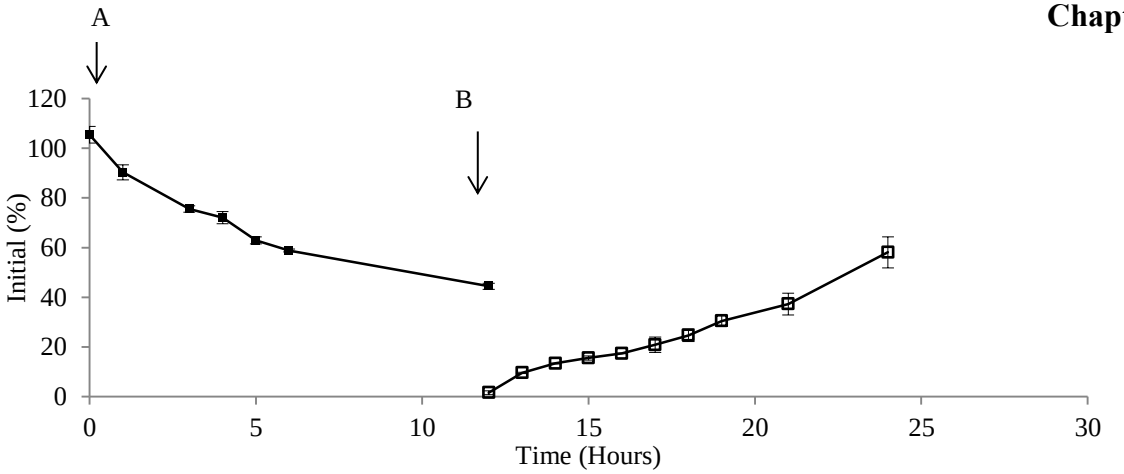


Figure 3.11 Mean levels of diclofenac-β-cyclodextrin (■) and diclofenac (□) in the presence of α-amylases from A*spergillus oryzae* (40 units/mL) and carboxylic esterase (39 units/mL) in acetate buffer (pH 5.5) at 37 °C. The esterase (39 units/mL) was added 12 hours after the α-amylase. A- addition of the α-amylase; B- addition of the esterase.

These results show that, if esterase is added 12 hours after digestion of the conjugate in presence of amylase, a higher quantity of diclofenac is released. Time of the experiment should be prolonged in order to conclude about the ability to completely release of diclofenac.

These results suggest that the mechanism of drug release from this kind of prodrug, with a cyclodextrin as a carrier, involves sequential drug liberation. This mechanism comprises an initial enzyme-mediated cleavage of the carrier backbone (cyclodextrin) towards the action of amylases, which allows reduction of the steric protection of the ester diclofenac-cyclodextrin bond. With the progressive fragmentation of cyclodextrin, small fragments will become substrates for esterases and other hydrolases residing within the colon. This sequential hydrolysis mechanism was demonstrated previously in the hydrolyses of the β-cyclodextrin-*n*-butyric acid conjugate and the β-cyclodextrin-5-fluorouracil conjugate catalysed by α-amylase (*Aspergillus oryzae*) and carboxylic esterase (porcine liver) (Hirayama, Ogata et al., 2000; Udo, Hokonohara et al., 2010).

However, it is important to note that this sequential mechanism cannot be assumed for all the cyclodextrin conjugates. Bacterial enzymatic degradation depends not only on the susceptibility of the chemical bond, but also on the struture of the molecule surrounding the susceptible bond. This was found previously through the study of naproxen-cyclodextrin conjugates, which was not degraded on rat colonic fluids due to the steric hindrance surrounding the ester bond caused by the naphthalene structure presented in naproxen (El-Kamel, A.M.Abdel-Aziz et al., 2008).

Overall, the combination of α-amylase, able to degrade cyclodextrin carrier, and an esterase can lead to release of diclofenac form this cyclodextrin-based prodrug. However, with the concentrations of enzymes used, the rate of release is not comparable with that observed in human faecal slurry. A faster degradation of cyclodextrin backbone needs to take place on the degradation process observed in the presence of amylase. This can be conducted with higher concentrations of enzymes as indicated by results presented in Figure 3.9.

3.6.2.2 Stability of diclofenac-β-cyclodextrin conjugate in the upper gastrointestinal tract

3.6.2.2.1 Simulated stomach and intestinal fluids

Incubation of conjugate in simulated gastric and intestinal fluids were taken as a starting point to study the stability in upper gastrointestinal tract. Results from these *in vitro* studies revealed that the conjugate is not susceptible to be hydrolyzed in these simulated fluids.

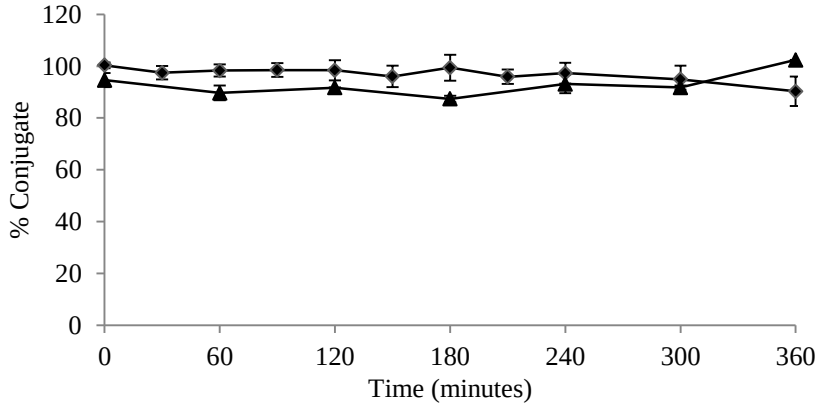


Figure 3.12 Mean levels of diclofenac-β-cyclodextrin conjugate in simulated gastric fluid (♦) and in simulated intestinal fluid (▲). Each point represents mean ± S.D. (n = 3).

As expected, once this conjugate does not present a peptide structure, pepsin (EC.3.4.23.1) is not able to affect the stability of the conjugate. Additionally, pancreatic enzymes do not affect the integrity of the conjugate.

These results are the first indication that conjugate may resist to the attack of digestive enzymes in gastric and intestinal media and reach the lower intestine intact.

3.6.3 *Stability of diclofenac-β-cyclodextrin conjugate in animal fluids*

3.6.3.1 Stability in gastric and intestinal fluids

Conjugate was incubated with stomach and intestinal fluids of each animal model. Figure 3.13 shows the stability of the conjugate after incubations with fluids of animals (rat, pig and rabbit) from stomach and small intestine (duodenum, jejunum and ileum). Results show that conjugate is stable in the contents of stomach and small intestine. The profile obtained with these biological fluids is the same obtained using simulated gastric and intestinal fluids, although these fluids from animals are enriched by many elements that are not present in the artificial fluids. These results with animal fluids reinforce the idea provided by simulated fluids relative to the stability of conjugate in the upper gastrointestinal tract.

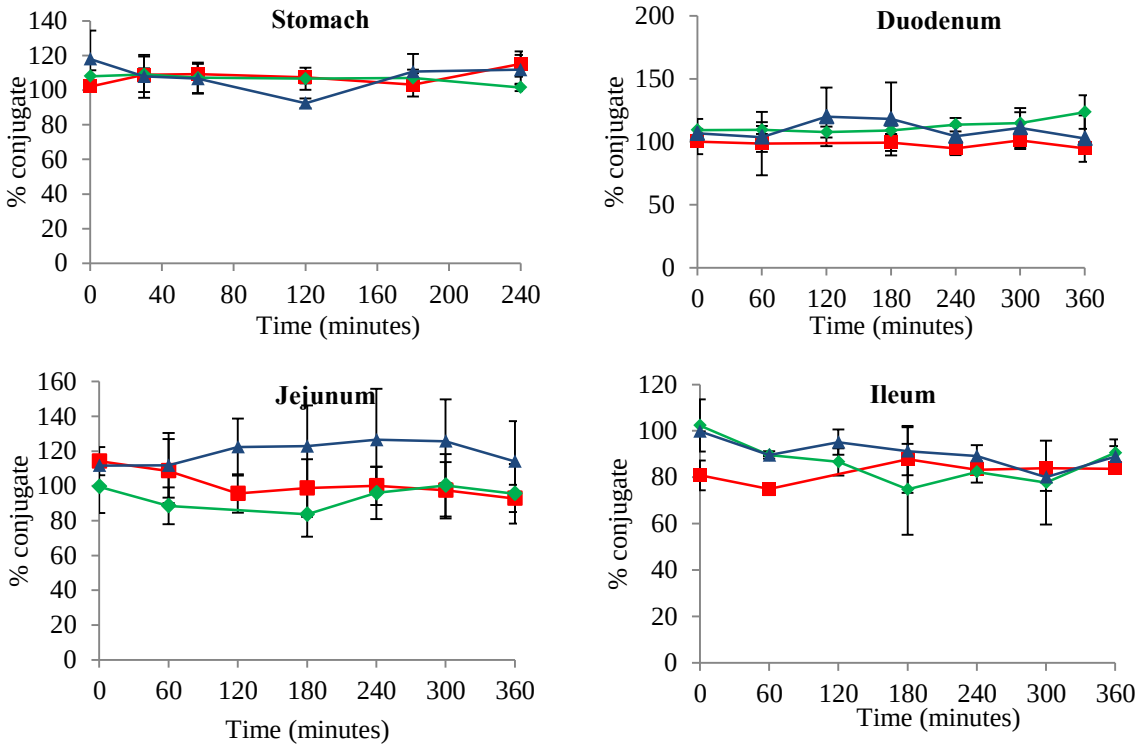


Figure 3.13 Stability of diclofenac-β-cyclodextrin in stomach, duodenum, jejunum and ileum contents from pig (■), rat (▲) and rabbit (♦). Each point represents mean ± S.D. (n = 3).

A previous study involving drugs with amide and/or ester bonds concluded that intestinal juice from pig had a major capacity to mimicry the human conditions than the simulated intestinal fluid, when oseltamivir was tested. The higher metabolism in intestinal juice pig

than in simulated intestinal fluid was also observed for atazanavir carbamate and diloxanide furoate. The higher metabolism in collected juice was attributable to its enzymatic activity (Sarti, Barthelmes et al., 2011).

Moreover, no differences were observed between the fluids of the upper GI tract of different animals, which mean that the conjugate has the same behaviour independently on the specie. Therefore, animal fluids can be replaced with simulated fluids to carry out the stability studies on the upper gastrointestinal tract of this conjugate.

It is important to note that, despite the high levels of bacteria in rat stomach, these do not affect conjugate stability. This might be due to the inexistence e/or inability of enzymatic bacteria capable to operate on the conjugate. Otherwise, it is known that rabbits practice caecoprophagy, which is associated with fermentation at the level of the stomach. However, fermentation only exists when caecoprophes are present and it does not occur in the gastric media. Additionally, only the supernatant obtained after centrifugation of fluids was used, which avoided destruction and mixture of the caecoprophes with the gastric fluid. Moreover, these studies were carried out using the supernatant of the fluids obtained after centrifugation and some enzymes can be bound to particulate matter left in the pellet (Ikesue, Kopečkovà et al., 1993). For this reason some authors instead of the centrifugation, proceed to dilution of fluids before stability experiments. However, a study conducted for determination of α-amylase activity in the supernatants of fluids used in these stability tests showed that a huge activity of amylase is observed in stomach of rats when compared with pigs and rabbits (Appendix D).

Enzymes in the luminal fluids can also come from dead gut wall cells, which will be abraded into the intestine. Therefore, sometimes it is worth to include studies with microssomes to be sure about the stability in intestinal mucous (Borde, Karlsson et al., 2012). However, previous studies with intestine homogenates of rats without contents had shown negligible degradation of the cyclodextrin prodrugs (Hirayama, Ogata et al., 2000; Kamada, Hirayama et al., 2002).

Results of diclofenac-β-cyclodextrin are according to those reported before which investigated the stability of cyclodextrin conjugates in fluids from rats. For instance, butyric acid β-cyclodextrin was stable with any release of drug in stomach and intestinal contents for 8 hours (Hirayma, Ogata et al., 2000). Okamato *et al.* demonstrated that in contents from

stomach and small intestine of rats, the cyclodextrin-5-ASA released 5-ASA only in small amounts (< 12%) during 48 hours (Zou, Okamoto et al., 2005). Comparing the stability of ethyl 4-biphenylylacetate with the stability of the equivalent cyclodextrin prodrug, the first was rapidly hydrolysed in contents from stomach and small intestine of rat, while only 10% of the cyclodextrin ester prodrugs were hydrolysed in the same contents (Uekama, Minami et al., 1997a). Another study that compares the stability between prednisolone-succinate-α-cyclodextrin and prednisolone succinate showed that in case of cyclodextrin prodrug the quantity remained after 6 hours of incubation was 80-90%, while prednisolone succinate alone was rapidly hydrolysed (Hideki Yano, Fumitoshi Hirayama et al., 2002). These studies demonstrated that the type of carrier influences the stability of the ester prodrugs and apparently the size of the carrier is determinant for the stability of the ester prodrugs in fluids from the upper GI tract. Thus, these authors justified the resistance of the conjugate against the ester hydrolysis in the contents from the stomach and small intestine due to a large steric effect of the bulky cyclodextrin moiety (Uekama, Minami et al., 1997a; Hideki Yano, Fumitoshi Hirayama et al., 2002).

3.6.3.1.1 Stability studies in caecum and colonic fluids

Results of stability of the conjugate and sulfasalazine in caecum fluids from rat, pig and rabbit and from anterior colon of pig (colon A) are represented in Figure 3.14 and Figure 3.15. These show that caecum and colon A pig fluids do not degrade the conjugate. Sulfasalazine is also not metabolised. This suggests an absence of enzymatic activity or inactivity of the microbiota at least for 6 hours of experiment in these pig fluids. Contrarily, in the case of rat and rabbit fluids, it is observed degradation of both prodrugs.

The degradation of sulfasalazine is faster than the degradation of conjugate. In caecum rat fluids, sulfasalazine is completely degraded in 30 minutes of incubation, while in the case of conjugate 60% is degraded after 6 hours of incubation. With rabbits, after 5 hours of incubation in caecum fluid, 60% of the conjugate was metabolised. For sulfasalazine, the degradation occurs with a high rate in the first 2 hours of incubation but after the % of prodrug remained constant, around 18%. These profiles of diclofenac-β-cyclodextrin conjugate and sulfasalazine degradation in caecum fluids show that the metabolism of both prodrugs is variable between these three species.

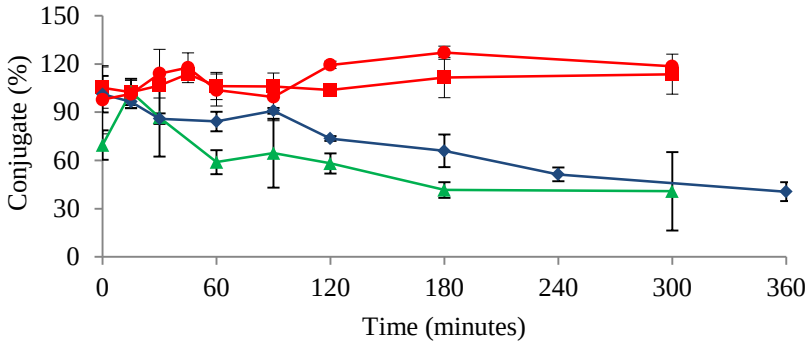


Figure 3.14 Stability of diclofenac-β-cyclodextrin in caecum contents from pig (■), rabbit (▲), rat (♦) and in pig colon A fluids (●). Each point represents mean ± S.D. (n = 3).

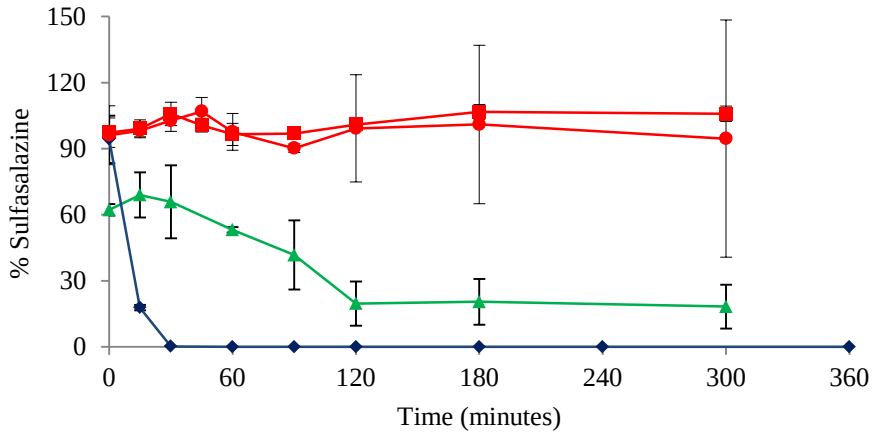


Figure 3.15 Stability of sulfasalazine in caecum contents from pig (■), rabbit (▲) and rat (♦) and in pig colon A fluids (●). Each point represents mean ± S.D. (n = 3).

It was observed that both prodrugs suffer degradation in colonic fluids. Profiles of degradation of conjugate and sulfasalazine are represented in Figure 3.16 and Figure 3.17, respectively.

One more time, the degradation of sulfasalazine is faster than the degradation of conjugate. In pig fluids, after 6 hours of incubation, 60% suffered degradation while in case of sulfasalazine 70% was degraded considering the same time of incubation In case of rabbits, the percentage of conjugate remained after 6 hours of incubation in colonic fluids is 24%, while sulfasalazine is completely metabolised. The fastest rate of degradation in colonic fluids was observed in rats, herein sulfasalazine and conjugate were degraded completely after 45 minutes and 3 hours, respectively.

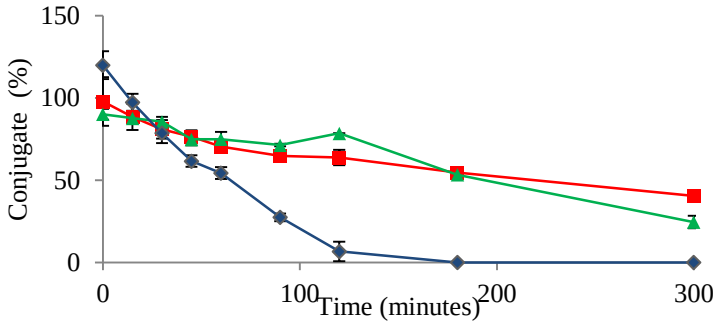


Figure 3.16 Stability of diclofenac-β-cyclodextrin conjugate in colon contents prepared as a faecal slurry from pig (■), rabbit (▲) and rat (♦). Each point represents mean ± S.D. (n = 3).

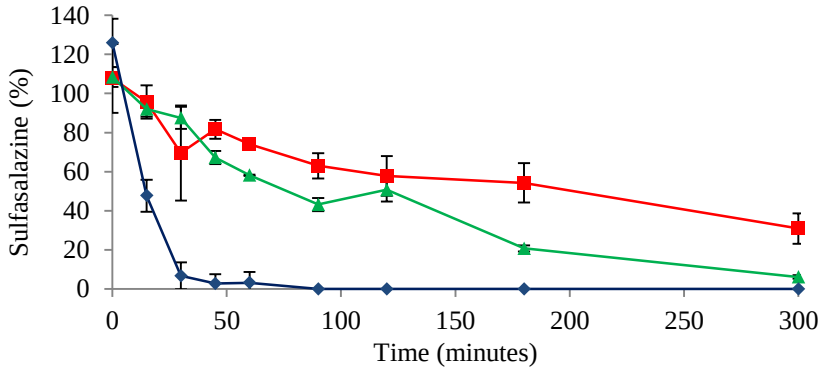


Figure 3.17 Stability of sulfasalazine in colon contents prepared as a faecal slurry from pig (■), rabbit (▲) and rat (♦). Each point represents mean ±S.D. (n = 3).

Comparing the metabolism in caecum and colonic fluids, the degradation of both prodrugs is faster in colonic than in caecum fluids in all the animals. In case of pigs, Butine and Leedle reported a higher microbial counts in the colon than in caecum (Butine and Leedle, 1989). But, Bach Knudsen *et al*. found that the densities of microorganisms in the caecum and colon appear to be quite similar (Bach Knudsen and Hansen, 1991). In terms of microbial activity, a study that compared the *in vitro* fermentation between 3 different portions of gastrointestinal tract (caecum, colon and rectum) of pig found that fermentation is slower in caecum (Bauer, Williams Barbara et al., 2004) which is according to results present here.

However, other studies found a reverse microbial activity, i.e. higher in caecum than in colon of pigs. For instance, Jensen and Jørgensen, found out that the highest microbial activity (highest bacterium counts, highest ATP concentration, low pH) was found on the caecum and proximal colon, where substrate availability is greatest (Jensen and Jørgensen, 1994). Other study that determined the metabolic activity by ATP concentrations found that it is less in colon than in caecum, which occurs probably due to energy becoming limiting during movement of digesta through the large intestine (Bach Knudsen and Hansen, 1991). Also Awati *et al.* found higher volatile fatty acids concentrations in the caecum than in the distal colon of pigs (Awati, Williams et al., 2006), which is associated with the higher quantity of substrate.

The controversy between studies led to speculate that the metabolic activity measured depends not only on the fermented subtract and the respective end products measured, but also depends on the fed state of the animals. These pigs had *ad libitum* access to food on the moment of collection of the fluids, and therefore the microbiota had enough substrate available. Thus, when an external substrate is added to the media, even if the metabolic activity is high, it could not be the preferable substrate for the microbiota, which consequently implies a slow rate of digestion. Thus, the activity of the intestinal ecosystem is a reflection of the relative availability for fermentation in each compartment and depends on the type of substrate. This can explain the highest rate of metabolism of both prodrugs in the colon than in caecum, whereby the prodrugs work as a substrate in the absence or lack of other substrate for the microbial enzyme. The effect of food on the metabolism of both prodrugs is discussed further in Chapter IV.

Moreover, degradation in colonic fluids is probably faster, given that colonic fluids were prepared using all the amount of fluids, and therefore all the bacteria (even diluted) were presented. Otherwise, in case of caecum fluids, the slow release can be associated with the lack of some enzymes in the supernatant used. The centrifugation can lead to loss of enzymes that can be attached to the complex mixture digested contents that were precipitated after centrifugation, as referred above. Indeed, colonic fluids suffer dilution before the experiment, while the caecum was not subjected to any dilution. Thus both centrifugation and dilution of fluids may lead to an underestimation of the rate of metabolism *in vivo*. However, the thickness of the crude fluids makes it difficult to work without previous centrifugation or dilution.

Overall, hydrolysis rates of these prodrugs by luminal fluids on the lower gastrointestinal tract were different between species. However, bacterial strains seem to commonly inhabit the gut of laboratory animals and humans, since similar metabolic activity was noted in human faecal slurries, and colonic fluids form pigs, rabbits and rats. Bacteroides (Bacteroidetes Phylum) are believed to be the main species responsible for degradation of the cyclodextrin carrier. Bacteroides genus is able to induce enzymes adapting to the available substrates. They are able to grow on cyclodextrin as the sole carbon source. Also cyclodextrinase were isolated from two selected Bacteroides genus (Antenucci and Palmer, 1984). Moreover, species from Proteobacteria Phylum isolated from colon of pig *(P. mirabilis* and *E. fergusonii)* demonstrated to increase the release of bisoprolol from a film-incorporated β-cyclodextrin after pre-incubation in β-cyclodextrin containing nutrient medium (Fetznera, Bo¨Hmb et al., 2004).

The stability of the conjugate was not assessed using animal faeces. However, previous studies in rabbits had concluded that faeces could be used as an alternative source of inoculum to caecum contents to study *in vitro* caecum fermentation (Bovera, D'urso et al., 2006). Other study with rabbits confirmed that *in vitro* fermentation characteristics of faeces were highly related to those of caecum content by measurement of the degraded organic matter, potential gas production; volatile fatty acids and NH_3 production (Bovera, D'urso et al., 2009). Other authors also pointed out that using faeces from pig to perform *in vitro* fermentation studies can overestimate the microbial activity of the caecum (Bauer, Williams Barbara et al., 2004). Otherwise, in the case of rats it was reported that the faecal material does not provide accurate information concerning the intestinal microbiota, because significant epithelial microbial associations occur in the proximal digestive tract (Tannock, 1999). Interestingly, rat caecal contents have been widely utilized as alternative dissolution medium of human colon (Yang, 2008). This is because of the similarity colonic microbiota between human and rodent, when the number of *Bacteroides* and *Bifidobacteria* were counted (Hawksworth, Drasar et al., 1971). In fact, rat was the only animal used before in order to study the *in vitro* stability assays of cyclodextrin conjugates. In this work comparing the metabolism between animals, rat is the animal that allows a faster degradation rate of both prodrugs. Actually, amylase activity at the level of the lower GI tract is also higher in rats than in rabbits and pigs (Appendix D). It is worth to note that the substrate used on the determination of α-amylase was starch. Therefore, the substract was not cyclodextrin, which prevents the direct

correlation between rate of metabolism of cyclodextrin conjugate and amylase activity. However, after breakdown of the cyclic structure of cyclodextrin, the dextrin formed is susceptible to be degraded by the same α-amylase that degrades starch. Therefore, the higher activity of α-amylase in rats can be responsible for the faster metabolism of the conjugate in this animal model.

3.7 Conclusion

This Chapter finds out that this conjugate can deliver diclofenac specifically at the level of the colon. Cyclodextrin can function as a colon targeting carrier of diclofenac when an ester linkage is formed between them.

The conjugate is degraded at the level of the lower intestine with simultaneous release of diclofenac as demonstrated by results in human faecal slurries. This stability behaviour is due to specific hydrolysis of β-cyclodextrin by microbiota existent on the lower intestine. These results prove that gut microbiota is the key factor that determines the hydrolysis of the conjugate. The rate of degradation relied on the he synergistic interaction between the prodrug and the gut microbiota. Enzymes able to degrade cyclodextrins to linear dextrins are essential to initiate the metabolism of the conjugate at the level of the colon.

The conjugate is completely stable in simulated gastric and intestinal fluids, and also in collected fluids from the upper GI tract of animals. Digestive enzymes are not involved in the degradation of diclofenac-β-cyclodextrin.

Stability in fluids from the lower intestine of animals demonstrated that the faster metabolism of diclofenac-β-cyclodextrin was observed in rat fluids and therefore rat is presented as an adequate animal model to perform further studies. Comparing with sulfasalazine, the metabolism of sulfasalazine was always higher than that observed for conjugate.

These preliminary results are promising, and suggest that diclofenac-β-cyclodextrin is able to release diclofenac, in the colon. *In vivo* studies are now necessary to have the *in vivo* proof of concept.

CHAPTER IV

INFLUENCE OF FOOD ON GUT FLUIDS AND METABOLISM OF DICLOFENAC-β-CYCLODEXTRIN VERSUS SULFASALAZINE IN RATS

4 INFLUENCE OF FOOD ON GUT FLUIDS AND METABOLISM OF DICLOFENAC-BETA-CYCLODEXTRIN *VERSUS* SULFASALAZINE IN RATS

4.1 Overview

A screening of the *in vitro* performance of diclofenac-β-cyclodextrin conjugate in the different simulated gastrointestinal fluids, including different animal fluids, has been reported in Chapter III. Results showed that this prodrug is specifically degraded by microbiota in the large intestine, allowing diclofenac to be released into the colon.

In respect to oral administration, it is known that food can affect the bioavailability of drugs. Generally patients are advised to take their medicines with food or on an empty stomach. However, these are imprecise instructions that are open to individual interpretations, and consequently are expected to contribute to a greater variability and lower therapeutic efficacy. Research on feeding regimens, such as timing of meals in relation to drug administration is necessary to increase the understanding of their influence on drug disposition.

The aim of this study is to understand how different feeding regimens can affect the physiological conditions in the gastrointestinal tract and to understand how food intake influences the degradation of the conjugate in the large intestine. Concomitantly with diclofenac-β-cyclodextrin conjugate, the effect of feeding regimens on the metabolism of sulfasalazine - a well-known commercially available colonic prodrug of reference - was also studied.

In vitro studies (Chapter III) demonstrated that rats provided the faster metabolism of both prodrugs, diclofenac-β-cyclodextrin and sulfasalazine. Therefore, this was the animal model used in this Chapter to access the influence of different feeding states on the metabolism of prodrugs.

4.2 Introduction

The presence of food in the gastrointestinal tract reduces gastrointestinal motility, thereby delaying the arrival of a colonic drug carrier to its site of action (Mittelstadt, Hemenway et al.,

2005; Varum, Merchant et al., 2010; Varum, Hatton et al., 2013). Food intake also influences the amount of water in the gut that is available for drug dissolution prior to absorption. Food also influences pH of gut content, which as well as controlling drug release from pH-responsive drug carriers, also influences the ionisation of weakly acidic/basic drugs and thus their aqueous solubility, stability and absorption (Stella, Borchardt et al., 2007; Varum, Merchant et al., 2010). Food also influences the performance of colonic prodrugs, whose conversion to drugs relies on gut bacterial enzymes. Bacterial activity in the colon depends on the quantity and quality of available substrates for fermentation, and determines the intensity and direction of gut bacterial metabolism of prodrugs and thereby drug absorption (Agorama, Woltosza et al., 2001; Mountzourisa, Kotzampassib et al., 2009).

Diclofenac has been studied as one of the drugs whose pharmacokinetic parameters are influenced by the presence of food. The administration of an enteric coated diclofenac sodium formulation (50 mg) immediately after a standard breakfast showed that food does not have significant effect on the extent of diclofenac absorption when compared with the fasted state. However, delay in the onset of absorption (varying from 2.5 to 12 hours in fed and from 1.5 to 2.75 hours in fasted state) and a reduction in peak plasma levels of approximately 30% was observed in the presence of food (Willis, Kendall et al., 1981). Other study conducted in humans with diclofenac hydrogel beads (150 mg) originated comparable effects on the presence of food. The administration immediately after breakfast did not affe**c**t the bioavailability, however the C_{max} decreases 38% and the T_{max} increases three fold in the fed state comparing with the fasted state (Thakker, Mangat et al., 1992). Billa *et al.* showed that after administration of gum xanthan tablets of sodium diclofenac (100 mg) in healthy men in fed state, AUC was not significantly affected and the T_{max} increased (T_{max} values 2.6±0.3 hours in fasted state and 5.5 ± 0.8 hours in fed state) (Billa, Yuen et al., 2000). This is consistent with the findings of Willis *et al.* (1981), Thakker *et al.* (1992) and Chan *et al.* (1990) (Willis, Kendall et al., 1981; Chan, Mojaverian et al., 1990; Thakker, Mangat et al., 1992). Moreover, no differences were observed between the AUC of gum xanthan tablets and the buffer solution (pH = 7) of diclofenac in both dietary stats, in agreement with the findings of Riad *et al.* (1989) (Riad, Sawchuk et al., 1995; Billa, Yuen et al., 2000).

On the other hand, Billa *et al.*, found out that the C_{max} of diclofenac is higher in fed state (C_{max} = 347±112 ng/ml) than in fasted state (C_{max} = 661.5±158.5 ng/ml), although the gastric emptying time was longer in presence of food. The faster release and absorption of diclofenac

was attributable to an interaction between food and the formulation. In fed state, the longer residence of the tablets in stomach leaded to a loss of the integrity of the xanthan gum matrice due to the low pH, allowing rapid influx of water into the matrix system with a faster rate of drug release (Billa, Yuen et al., 2000).

Additionally, the pharmacokinetic parameters are dependent on the type of formulation, as demonstrated by Chan *et al.* who found that gastric emptying of sodium diclofenac, either in solution or as a precipitate, occurred more quickly than the emptying of Heidelberg capsule (Chan, Mojaverian et al., 1990). Another author, Zmeili *et al.* (1996) reported no significant change in C_{max} in the fasted and the fed states with their enteric-coated controlled release tablet (Zmeili, Hasan et al., 1996).

These studies proved that the presence of food affects the rate of absorption and the concentrations of diclofenac in plasma, which depends on the the physical properties of the dosage form, and also depends on the amount and composition of the meal which affects the gastric emptying time (Billa, Yuen et al., 2000). However, the presence of food has no influence on the extent of diclofenac absorption, independent of the formulation.

Given the critical influence of the fasted/fed state on the performance of colonic prodrugs as described above, we determined the influence of different feeding regimens on gastro-intestinal contents, pH and metabolism of the colonic prodrug, in order to establish the most appropriate fasted/fed state that should be employed for its *in vivo* assessment in rats.

The study was conducted in different feeding regimens: fed *ad libitum*, 12-hour fast, 12-hour fast followed by one hour feeding, which was itself followed by either 30 minutes or 4 hours of fast, prior to the animals being killed, and measurements being taken. These four regimens were selected for a number of reasons: firstly, as stated above, most oral drug delivery experiments are conducted on overnight-fasted rats; secondly, a control of the 12-hour fast i.e. animals being fed *ad libitum*; thirdly, a 4-hour fast after feeding, to ensure complete gastric emptying of food ingested (Booth, Gibson et al., 1986), fourthly, a control experiment for the latter, i.e. 30 minutes fast after feeding assures that the ingested food has not arrived in the lower intestine (caecum and colon) (Brown, Greenburgh et al., 1994).

The rat is an appropriate model to use in early drug development; its mean intestinal transit time is comparable to that in humans despite the different gastro-intestinal lengths, transit

time is significantly shorter in the fasted state compared to the fed state, as in man, and its gastrointestinal motility is under the control of the migrating myoelectric complex (MMC), like in man (Tuleu, Andrieux et al., 1999; Mittelstadt, Hemenway et al., 2005). Rats are thus widely used as an *in vivo* model and have been used to assess different colonic prodrugs, including cyclodextrin-based ones (Minami, Hirayama et al., 1998; Kamada, Hirayama et al., 2002).

As far as we know, the effect of fasting on the metabolism of cyclodextrins and the respective conjugates has not been subject of investigation. Otherwise, few studies have looked at the effect of food on the metabolism of other colonic prodrugs. One of the studies reported that metronidazole and olsalazine were found to be practically stable in the material from the contents of the ascending colon from human collected in the fed state. Otherwise, olsalazine was degraded up to 20% to 5-ASA within 10 minutes in the fasted ascending colon (Vertzoni, Carlsson et al., 2011). However, previous study in humans had shown that the systematic availability of olsalazine was not affected by food (Ryde and Ahnfelt, 1988). These results leaded Vertzoni *et al.* to conclude that the metabolism of olsalazine was limited in the ascending colon and became higher in descending colon both in fasted and fed state (Vertzoni, Carlsson et al., 2011). Another study reported that fasted rats had lower activities of α-galactosidades, α-glucosidades, and β-glucosidades and higher activities of β-galactosidase and azoreductase (Mountzourisa, Kotzampassib et al., 2009).

4.3 Aims and Objectives

This Chapter aims to investigate the influence of feeding regimen in Wistar rats on:

i) Distribution and pH contents along the gut;
ii) Metabolism of diclofenac-β-cyclodextrin within the caecal and colonic contents, using sulfasalazine as a control.

This enables an understanding of how the regimen state affect the pharmacokinetics of prodrugs and enable to choose the most favorable feeding state to perform the *in vivo* studies.

4.4 Materials and methods

4.4.1 *Reagents and chemicals*

Sodium diclofenac (M_W=318.14 g/mol), sulfasalazine (M_W=398.394 g/mol) were purchased from Sigma. Diclofenac-β-cyclodextrin (M_W=1411 g/mol) was synthesized according to the method reported in Chapter II.

Sodium chloride, potassium hydroxide, sodium hydroxide, potassium dihydrogen phosphate, HPLC grades acetonitrile, methanol and water were purchased from Fisher Scientifics. Peptone water and yeast extract were obtained from Oxoid Limited (Hampshire, UK). Magnesium sulphate heptahydrate and calcium chloride hexahydrate were obtained from VWR (Leicestershire, UK). Trifluoroacetic acid (TFA) and dimethylformamide (DMF), sodium bicarbonate, haemin, l-cysteine HCl, vitamin K and resazurin were obtained from Sigma Aldrich (Dorset, UK). All other chemicals and solvents were of HPLC reagent grade and were used without further purification. Phosphate buffer saline (PBS) pH 6.0 and pH 6.8 were prepared according to the USPXXIV.

4.4.2 *Animals*

All the procedures had been approved by the School's Ethical Review Committee and were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. Studies were performed using healthy adult male Wistar rats (8 weeks, 240-250 g) purchased from Harlan Olac Ltd. (Oxfordshire, UK). All animals were housed in rooms with controlled conditions: 20 ^{o}C, 40-60% humidity, 15-20 air changes per hour. Animals underwent a period of acclimatization, with free access to standard rat chow and water for 7 days prior the experiment. Twelve hours before the beginning of each experiment, the animals were housed in individual metabolic cages, whose floors were perforated to restrict the ability of animals to eat their own faeces and allowed the collection of their urine and faeces. Water was available *ad libitum* through the experiment.

4.4.3 *Feeding regimens*

Four Groups (A, B, C and D) of 5 rats were given different food intake regimens. Rats from Groups A, B and C were fasted for 12 hours overnight. Subsequently, Group A rats were killed. Groups B and C rats were allowed to feed for one hour, after which they were fasted

for either 30 minutes (Group B) or 4 hours (Group C), before being killed. Group D rats were not fasted at all, and were given access to food *ad libitum*. The different feeding regimens are shown in Figure 4.1.

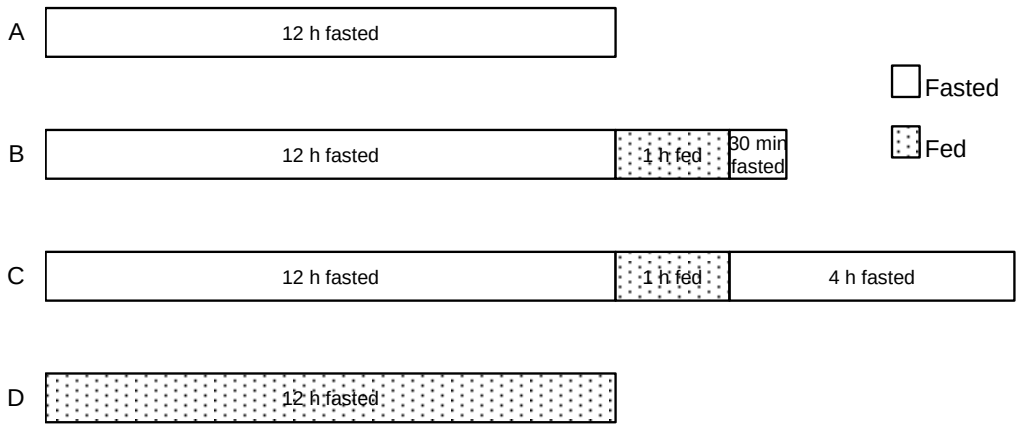


Figure 4.1 Schematic representation of the different feeding regimens of rat groups A, B, C and D. A: 12-hour fast; B: 12-hour fast followed by 1-hour feeding, followed by 30-minutes fast; C: 12-hour fast, followed by1-hour feeding, followed by 4-hour fast; D: fed *ad libitum*.

Before starting the experiment, all animals were weighted. After 12 hours of fasting or feeding, different parameters were measured including: the volume of the water consumed, the volume of urine produced, the mass of feces produced, and the body weight loss.

Animals were killed following a Schedule One Method (CO_2 asphyxiation), after which the intestinal tract was removed. The pH and mass of gut contents were determined as follows.

4.4.4 *Determination of pH and mass of the gastrointestinal luminal contents*

The pH of the contents was measured *in situ* by placing the pH probe (H160 Portable pH Meter, Hach, Düsseldorf, Germany) within the luminal contents of each gastrointestinal section. It was measured at the anterior (labelled I in Figures) and posterior (except for colon), (labeled II in Figures) of each section of the stomach, small intestine (divided into three sections approximating to the duodenum, jejunum and ileum), caecum and colon before the gut contents were collected into previously weighed vials. The wet masses were recorded, and

the vials were stored at – 80 °C. The pH of the distal part of colon contents could not be reliably measured due to its solid nature.

4.4.5 *Determination of stability of prodrugs (diclofenac-β-cyclodextrin and sulfasalazine), in caecal and colonic contents*

Stability tests were performed inside an anaerobic workstation (Electrotek 500TG workstation, Electrotek, UK) at 37 °C and 70% RH. The caecum and colonic contents from each group of rats were mixed with PBS – of differing pHs as explained below - in order to obtain a 40% w/w slurry as prepared in Chapter III. The pH of the PBS differed for the different samples, but matched the *in situ* measured pH in the different gastrointestinal sections (section above), in order to maintain the pH of the gut contents. Thus, gut contents from Groups A and B rats were mixed with PBS pH 6.8, while those from Groups C and D rats were mixed with PBS pH 6.0. The slurries were then homogenized using a glass rod and sieved through an open mesh fabric (Sefar NitexTM, pore size 350 µm) to remove any unhomogenised fibrous material. The sieved faecal slurry was then diluted 50% (w/w) with basal medium containing peptone water, yeast extract, NaCl, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 6H_2O$, $NaHCO_3$, haemin, l-cysteine HCl, bile salts, tween 80, vitamin K and resazurin, was described previously in Chapter III (Basit and Lacey, 2001; Basit, Newton et al., 2002).

Two solutions of each prodrug sulfasalazine (5 mg/mL) and diclofenac-β-cyclodextrin (2.4 mg/mL) were prepared in PBS at pH 6.8 and pH 6.0, both containing 4% (v/v) of dimethylformamide. Subsequently, 100 µL of these solutions were mixed with 900 µL of caecal or colonic fluids prepared above; the fluids from Groups A and B rats being mixed with prodrug solutions at pH 6.8, while those from Groups C and D rats were mixed with prodrug solutions at pH 6.0 in order to maintain the pH constant (Table 4.1). The final concentrations of the conjugate, sulfasalazine and DMF were 0.5 mg/mL, 0.24 mg/mL and 0.4% (v/v), respectively.

Thereafter, these mixtures were incubated and shaken at 100 rpm (VXRbasic Vibrax®, Leicestershire, UK), with 50 µL aliquots being withdrawn at times 0, 15, 30, 60, 90, 120, 180, 240, 360, 400, 600, 720 and 1440 minutes. The aliquots were immediately mixed with 100 µL of methanol and centrifuged at 10 000 rpm for 10 minutes at room temperature, after which the supernatant was removed and analyzed via HPLC for prodrugs concentrations.

Table 4.1 Schematic representations of procedure to perform stability tests of conjugate and sulfasalazine in caecum and colon fluids from rats.

[Drug]	Phosphate buffer	Group	Contents	Incubation	[drug] incubation	Sampling Volume	Final [drugs]
5 mg/mL of sulfasalazine and 2.4 mg /mL of diclofenac-β-cyclodextrin	pH 6.8	A pH~6.8	caecum	100 µL drug solution + 900 µL of fluid	0.5mg/mL of conjugate and 0.24mg/mL of of diclofenac-β-cyclodextrin	50 µL (0.4% DMF)	16.7 µg/mL of sulfasalazine and 80 µg/mL of diclofenac-β-cyclodextrin
			colon				
		B pH~6.8	caecum				
			colon				
	pH 5.9	C pH~5.9	caecum				
		D pH~5.9	colon				

4.4.6 *HPLC analysis*

All HPLC runs were performed using an Agilent 1100 series system equipped with a UV detector and a XTerra reverse phase C-18 column with 5 µm particle size, 4.6 mm internal diameter and 250 mm length. The mobile phase (consisting of acetonitrile and 0.1% trifluoroacetic acid (TFA) in water) was pumped at a flow rate of 1 mL/min. A gradient system of 0.1% TFA in water (A) and acetonitrile (B) was followed: 0-15 minutes 25-60% B; 15-22 minutes 60-25% B. The sample injection volume was 20 µL and detection wavelength was 254 nm at 30 ºC. Each measurement was performed in triplicate. Results were acquired and processed with the Agilent Chemstation Data System Software 7.

4.4.7 *Data analysis*

Statistically significant differences in the total mass of contents between Groups and in the mass and pH of contents per section among Groups were evaluated using One-way analysis of variance (ANOVA), followed by Tukey test. General linear model (repeated measurements) was used to assess the differences of pH contents between Groups with different regimens of food intake (A, B, C and D). The relationship between mass and pH was investigated using Pearson Correlation Coefficient. Degradation kinetics of diclofenac-β-cycodextrin were determined by fitting the percent prodrug remaining *versus* incubation time curves to a first-order kinetic model, and subsequently calculating reaction rate constant (K) and half-life ($t_{1/2}$). Statistically significant differences in the rate constant and half-life of diclofenac-β-

cyclodextrin conjugate between Groups were analysed using Kruskal–Wallis test, with Nemenyi's post-hoc analysis. All tests, apart from Nemenyi's test were carried out using SPSS 21.0 for Windows®. The Nemenyi test was conducted as described (Jones, 2002). Results were considered statistically significant when $p < 0.05$.

4.5 Results and discussion

4.5.1 *Influence of feeding regimens on body weight of the rats, mass of faeces and volume of urine*

The changes in body weight of the rats, mass of faeces and volume of urine produced, quantity of food and volume of water consumed per group were summarized in the Table 4.2. In all the animals kept fasted overnight (A, B, C and D) it was verified a loss of body weight (~5%) after 12 hours. This loss of weight was expected in these fasted overnight animals, however animals from Group D also demonstrated weight loss. Moreover, in Group D, loss of weight is higher (~12%), herein it was not expected since these rats were fed *ad libitum*. This body weight loss can be explained due to the higher production of feces observed in this group of rats. This was already observed before in a study with rats on an *ad libitum* diet (Jeffrey, Burrows et al., 1987). The same study had observed that rats on a controlled diet maintained their body weight.

After feeding, the animals from Group B and C did not show any production of faeces. This can mean that the food ingested before 12 hours of fasting did not have enough time to be digested and consequently to be excreted by the gastrointestinal tract of rats. It is important to note that rats eat only small part of their food during the day and and consume 80% of their total daily food intake at night, taking 5-8 meals during the night, hence during the feeding of the animals the room was kept dark (Nebendahl, 2000).

Measurements of the solid and water contents in fed and fasted female Wistar rats had shown that the water amount was also higher in the fed state (7.8 ± 1.5 mL) against 3.2±1.8 mL in the fasted state, attributable to the increase of secretions and water bound with the ingested food (Mcconnell, Basit et al., 2008). The higher quantity in fed state can be favourable to the dissolution of drugs or formulations. However, possible interactions of food with drugs have to be considered.

As the photographs of each section of the gastrointestinal tract (Figure 4.2) show, the animals from Group B (Figure 4.2-II), C (Figure 4.2-III) and D (Figure 4.2-IV) present the stomach and the colon full comparing with the fasted animals (A) (Figure 4.2-I).

Moreover, when the stomach is full it is possible to distinguish between the glandular and the non-glandular part. Herein, the non-glandular part is thin and translucent and the glandular is opaque, muscular and reddish involving the fundic and pyloric regions (Ghoshal and Bal, 1989).

Table 4.2 Summary of the parameters measured in rats from different Groups of rats (A, B, C and D). The values represent the mean of 5 rats per group ± SD.

	Time = 0	After 12 hours					After 1 hour of feeding followed by fasting				
Group	**body weight (grams)**	**mass of feces (grams)**	**volume of urine (mL)**	**water consumed (mL)**	**weight of food consumed (grams)**	**Body weight (grams)**	**water consumed (mL)**	**volume of urine (mL)**	**mass of faeces (grams)**	**weight of food consumed (grams)**	**Body weight (grams)**
A	271.3 ± 12.85	5.44 ± 0.90	17.6 ± 2.42	20.7 ± 5.20	_	256.4 ± 10.07	_	_	_	_	_
B	265.52 ± 8.72	2.98 ± 0.47	20.6 ± 3.98	19.22 ± 3.54	_	251.6 ±6.67	8.1 ± 3.11	1.2 ± 0.75	0	3.74 ± 1.72	258.5 ± 9.71
C	246.52 ± 8.72	2.42 ± 0.61	17.8 ± 3.12	14.02 ± 3.05	_	232.96 ± 4.13	25.72 ± 17.89	17.6 ± 16.75	0	3.9 ± 2.66	236.68 ± 2.99
D	259.39 ± 18.57	6.24 ± 2.36	16.4 ± 6.86	25.04 ±10.62	74.78 ± 4.60	229.98 ±7.60	_	_	_	_	

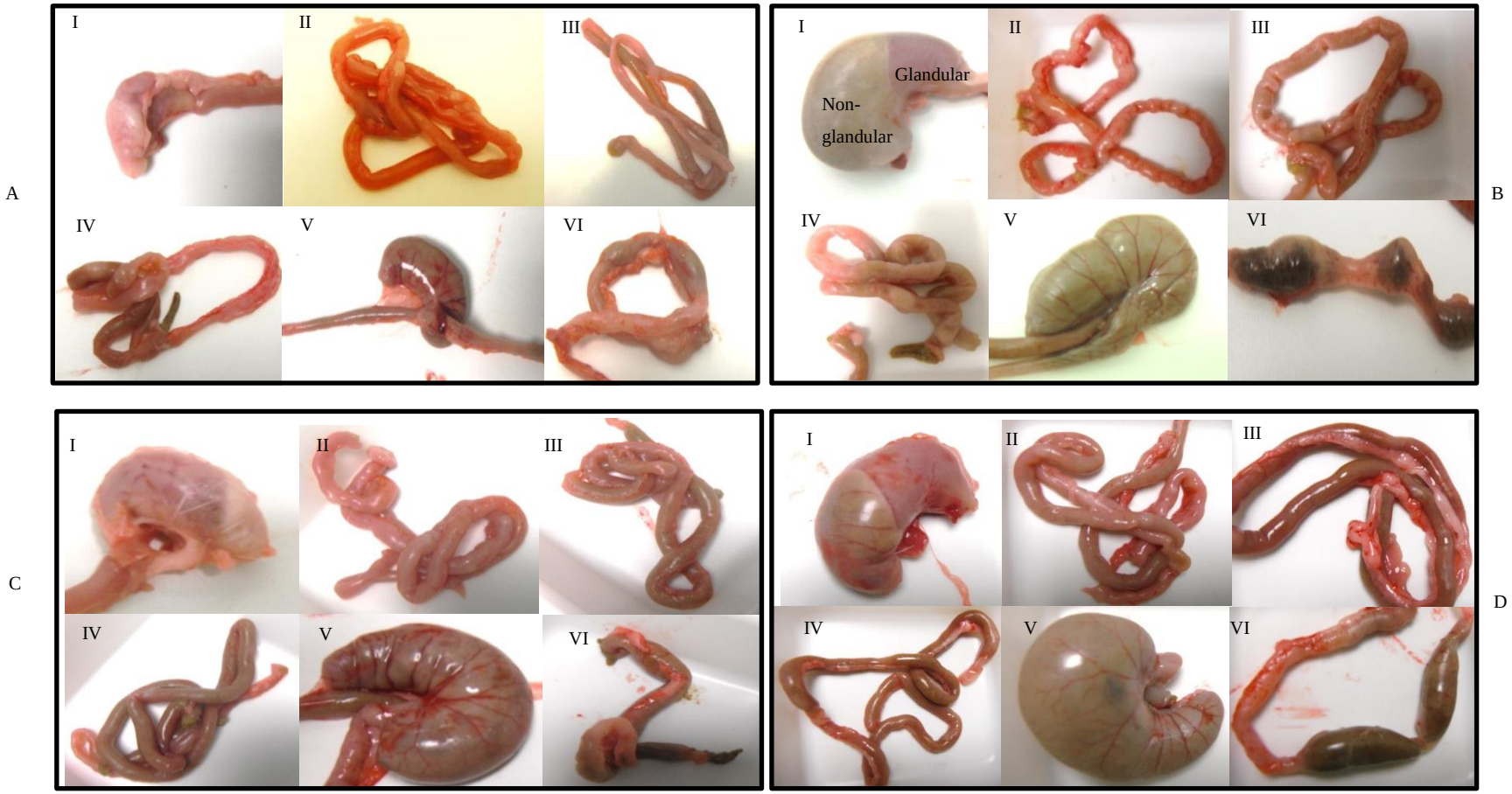


Figure 4.2 Photographs of different sections of the gastrointestinal tract of rats:(I) Stomach; (II) Duodenum; (III) Jejunum; (IV) Ileum; (V) Cecum; (VI) Colon, from Groups A, B, C and D.

4.5.2 *Influence of feeding regimens on the mass of gastrointestinal contents*

It was observed that the aspect of the contents was variable along the gastrointestinal tract of the same rat and between rats of different groups. Basically, at the level of the stomach, the contents had a yellow color and food present seemed almost intact. At the level of the small intestine, contents were liquid and viscous and had a dark brown color. In caecum, contents were semi-solid with a dark brown color. This color was less intense in animals from Group C, giving the impression that food did not have enough time to be completely fermented. In the proximal colon, contents were generally semi-solid, while in the distal colon, when presented, they were solid in form of pellets. As it was reported, the initial part of colon is the site of initial dehydration of the contents and the distal portion the responsible for the formation of faecal pellets (Ferre and Ruckebusch, 1985).

As expected, feeding regimens influenced the total mass of gastrointestinal contents (Figure 4.3), and these were statistically different among the four Groups (ANOVA, $p<0.05$).

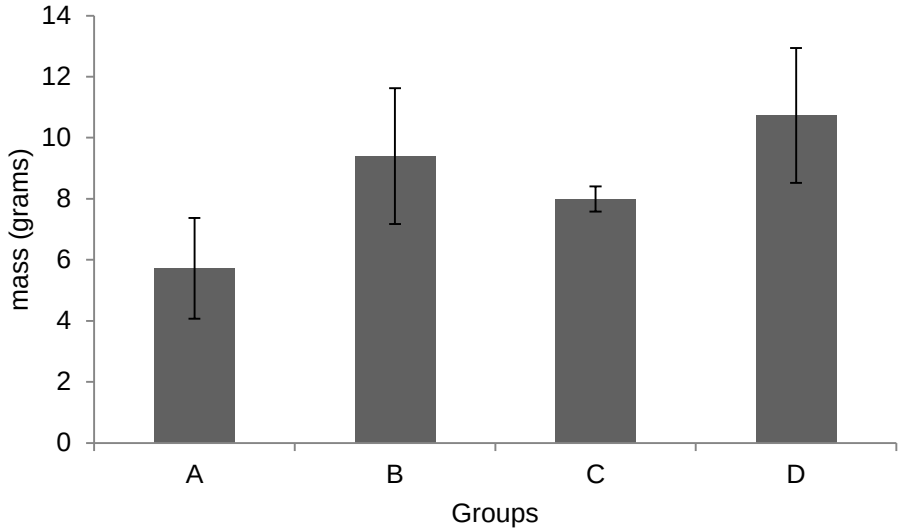


Figure 4.3 Total mass of gastrointestinal contents in healthy male rats in different Groups. A: 12 hours fast, B: 12 hours fast then 1 hour fed then 30 minutes fast, C: 12 hours fast then 1 hour fed then 4 hours fast; D; fed. Each bar represents mean ± S.D, n = 5.

The total gut content weights of the fed Group D was almost twice from that of the 12-hour fasted Group A rats (post hoc Tukey, $p<0.05$). This result in male Wistar rats reflects the previous report in female Wistar rats, where similar values for gut contents are reported

(Mcconnell, Basit et al., 2008). When Group B rats were allowed to feed for 1 hour, they ate sufficiently during the hour, such that their total gut contents mass were similar to those in Group D that were allowed food *ad libitum* throughout the experimental duration ($p>0.05$). On the other hand, Group C rats, which were fed for 1 hour and fasted for 4 hours prior to being killed, had similar mass of gut contents as Group A rats ($p>0.05$).

Analysis of the distribution of gastrointestinal contents (Figure 4.4 and Figure 4.5) shows the influence of feeding/fasting states and timings. Groups A and C (which were fasted for substantial durations prior to being killed) have similar profiles to each other (Figure 4.4).

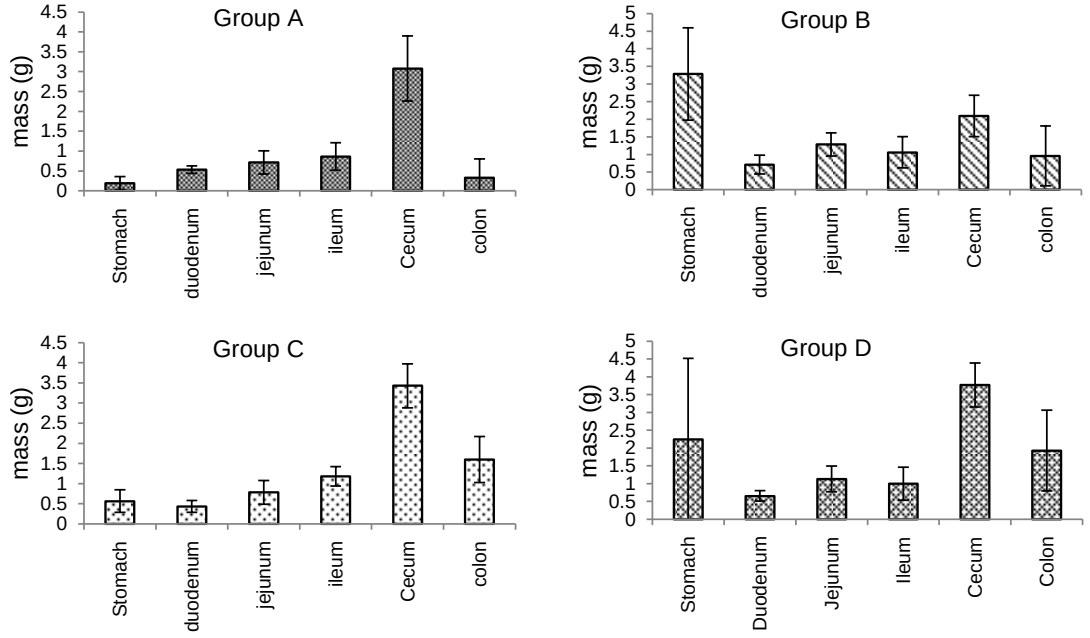


Figure 4.4 Mass of luminal contents in different gastrointestinal sections in rat Groups. Each bar represents mean ± S.D. n = 5.

In all Groups, the major quantity of fluids was found in the caecum, except for Group B where the highest quantity of fluids was found in stomach. Groups B and D (which had short/no fasting times prior to being killed) also had similar profiles to each other. The major difference in the four profiles is the large stomach contents in Groups B and D, compared to minimal stomach contents in Groups A and C. In contrast, the small intestinal contents measured in the duodenum, jejunum and ileum are low and similar across all groups.

Fasted animals (Group A) have minimal stomach contents, slowly increasing contents in the duodenum, jejunum and ileum, with most of the gut contents being located in the caecum, and

the mass of contents dropping in the colon. A 12-hour fast in Group A rats (Figure 4.1) means that any food eaten prior to the fast has moved down the gastro-intestinal tract to the caecum. However, it is important to note that, as reported before in a study with Sprague Dawley rats, the quantity of food remaining in the stomach after an overnight fasting is variable and depends on the diet type and on the diet regime (Jeffrey, Burrows et al., 1987).

In contrast, the profile for the fed Group D rats is very different compared to that of Group A, with larger masses in the stomach and in the colon of the fed animals.

Greater variability in the gut (especially stomach) contents is also seen in Group D rats fed *ad libitum*. This variability in the quantity of food remaining in the stomach can be attributable to the house condition, caged singly, which may cause stress to the rat (Nebendahl, 2000).

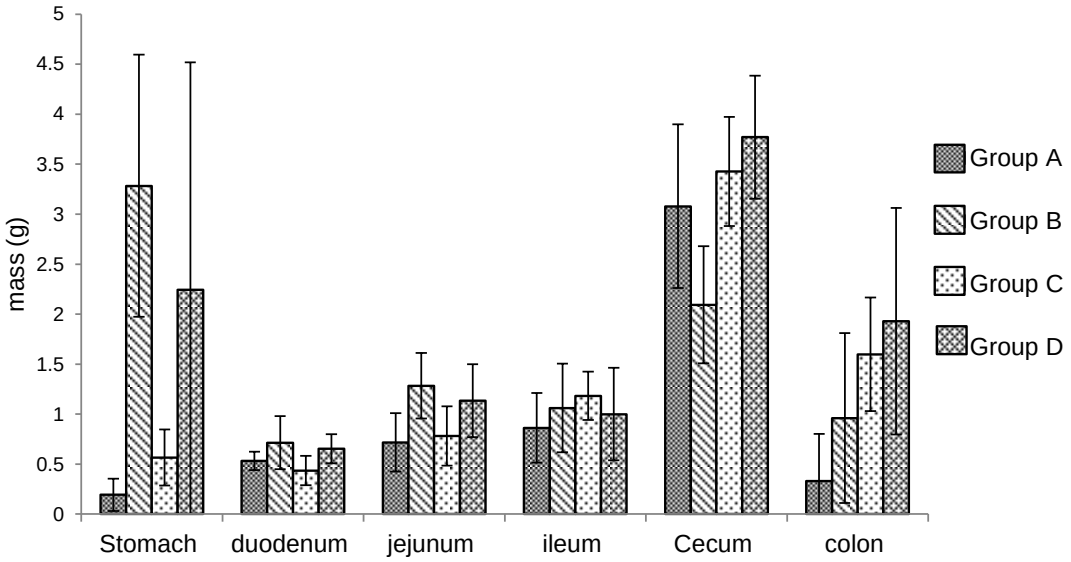


Figure 4.5 Mass of luminal contents in the different gastrointestinal sections in the rat Groups. The data shown in Figure 4.4 has been replotted to assist comparison of the different gastrointestinal regions. Each bar represents mean ± S.D, n = 5.

Rats with full/partially full stomachs are expected to show variable gastric emptying times, leading to variable drug release from the drug carrier, and hence greater variability in the latter's performance. This explains why most *in vivo* experiments in laboratory animals are conducted in the fasted state when oral drug absorption is assessed.

The similar profiles of Groups B and D rats show that a 30 minutes fast after feeding is obviously not sufficient for gastric emptying. In contrast, the similarity of profiles of Group C

rats to those of Group A shows that gastric emptying has taken place after 4 hours. The Group A and C profiles indicate that during the 5 extra experimental hours undergone by Group C rats, the gastrointestinal contents moved down the gut, such that it was observed more of it in the colon.

4.5.3 *In situ pH of the gastrointestinal contents*

The pH of the contents measured along the gastrointestinal tract for the different Groups of rats is shown in Figure 4.6

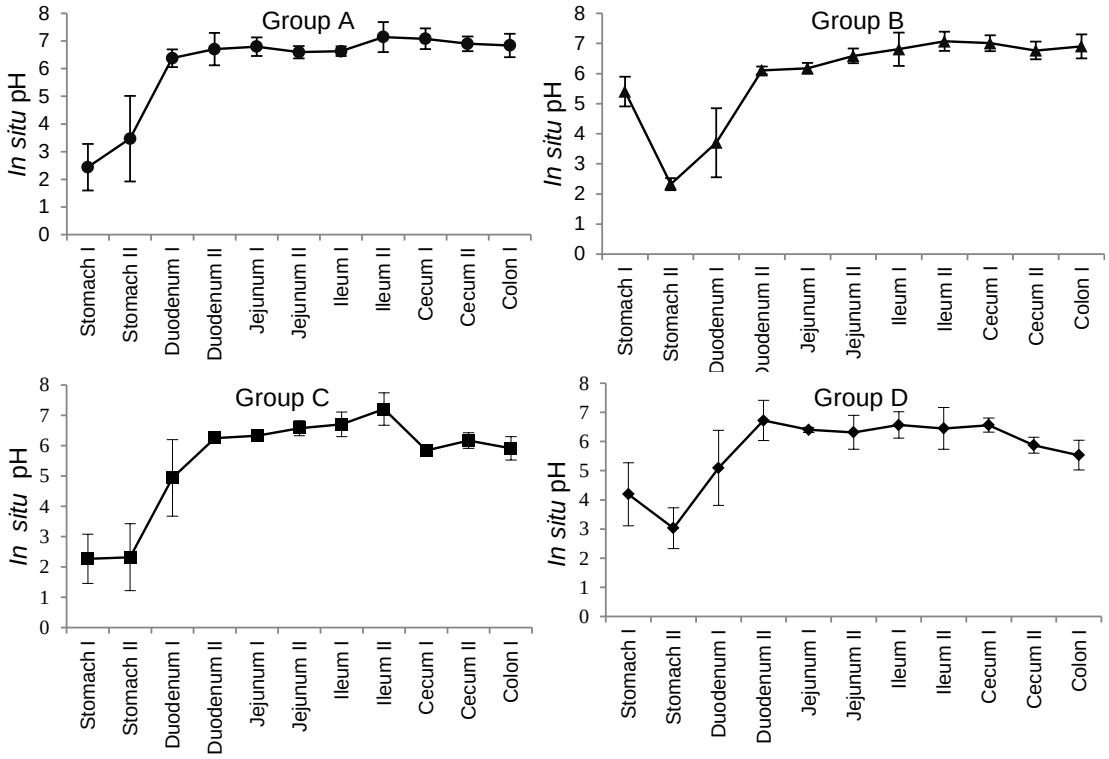


Figure 4.6 *In situ* pH of gastrointestinal contents in the different sections of the gastrointestinal tract in the rat groups. I and II refer to the anterior and posterior parts respectively. Each point represents mean ± S.D, n = 5.

Profiles are as expected and reflect those previously reported in rats (Mcconnell, Basit et al., 2008) and man (Dressman, Berardi et al., 1990). The pH value is variable in the stomach depending on food intake and its buffering/dilution effect on gastric HCl, rising in the small intestine due to pancreatic juice secretion, and slightly falling in the large intestine due to the production of short chain fatty acids (SCFA) by bacterial fermentation of dietary fiber. The largest differences among the Groups occur in the early sections of the gastrointestinal tract (the stomach) with smaller differences in the caecum and colon (Figure 4.7). In contrast, the

small intestinal pH is immune to the effects of fasting/feeding regimens. Similarities between pH profiles for Groups A and C rats (repeated measures ANOVA, $p>0.05$), and those of Groups B and D rats (repeated measures ANOVA, $p>0.05$) shown in Figure 4.7, reflect the influence of feeding/fasting states and times.

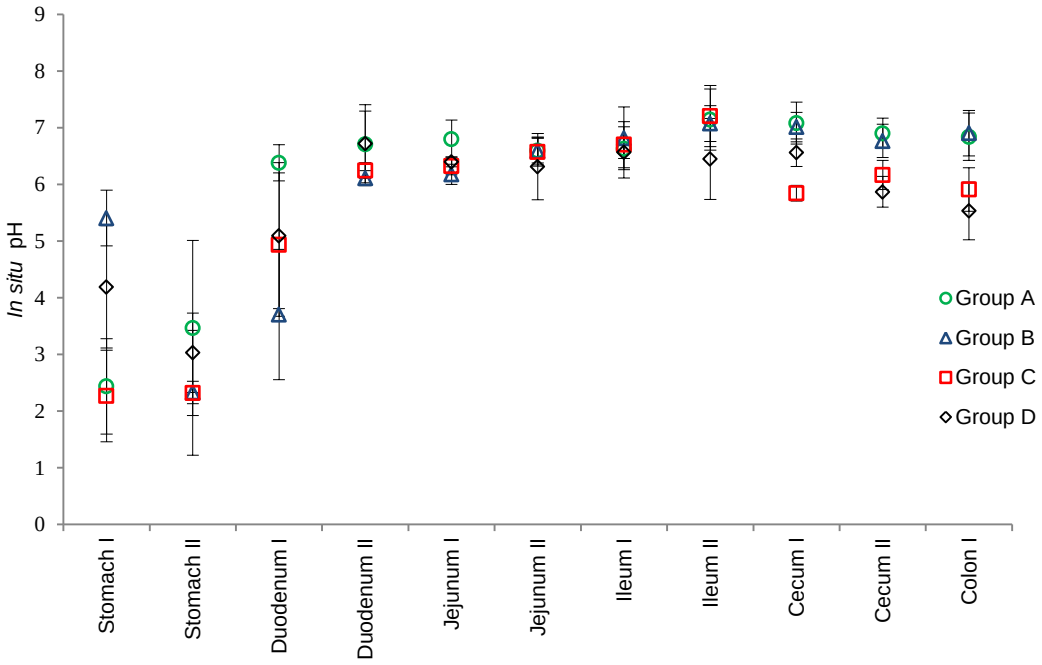


Figure 4.7 *In situ* pH of gastrointestinal contents along the gastrointestinal tract of rat Groups A(○), B(Δ), C(□) and D(◊). I and II refer to the anterior and posterior parts, respectively. Same data as in Figure 6 has been re-plotted to ease comparisons. Each point represents mean ± S.D, n = 5.

Groups A and C rats which have no or limited food in the stomach show a low pH in the first part of the stomach (forestomach) compared to Groups B and D rats, which have more food (see Figure 4.5 for food contents). Indeed, a correlation of 0.7 (Pearson, $p<0.05$) was found between the mass of the stomach contents and pH in stomach I in rats, reflecting the buffering and dilution effects of food in the stomach (Evans, Pye et al., 1988 ; Fallingborg, Christensen et al., 1989). In contrast to stomach I, the pH in the lower part of the stomach (Stomach II) was low in all Groups and was independent of food presence (Pearson, $p>0.05$). This correlates with previous reports that in rats, the non-glandular stomach I (forestomach) is used for storage and mechanical digestion of food (Ghoshal and Bal, 1989), whose

presence/absence is the principal factor responsible for the local pH (Ward and Coates, 1987), while the pH in the glandular HCl-secreting Stomach II is influenced mainly by microbial products, despite HCl-secretion being stimulated by the presence of food (Ward and Coates, 1987). It must be noted that in man, the whole stomach is glandular and harbours few bacteria, in contrast to the large bacterial numbers in rats (Kararli, 1995).

As mentioned above, the pH profiles of the four groups of rats diverge at the large intestinal caecal and colonic fractions. Although differences are small, the lowest pHs are seen in Groups C and D, with the highest in Groups A and B. Comparison of Figures 4.4 and 4.6 suggest a correlation between mass contents and pH measured. Indeed a strong correlation ($r = -0.9$, Person $p<0.05$) was found between the mass contents and the pH in the first part of the colon (Colon I) when all the data was analysed, i.e. $n=20$. This reflected in the production of bacterial fermentation byproducts, the short chain fatty acids (acetate, propionate and butyrate) (Scott, Gratz et al., 2013). With a greater mass of dietary fiber, a greater bacterial metabolism and production of short fatty acids is observed (Ferguson, Tasman-Jones et al., 2000; Paturi, Butts et al., 2012).

A higher pH in the large intestine of Group A fasted rats (compared to Group D fed rats) was observed in previous reports both in rats (Mcconnell, Basit et al., 2008) and in men (Evans, Pye et al., 1988 ; Fallingborg, Christensen et al., 1989) and may be explained by their lower concentrations of SCFA compared to rats fed *ad libitum* (Mountzourisa, Kotzampassib et al., 2009). Similar pH in large intestine in Groups A and B suggest that food ingested by Group B rats during the 1 hour feeding has not travelled down the gastro-intestinal tract during the 30 minutes fast prior to measurement. Meanwhile, pH in the lower large intestine in Group C rats suggests that food ingested prior to a 4-hour fast has travelled down the gastro-intestinal tract to some extent. This shows the importance of the fasting/fed states and feeding regimens when evaluating colonic drug carriers in the rat *in vivo* model. The fasted rat (most commonly used model) may demonstrate a poor performance of a pH-controlled colonic drug carrier, due to an insufficiently low pH in the colon, rather than due to a poor formulation. On the other hand, while the fed rat may possess the correct (low) colonic pH required for drug release from such a pH-controlled carrier, variable feeding by a group of animals could lead to variability in gut contents, transit times, drug release and absorption profiles, which could in turn mask the true potential of the colonic drug carrier.

Table 4.3 The pH values, measured *in situ*, of the contents of the different gastrointestinal sections in rat Groups A, B, C and D. I and II refer to anterior and posterior parts respectively. Mean ± SD are shown, n=5.

Gastrointestinal Section	pH ± SD			
	Group			
	A	B	C	D
Stomach I	2.44 ± 0.84	5.41 ± 0.49	2.27 ± 0.81	4.19 ± 1.08
Stomach II	3.47 ± 1.55	2.33 ± 0.20	2.32 ± 1.10	3.03 ± 0.70
Duodenum I	6.38 ± 0.32	3.70 ± 1.15	4.94 ± 1.26	5.10 ± 1.29
Duodenum II	6.71 ± 0.59	6.11 ± 0.13	6.25 ± 0.14	6.72 ± 0.69
Jejunum I	6.80 ± 0.34	6.18 ± 0.18	6.32 ± 0.16	6.40 ± 0.09
Jejunum II	6.60 ± 0.22	6.59 ± 0.25	6.57 ± 0.25	6.31 ± 0.58
Ileum I	6.63 ± 0.18	6.81 ± 0.55	6.70 ± 0.40	6.57 ± 0.45
Ileum II	7.15 ± 0.54	7.07 ± 0.32	7.21 ± 0.54	6.45 ± 0.71
Caecum I	7.08 ± 0.37	7.01 ± 0.26	5.85 ± 0.14	6.56 ± 0.24
Caecum II	6.90 ± 0.27	6.77 ± 0.29	6.17 ± 0.26	5.87 ± 0.27
Colon I	6.84 ± 0.42	6.91 ± 0.40	5.91 ± 0.38	5.53 ± 0.51

pH directly affects the population of different groups of bacteria, and hence affects the end products of fermentation (Scott, Duncan et al., 2008). Studies with a continuous flow fermenter have shown that a pH value of 5.5 gave fourfold higher butyrate concentrations than a pH of 6.5. This increase of butyrate production was accompanied by acetate consumption. At pH 6.5, there is a decrease in butyrate and a two-fold increase of the propionate production. Other studies with human faecal microbial have shown that lactate is rapidly converted to acetate, butyrate and propionate at pH as low as 5.9, but at pH 5.2 reduced the utilization of lactate while its production was maintained (Belenguer, Duncan et al., 2007).

Bacteroides are the main bacteria involved in the degradation of cyclodextrins (Antenucci and Palmer, 1984), and therefore the abundance of these species in the lower intestine will affect the rate of fermentation of the conjugate at this level. The growth of *Bacteroides* occurs ideally at pH 6.5, coinciding with propionate formation. When the pH is not favorable to *Bacteroides*, pH 5.5 the butyrate producers dominate and leads to formation of butyrate (Walker, Duncan et al., 2005).

Moreover, it is important to note that the rate of production of short chain fatty acids (which influence the pH) are also associated with the rate of transit time. Basically the reduction of SCFA production leads to a slower transit time, related with the increase of absorption as the digesta takes longer to pass through the colon and partially by decrease of bacterial number. (Stephen, Wiggins et al., 1987). Contrarily, the increase of SCFA increases the rate of transit time (Richardson, Delbridge et al., 1991).

4.5.4 *Evaluation of the stability of diclofenac-β-cyclodextrin and sulfasalazine in caecum and colon contents*

The stability of prodrugs in caecal and colonic fluids is shown in Figure 4.8. Moreover Figure 6 indicates that the disappearance of diclofenac-β-cyclodextrin coincides with the appearance of free diclofenac in each release medium; therefore confirming the prodrug is able to liberate the drug in a colonic environment. It can be seen that, in all the animal Groups:

1. Degradation of sulfasalazine is much faster than that of diclofenac-β-cyclodextrin in both milieus. Sulfasalazine is degraded by azoreductases, which are produced by many different bacterial species in the large intestine. The supply of the enzyme azoreductase being almost unlimited, degradation of sulfasalazine can take place without delay, and does not seem to be influenced by the feeding regimen. In fact, the degradation of sulfasalazine was so fast that the degradation profiles have few time points, and curves were not fitted for further analysis of reaction rates and half-lives.

In contrast, the metabolism of cyclodextrin conjugate is much more complex, and involves two types of enzymes - amylase and esterase. The esterase can only act after the amylase has started degrading the cyclodextrin carrier, as reported previously (Hirayma, Ogata et al., 2000). Moreover, the compounds formed in the initial stages of amylase-degradation of cyclodextrin - high-membered maltooligomers (maltohexaose, maltopentaose, maltotetraose) - are themselves substrates for the amylases, and can therefore act as competitive inhibitors of the enzymatic reaction (Suetsugu, Koyama et al. 1974; Jodal, Kandra et al. 1984). In addition, while the lower-membered maltooligomers (glucose, maltose and maltotriose) formed during the reaction are not substrates for the amylase, they can become “non-competitive” enzyme inhibitors by linking to the enzyme protein (Jodal, Kandra et al. 1984). Thus, the diclofenac-cyclodextrin conjugate shows potential as a sustained-release formulation.

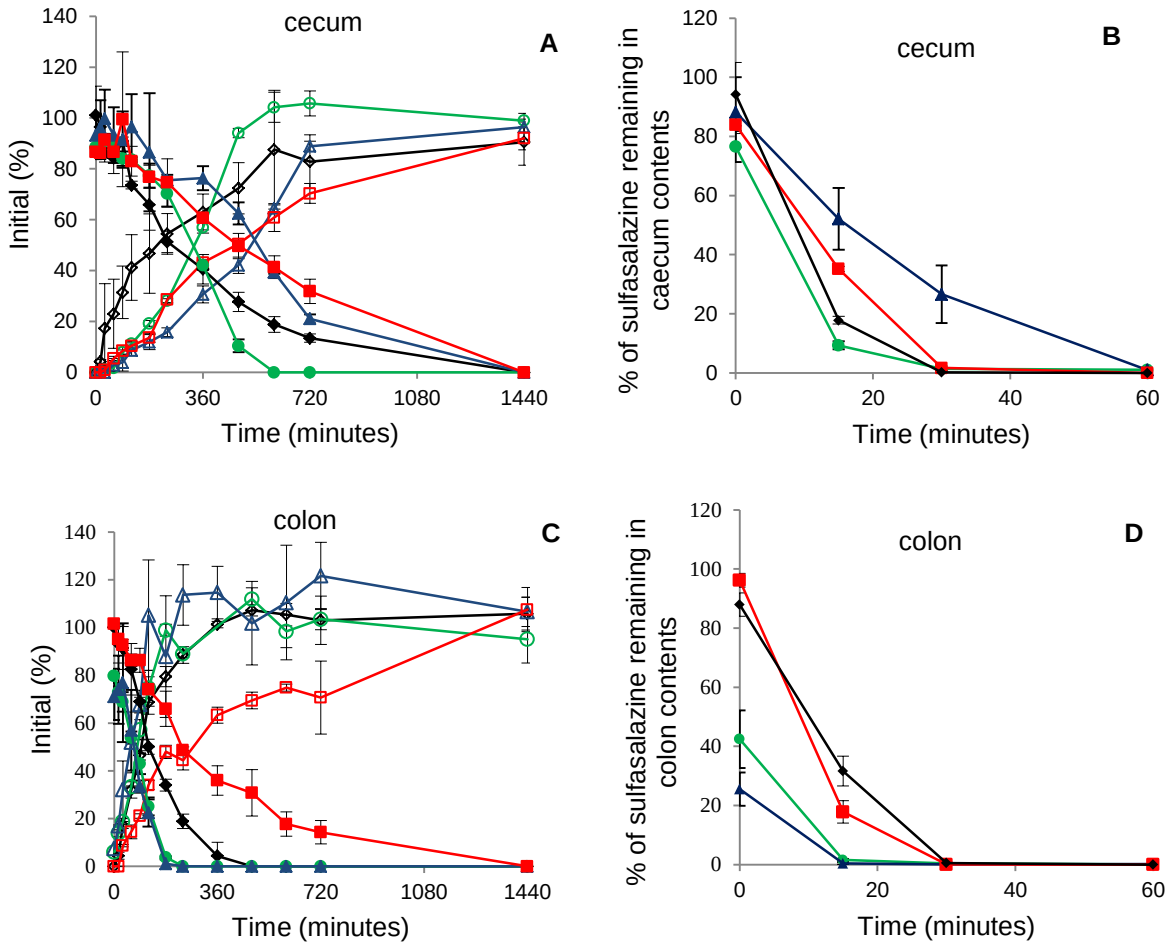


Figure 4.8 Mean levels of diclofenac-β-cyclodextrin and sulfasalazine remaining when prodrugs were incubated in caecal and colonic contents from rats from Groups A (○), B (Δ), C (□), and D (◊). Each point represents mean ± S.D, n = 3.

2. The degradation of both prodrugs is faster in colonic contents as compared to caecal contents (Figure 4.4). This could be due to a number of reasons; such as the caecal slurry having a lower bacterial concentration (due to its greater liquid content), or the caecal slurry being more nutrient-rich (the caecum being the main site of bacterial fermentation), such that less prodrug is metabolized by the bacteria as a source of substrate.

Table 4.4 Degradation rate (k, min^{-1}) and half-life ($t_{1/2}$, min) for diclofenac-β-cyclodextrin in caecal and colonic contents of rat Groups A, B, C and D.

	CAECUM		COLON	
Groups	k (min^{-1})	$t_{1/2}$ (min)	k (min^{-1})	$t_{1/2}$ (min)
A	0.004 ± 0.001	185 ± 22	0.016 ± 0.001	44 ± 2
B	0.002 ± 0.000	397 ± 56	0.013 ± 0.005	60 ± 22
C	0.001 ± 0.000	490 ± 76	0.003 ± 0.001	250 ± 43
D	0.003 ± 0.000	249 ± 27	0.008 ± 0.002	90 ± 16

3. The rate of degradation of CD-drug conjugate by bacterial enzymes was influenced by the feeding regimen in the colon (Figure 4.8). Degradation was fastest from Groups A and B fasted rats, followed by Group D, followed by Group C (Table 1). This order of degradation rates K was statistically significant ($p<0.05$, Kruskal Wallis, followed by Nemenyi's test). The fast prodrug degradation in Group A rats (which had been fasted for 12 hours) could be due to a lack of nutrient in the colon for the bacterial enzymes to act on, which therefore act exclusively on the prodrug, degrading the latter. Similarly, in Group B rats, the colon is nutrient-poor, and exclusive enzyme action on the prodrug leads to the fast degradation of the latter. Although Group B rats were fed for one hour (Figure 4.1), food did not have time to move down to the colon by the time rats were killed, 30 minutes after the one-hour feeding time. In contrast, food and nutrient were present in the colon of Group D fed rats, and competition between nutrient and prodrug for enzyme action led to slower prodrug degradation. The slower rate of prodrug degradation in Group C rats (compared to Group D rats) shows an even greater amount of nutrient in the colon of Group C rats, and hence greater competition of enzyme action. It is possible that the bolus intake of food by Group C rats during the one hour feeding arrives in the colon at some point during the four hours of fasting, such that there is overwhelming competition for the enzyme.

The dilution of the contents from Group A and B were processed at pH 6.8, while the dilution of contents from group C and D at pH 6.0, according to the mean value of the pH measured anteriorly (Table 4.3). Thus these pH differences on the incubation media also affect the activity of enzymes, which can explain the faster metabolism in fasted state.

In the fed state is expected a decrease on the activity of Bacteroids and consequently on the metabolism of cyclodextrin carrier due to the diminuish of pH associated with the production of SCFA, which can also reinforce the explanation associate with the decrease of metabolism in fed state.

Overall, it is interesting to note that the rates of degradation of diclofenac-β-cyclodextrin in colonic contents in Group A and B is close to the rate of degradation observed in the human faecal slurries collected from individuals without any food intake control, as reported previously in Chapter II.

In contrast to the obvious influence of feeding regimen on prodrug degradation in the colon, the influence was less obvious in the caecum. The degradation curves for all rat groups have similar profiles, especially at the beginning of the *in vitro* degradation reaction (Figure 4.8). Caecum has such a high content of material in all rat groups (Figure 4.5) that feeding/fasting did not seem to alter the nutrient content and subsequently, any competition between nutrient and prodrug for enzyme action. One point to note though is the completion of prodrug degradation in Group A fasted rats caecal contents at 600 minutes (Figure 4.8) in contrast to the other groups. This correlates with the fact that Group A fasted rats had a lowest amount of nutrient (and hence competition for enzyme action) in their caecum.

4.6 Conclusion

This study demonstrates the importance of feeding regimens, specifically the timing of meal ingestion, on the gastrointestinal conditions in rats and how this influences the metabolism of colonic prodrugs, namely diclofenac-β-cyclodextrin. In addition to changes in the distribution of gut contents along the GI tract, which directly affects the gastrointestinal transit time, different feeding regimens are accompanied by changes in the pH of gut contents, specifically in the stomach and large intestine. Moreover, differential gut contents in the large intestine have an impact on the microbiota activity, which affects the rate of diclofenac-β-cyclodextrin metabolism, and hence drug release and absorption. We also showed that the different feeding regimens did not seem to im-pact on the metabolism of sulfasalazine, which was rapidly metabolized. Thus, we conclude that while the feeding regimen influences the performance of the colonic prodrug, diclofenac-β-cyclodextrin, that influence has to be measured for each prodrug individually, given the different metabolism pathways of different colonic carriers.

CHAPTER V

DICLOFENAC-β-CYCLODEXTRIN *VERSUS* SULFASALAZINE FOR COLONIC DRUG TARGETING: *IN VIVO* PERFORMANCE IN RATS

5 DICLOFENAC-BETA-CYCLODEXTRIN *VERSUS* SULFASALAZINE FOR COLONIC DRUG TARGETING: *IN VIVO* PERFORMANCE IN RATS

5.1 Overview

The influence of feeding regimen on the performance of diclofenac-β-cyclodextrin was studied previously in rats (Chapter IV). Results showed that its metabolism depends on the feeding regimen, herein fasted state, leading to a faster metabolism of this conjugate.

An *in vivo* proof of concept is required not only to confirm the ability of the conjugate to provide targeting of diclofenac on the large intestine, but also to study the bioavailability of diclofenac when absorption takes place at the colonic level. Based on results from the stability experiments of both prodrugs, and considering the greater variability on the gastrointestinal transit time caused by food intake, oral administration was conducted in the fasted state. The main advantage of conducting these studies under fed conditions would be the elimination of the additional step of food removal, which could reduce potential stress on the animals caused by fasting (Mittelstadt, Hemenway et al., 2005).

Sulfasalazine is a prodrug activated at a colonic level, as previously described, releasing 5-aminosalicylic acid (mesalazine) and sulfapyridine. A delay between the oral intake of sulfasalazine and the appearance of the respective metabolites in the blood circulation is expected. When administered concomitantly with other potential colonic prodrugs, this prodrug works as a control, providing us with an idea of the time necessary for it to reach the colon by the appearance of its metabolites in plasma.

Hence, this Chapter presents a comparative *in vivo* study in fasted rats that considers oral administration of a suspension of prodrugs, diclofenac-β-cyclodextrin and sulfasalazine to a group of rats (Group I), and also the simultaneous quantification analysis of the respective active drugs diclofenac and sulfapyridine in plasma. Moreover, a control group of animals (Group II) was dosed orally with sulfapyridine and diclofenac. The comparison of pharmacokinetic parameters between both groups, namely plasmatic concentrations and time of absorption, allowed determining the ability of the conjugate to release diclofenac in the large intestine. The dose of drugs administrated to Group II was equivalent to the dose of free

drugs administered to Group I; this was usefull to assess differences in the bioavailability between each drug and the respective colonic prodrug.

5.2 Introduction

When administered orally, diclofenac absorption is rapid and complete in humans and in animal models, namely rats (Peris-Ribera, Torres-Molina et al., 1991; Giagoudakis and Markantonis, 1998). Diclofenac is poorly soluble in acidic media (pKa = 4), and therefore its release, dissolution and absorption is favorable when pH is high. On the other hand, when the pH is low, this drug will precipitate or will not be released (Chan, Mojaverian et al., 1990; Riad, Sawchuk et al., 1995). However, diclofenac suffers first-pass metabolism, such that ~50% of the drug reaches the systemic circulation in the unchanged form. The plasma half-life time is between 1 to 2 hours (Davies and Anderson, 1997).

When it reaches the colon, this drug is not degraded and is additionally well absorbed (Tannergren, Bergendal et al., 2008). Nevertheless, when diclofenac reaches the colon, namely in the form of diclofenac-β-cyclodextrin, its absorption will depend on the rate of drug release, which is directly depends on the activity of microbiota. Moreover, in the form of the conjugate, a longer time is expected between the time of oral intake of diclofenac-β-cyclodextrin and the appearance of diclofenac in blood when compared with the administration of free diclofenac.

Sulfasalazine is a well-known colonic prodrug available on the market. When administered orally, approximately 10-30% of the sulfasalazine is absorbed unchanged, with the remaining is cleaved in 5-aminosalicylic acid (mesalazine) and sulfapyridine when reaching the colon. After cleavage, about 60-80% of the available sulfapyridine is absorbed and mainly excreted in the urine, whereas the mesalazine moiety is not so well absorbed being mainly excreted in faeces (Azad Khan and Truelove, 1980; Buggé, Gautam et al., 1990). Therefore, sulfasalasine metabolites can be used as a marker of colonic targeting. Due to the low levels of mesalazine in plasma and consequently the requirement of a more sensitive method for its quantification when compared with sulfapyridine, being this last one used as an indicator of colonic delivery (Lee, Zhang et al., 2012). As reported previously, the first appearance of sulfapyridine in plasma can be used as a marker to determine the transit time through the small intestine (Sjödin, Visser et al., 2011).

As in Chapter IV, the rat was chosen as an animal model to perform the *in vivo* studies. Rat has been used widely as an animal model mainly due to its size and cost effectiveness. Additionally, the mean intestinal transit time in rats seemed comparable with humans in the order of 20 to 30 hours, even though the lengths of their small gut are different (Gruber, Longer et al., 1987; Kararli, 1995; Tuleu, Andrieux et al., 1999) Additionally, rats have been used to performed *in vivo* studies with diclofenac (Reyes-Gordillo, Muriel et al., 2007; León-Reyes, Castañeda-Hernández et al., 2009) and sulfasalazine (Chungi, Dittert et al., 1989 ; Lee, Zhang et al., 2012). They were particularly suited for the determination of pharmacokinetic parameters after oral administration of powder or liquid formulations (Kararli, 1995). Furthermore, rats have also been used to assess the performance of different colonic delivery prodrugs, including those that use cyclodextrins as a carrier.

5.3 Aims and Objectives

This Chapter aims to perform *in vivo* studies in order to:

i) Assess the ability of diclofenac-β-cyclodextrin to release diclofenac into the large intestine using sulfapyridine (sulfasalazine metabolite) as a marker of colonic absorption;

ii) Compare the pharmacokinetic parameters between drugs and prodrugs.

5.4 Materials and methods

5.4.1 *Reagents and chemicals*

Diclofenac Sodium (M_W=318.14 g/mol), sulfasalazine (M_W=398.394 g/mol), sulfapyridine (M_W=249.29 g/mol), and trifluoroacetic acid (TFA) were purchased from Sigma. Diclofenac-β-cyclodextrin (M_W=1411 g/mol) was synthesised according to the method described in Chapter II. Sodium chloride, Microtainer tubes containing K_2EDTA, HPLC grades acetonitrile, methanol and water were purchased from Fisher Scientifics UK Limited. Phosphate buffer pH 7.4 was prepared according to the USPXXIV.

5.4.2 *In vivo study*

5.4.2.1 Animals

Adult male healthy Wistar rats (8 weeks, 240-250 g) were purchased from Harlan Olac Ltd. (Oxfordshire, UK). All the procedures had been approved by the School of Ethical Review Committee and were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. All animals were housed in rooms with controlled conditions: 20 ^{o}C, 40-60% humidity, 15-20 air changes per hour. Animals underwent a period of acclimatization with free access to standard rat chow and water for 7-days prior the experiment. Twelve hours before the beginning of each experiment, animals were housed in separate metabolic cages. The latter were perforated at the bottom, which restricted the ability of animals to eat their own faeces and allowed to collect their urine and faeces, separately. Water was available *ad libitum* through the experiment.

5.4.2.2 Drugs and Prodrugs suspensions

Two different suspensions were prepared freshly just before dosing in sodium chloride 0.9%. One of the suspensions contains diclofenac-β-cyclodextrin (17.7 mg/mL) and sulfasalazine (20 mg/mL) and the other contains sodium diclofenac (4 mg/mL) and sulfapyridine (12.5 mg/mL).

Doses and drug administration schedule of sodium diclofenac (20 mg/kg) and sulfasalazine (100 mg/kg) were selected based on previous reports (León-Reyes, Castañeda-Hernández et al., 2009; Zhang, Zheng et al., 2011; Lee, Zhang et al., 2012). Otherwise, the quantity of diclofenac-β-cyclodextrin (88.5 mg/kg) is equivalent to 20 mg/kg of diclofenac and the quantity of sulfapyridine is the correspondent to 100 mg/kg of sulfasalazine.

5.4.2.3 Experimental design

For this study, rats were assigned to two groups of 7: I (test) and II (control). Rats were fasted overnight (12 hours) and again throughout the first 12 hours of study, with free access to water. The first suspension containing prodrugs was administered to test rats (group I) and the second containing the respective drugs to control ones (group II). Oral administration (1.2

mL) was performed using a polypropylene syringe (3 mL) equipped with a 18-G ×3 curved gavage needle with 2.25-mm for p.o. in rats.

5.4.2.4 Blood sampling

Blood samples (250 - 300 µL) were collected via the tail after cutting 2 - 3 cm of the tail with a scalpel, accompanied with massage under anesthesia. As drops of blood appeared, they were collected into 500 µL microtainer tubes. The original wound in the tail was reopened by removing the clot to continue blood sampling at pre-determined time points, which were 30, 90, 120, 180, 240, 360, 480, 720 and 1440 minutes, for rats in group I (test) and 10 ,30, 45, 90, 120, 240, 360, 480, 720 and 1440 minutes in group II (control).

Plasma was obtained after centrifugation of blood samples at 13 000 rpm for 10 minutes at 4 ºC (Thermo Scientific Heraeus Fresco 17 refrigerated microcentrifuge). Plasma were transferred to labeled eppendorfs and kept at -20 ºC until analysis.

5.4.2.5 Sample preparation

Frozen plasma samples (100 µL) were allowed to thaw at room temperature. 100 µL of acetonitrile with 2.5 % (v/v) of KOH 0.2 M was added to the plasma sample and the mixture was mixed vigorously on a vortex mixer for 10 seconds. Samples were then centrifuged for 10 minutes at 10 000 rpm at 4 ºC. Subsequently, 100 µL of the supernatant were diluted with 20 µL of water and analyzed by HPLC.

5.4.3 *Simultaneous quantification of diclofenac and sulfapyridine in plasma by high-performance liquid chromatography with UV detection*

Diclofenac and sulfapyridine analysis was done by high-performance liquid chromatography (HPLC) using an Agilent 1100 series system equipped with a UV detector. Results were acquired and processed with the Agilent Chemstation Data System Software 7. HPLC analysis was conducted by using a XTerra reverse phase C-18 column with 5 µm particle size, 4.6 mm internal diameter and 250 mm length. Sample injection of 50 µL, detection wavelength 273 nm, flow rate of 1 mL/min at 40 ºC and a total run time of 24 minutes. A gradient system of 0.1% TFA in water (A) and acetonitrile (B) was followed: 0-15 minutes, 11-80% (B), 15-22 minutes 80-22% (B), 22-24 minutes 22-11% (B).

A stock solution of sodium diclofenac (50 mg/100mL) and sulfapyridne (50 mg/100 mL) was prepared adapting the recipe described by Doxsee *et al.* (Doxsee, Gout et al., 2007) following the next steps. First, 50mg of sulfapyridine was dissolved in 5 mL of NaOH 0.1 M under stirring. When completely dissolved, 70 mL of phosphate buffer pH 7.4 was added continuously under stirring. After the decrease in pH, 100 µL of HCl 2 M was then added to the solution followed by addition of 53.7 mg of sodium diclofenac equivalent to 50 mg of diclofenac free acid. Finally, phosphate buffer pH 7.4 was added to make up the solution to 100 mL. Appropriate dilutions of the stock solution in phosphate buffer 7.4 were performed in order to obtain standard working solutions with the next concentrations: 2.5, 5, 8, 10, 30, 50, 150 and 200 µg/mL. All these solutions were refrigerated at - 4 ºC and protected from light.

Appropriate solutions of diclofenac and sulfapyridine in plasma were prepared in the range of 0.25 µg/mL to 20 µg/mL. 90 µL of blank plasma was spiked with 10 µL of standard working solutions, followed by the extraction of both drugs as described above (2.2.2). Plasma was obtained by centrifugation of blood collected by cardiac puncture from rats at 13 000 rpm for 10 minutes at 4 ºC and keep in the freezer at -20ºC.

5.4.3.1 Validation of the analytical method

The optimized analytical method was validated with the assessment of selectivity, linearity, accuracy, precision, recovery, limits of detection (LOD) and lower limit of quantification (LLOQ).

- ***Selectivity***

To evaluate the **s**electivity, the capacity of the method to differentiate and quantify diclofenac and sulfayridine in presence of the other components of the plasma, blanks samples of plasma were run using plasma from different rats (n=6).

- ***Limits of detection (LLOD) and quantification (LLOQ)***

Separate plasma samples of low concentrations were prepared to investigate the LLOD, which is as the lowest concentration of diclofenac and sulfapyridine that could be detected but not necessarily quantified in the plasma. It was determined by visual evaluation of the minimum level at which the analytes can be reliably detected. LLOQ is defined as the lowest

concentration of diclofenac and sulfapyridine quantifiable with suitable precision and accuracy. LLOQ allows evaluating the sensitivity of the method.

- ***Linearity***

The linearity of the method was verified using diclofenac and sulfapyridine concentration range of 0.25 µg/mL to 20 µg/mL, prepared in plasma as described before.

- ***Accuracy and precision***

Precision indicates the closeness of agreement (i.e., the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample) and was determined by repeatability (intra-day) and intermediate precision (inter-day) for three consecutive days. Precision was calculated by the relative standard deviation [R.S.D.(%) = (standard deviation/average concentration)*100], which should be lower than 15%.

The accuracy of the method expresses the closeness of agreement between the nominal value and the value observed, calculated by the coefficient of variation [C.V.(%) = (observed concentration-nominal concentration)/nominal concentration], which must be within 15%.

The inter-day precision and accuracy of the method was assessed by analysing four different plasma concentration levels of 0.25, 1, 5 and 20 µg/mL on 3 different days, while the intra-day precision was measured using 6 determinations per concentration within the same day.

Recovery

Extraction efficiencies for diclofenac and sulfapyridine in plasma were determined by comparing replicate (n=6) peak area of extracted plasma samples to those obtained with non-plasmatic standards with the same concentration for the LLOQ 0.25 µg, intermediate concentration 1 µg/mL and high concentration 20 µg/mL. The extraction efficiency was determine by calculating the percentage recoveries [recovery (%) = (peak area of plasma standard) / (peak area of water standard)*100].

5.4.3.2 Pharmacokinetics analysis

Diclofenac and sulfapyridine concentrations in plasma (μg/mL) as a function of the time of administration (in hours) was plotted for both groups I and II. The maximum diclofenac and sulfapyridine concentration in the plasma (C_{max}) is the arithmetic mean of 7 rats, whereas the corresponding time (t_{max}) was read directly from the graph. The area under the diclofenac and sulfapyridine plasma concentration-time curve ($AUC_{0\text{-}24\ hours}$) was calculated using OriginPro 9.0.

5.4.3.3 Statistical analysis

Statistical analysis was performed using SPSS 21.0 for Windows®. Data was analysed using parametric tests. Maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}) and area under curve ($AUC_{0–24\ hours}$) for diclofenac and sulfapyridine between groups I and II were compared using the Student *t*-test.

5.5 Results and discussion

5.5.1 *Development of a HPLC method*

In order to investigate the behavior of sulfasalazine and conjugate after oral administration, the development of a suitable and validated method able to quantify the main compounds absorbed in the blood was necessary. Moreover, both drugs are metabolized in the liver, resulting in the formation of the corresponding metabolites which can also be considered for the development of the analytical method (Roškar and Lušin, 2012). Therefore, the choice of a proper analytical method to quantify drugs and/or their metabolites plays a significant role in the interpretation of pharmacokinetic data.

The most frequent method for quantitative determination of medicines in plasma is HPLC analysis coupled to different detectors such as mass spectrometry or a UV detector. Although some drawbacks are associated with the lack of sensitivity of the UV detector, namely when low concentrations need to be measured (Chmielewska, Konieczna et al., 2006), HPLC with UV detection has been widely used in quantification of diclofenac in plasma or serum samples from humans (Giagoudakis and Markantonis, 1998; Idkaidek, Amidon et al., 1998; Billa, Yuen et al., 2000; Su, Chou et al., 2003; J., N. et al., 2007; Lissy, Scallion et al., 2010;

Sarfraz, Sarfraz et al., 2011) and from rats (Yong, Oh et al., 2005; Huntjens, Strougo et al., 2008) (Tammara, Narurkar et al., 1994; Tabata, Yamaoka et al., 1995; Reyes-Gordillo, Muriel et al., 2007; Pathan, Karwa et al., 2010). Otherwise, HPLC techniques for the quantification of sulfasalazine, sulfapyridine and aminosalicylic acid had been reported for analysis of plasma samples from rats (Lanbeck and Lindström, 1978; Chungi, Rekhi et al., 1989; Yuen, Peh et al., 1997) and also from human serum (Buggé, Gautam et al., 1990; Lee, Waller et al., 2010).

However, until now, no technique for simultaneous analysis of these analytes have been reported. Herein, a new method for quantification of both prodrugs and respective metabolites in plasma was developed.

Regarding diclofenac-β-cyclodextrin, this prodrug is not absorbed into the blood, given the fact that cyclodextrins are poorly absorbed through biological membranes including those of the gastrointestinal tract, due to their high M_W (ranging from almost 1000 to > 2000 Da) (Loftsson, Jarho et al., 2005). Moreover, as *in vitro* studies showed, when conjugate reaches the colon, the degradation of cyclodextrin releases the drug directly without formation of any other intermediate compound resulting from cyclodextrin metabolism. Hence, diclofenac is the main compound that will be absorbed into the plasma and therefore quantified.

Otherwise, when sulfazalasine is administered orally, 15% is absorbed intact from the small intestine into the bloodstream without any activation. Neverthless, some of this will return to the intestine through the bile enterohepatic circulation. The great majority of this prodrug reaches the colon where the azo bond is cleaved, producing sulfapyridine and mesalazine (Lee, Waller et al., 2010). Mesalazine is poorly absorbed at the level of the colon, and as reported previously, it is not possible to quantify this in plasma after oral administration of 100mg/kg (Lee, Zhang et al., 2012). In other hand, sulfapyridine is well absorbed when administered to rats (Lee, Zhang et al., 2012), and was considered in the development of the method as the marker of sulfasalazine activation in the colon.

Initially, the HPLC method was developed to include the quantification of diclofenac, sulfasalazine and sulfapyridine in plasma. Due to the fact the metabolites resulting from drug hepatic metabolism are not available commercially, they were not quantified. Chromatographic conditions were optimized using extracted spiked blank plasma with the

analytes, and herein the resultant chromatograms showed a good resolution of the peaks. Afterwards, when the method was applied to extract plasma samples after oral administration of sulfasalazine and diclofenac-β-cyclodextrin in rats, chromatograms did not show any background interference at the retention time of sulfapyridine and diclofenac. However, chromatograms revealed some peak interference for the retention time of sulfasalazine, which corresponds to metabolites produced. Thus, due to the lack of time to continue the development of other methods able to quantify sulfasalazine without any interference, the HPLC method established was only towards the analysis of diclofenac and sulfapyridine.

Extraction conditions:

Given the small quantity of blood and consequently of plasma, the combination of the method of extraction, volume of injection and wavelength were determinedto allow good reproducibility and precision at low levels of concentration of analytes in plasma. Sample preparation is commonly carried out by (a) liquid-liquid extraction, (b) solid-phase extraction or (c) precipitation of plasma proteins (Ashri and Abdel-Rehim, 2011). Between these techniques, protein precipitation is a very simple, fast and straightforward method widely used to extract drugs from plasma. Acetonitrile is considered to be the most effective solvent for disrupting protein binding (Telepchak, August et al., 2004).

Initially, the ability of two water-miscible solvents, acetonitrile and methanol, was tested for protein precipitation. Results demonstrated that acetonitrile is the best organic plasma protein precipitant, particularly at a 1:1 (precipitant/plasma) volume ratio to extract diclofenac. However, in the case of sulfapyridine, acetonitrile did not provide reproducible results, namely for low concentrations of the drug in plasma. Consequently, acetonitrile alone was not enough to concomitantly extract both analytes, and further studies were performed. Sulfapyridine is a basic drug (pKa = 8.4), hence the addition of base helps its extraction into an organic solvent due to disruption of protein binding by eliminating the charge of the molecule (Prabu and Suriyaprakash, 2012). Thus, various concentrations of bases in acetonitrile were tested, namely NH_4OH and KOH, and it was found that the addition of 2.5% of KOH 0.2 M was a suitable choice to extract sulfapyridine from plasma**.** At the same time, the extraction of diclofenac is not affected by the presence of base in the solvent of extraction.

To avoid peak distortion in chromatograms, samples of the extracted solutions were diluted with water just before injection, allowing for reproducibility of the shape of the peak.

A representative chromatogram of blank plasma is shown in Figure 5.1 A.

A typical chromatogram produced by the developed HPLC method for the standard solutions of sulfapyridine and diclofenac at their limit of quantification is shown in Figure 5.2-B and Figure 5.3-C. Retention times for sulfapyridine and diclofenac were 5.8 and 16.2 minutes, respectively.

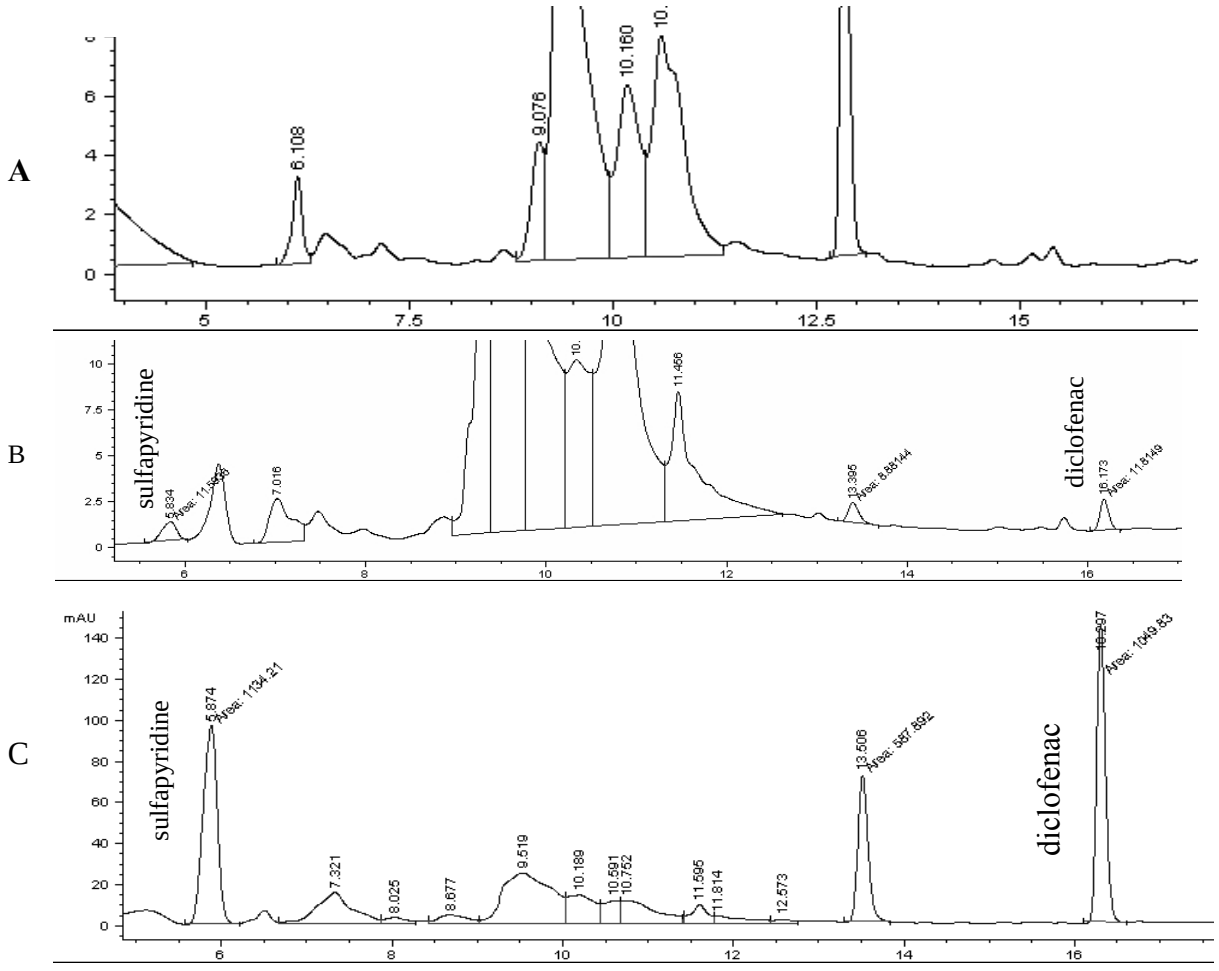


Figure 5.1 HPLC chromatograms of the (A) blank plasma, (B) spiked plasma at levels of low limit of quantification, (C) spiked plasma at levels of the upper limit of the calibration ranges.

5.5.2 *Validation of the analytical method*

For validation of the method, the main regulatory document from the United States Food and Drug Administration for Bioanalytical Method Validation (FDA 2001) was followed (Fda, 2001).

Eight-point calibration curves were constructed for sulfapyridine and diclofenac in plasma. Results of regression indicated good linearity (R^2 = 0.999) for both molecules (Table 5.1) in the wide concentration ranges of 0.25-20 µg/mL, previously selected considering the drugs concentrations observed in previous *in vivo* studies (Reyes-Gordillo, Muriel et al., 2007; León-Reyes, Castañeda-Hernández et al., 2009; Zhang, Zheng et al., 2011; Lee, Zhang et al., 2012).

Table 5.1 Regression statistics for diclofenac and sulfapyridine.

Analyte	Range (µg/mL)	*N*	slope	intercept	Correlation coefficient
Diclofenac	0.25-20	4	46.338±2.843	0.540±0.360	0.999±0.001
sulfapyridine	0.25-20	4	49.7935±0	-0.315±0.961	0.999±0.000

The accuracy values obtained for the four different fortification levels ranged from 2.65 and 11.81% for diclofenac, and between 0.85 and 2.20% for sulfapyridine.

Intra-day precision values ranged from 2.50% to 8.23 %, whereas the inter-day precision values were below 5%. The intra-day precision values ranged from 3.32 and 9.14% for diclofenac, and from 2.83 and 8.46% for sulfapyridine, whereas the inter-day precision ranged from 2.53 and 6.00% for diclofenac and from 2.71 and 7.87% for sulfapyridine. These results confirmed the precision and accuracy of the analytical method optimized in this study, which are in accordance with acceptance criteria described by FDA.

A colon-specific drug delivery system should be able to minimize drug release in the stomach and small intestine. In order to be certain of this, the analytical method used for the quantification of the drug must have low limits of detection and quantification. The LOD and LOQ of the method used in the present study were 0.05 and 0.25 µg/mL, respectively for both analytes, which is in line with previous studies for diclofenac and sulfapyridine (Zhang, Zheng et al., 2011; Lee, Zhang et al., 2012).

Table 5.2 Intra- and inter-day precision, accuracy, limit of detection and limit of quantification of diclofenac and sulfapyridine in rat plasma.

Concentrations µg/mL	**Precision (R.S.D., %)**		**Accuracy (AR, %)**	**LLOD µg/mL**	**LLOQ µg/mL**
Diclofenac	Intra-day	Inter-day	Intraday		
20	3.32	2.53	2.65		
5	3.94	3.08	3.17	0.1	0.25
1	9.14	6.00	11.81		
0.25	5.52	5.72	5.55		
Sulfapyridine	Intra-day	Inter-day	Intraday		
20	3.29	5.06	-2.9		
5	2.83	2.71	-0.85	0.1	0.25
1	6.02	6.09	1.46		
0.25	8.46	7.87	2.20		

The percentage recoveries (see Table 5.3) were 98.76%, 81.23% and 74.85% for the diclofenac 88.01%, 87.15% and 73.02% for the sulfapyridine at concentrations of 20 µg/mL, 1 µg/mL and 0.25 µg/mL, respectively.

Table 5.3 Recovery percentage of diclofenac and sulfapyridine from rat plasma.

Nominal concentration (µg/mL)	**Diclofenac**		**Sulfapyridine**	
	Recovery (%)	RSD	Recovery(%)	RSD
0.25	98.76	7.23	88.01	3.01
1	81.23	15.92	87.15	8.72
20	74.85	14.85	73.02	15.32

5.5.3 *Diclofenac and sulfapyridine plasma levels*

The mean plasma concentration–time profiles of diclofenac and sulfapyridine for the rats dosed orally with diclofenac-β-cyclodextrin (88.5 mg/kg) and sulfasalazine (100 mg/kg) in group I (test) are shown in Figure 5.2.

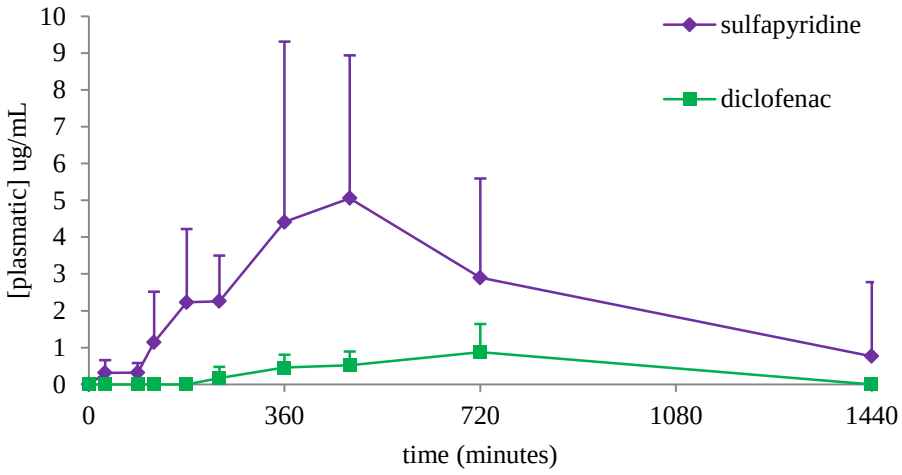


Figure 5.2 Concentration-time profiles (n=7) of diclofenac and sulfapyridine in Wistar rats after simultaneous oral administration of diclofenac-β-cyclodextrin (88.5 mg/kg) and sulfasalazine (100 mg/kg). Each point represents mean ± S.D., n = 7.

Figure 5.3 shows the diclofenac and sulfapyridine plasma concentrations-time profile obtained after oral administration of sodium diclofenac and sulfapyridine in a dose of 20 mg/kg and 62.5 mg/kg in group II.

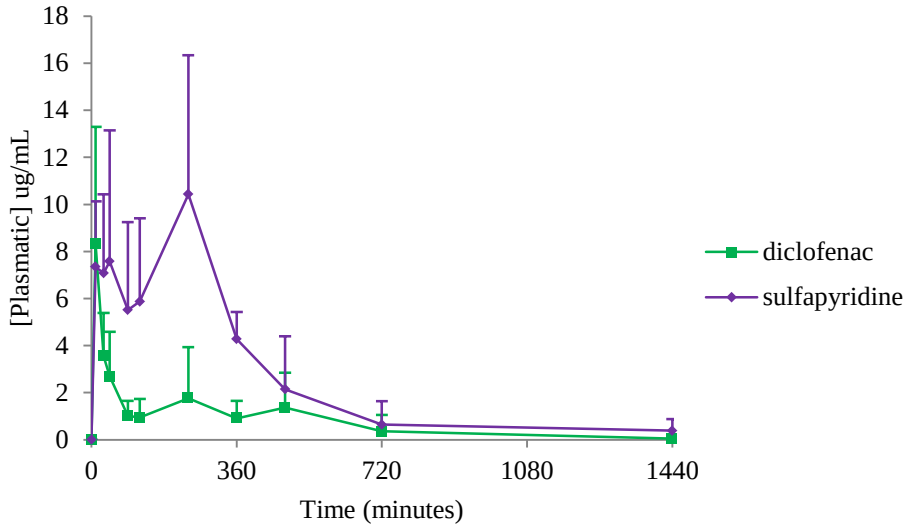


Figure 5.3 Concentration-time profiles (n=7) of diclofenac and sulfapyridine in Wistar rats after simultaneous oral administration of sodium diclofenac (20 mg/kg) and sulfapyridine (62.5 mg/kg). Each point represents mean ± S.D., n = 7.

The mean and the individual pharmacokinetics parameters: $AUC_{0-24\ h}$, C_{max}, and T_{max} are reported in Table 5.4 and for diclofenac and sulfapyridine, respectively.

Table 5.4 Pharmacokinetic parameters of diclofenac following oral administration of diclofenac-β-cyclodextrin (group I) and sodium diclofenac (group II) to rats (n=7).

Diclofenac											
Group	Parameter	Rats							Mean	SD	Range
		I	II	III	IV	V	VI	VII			
I	Cmax (µg/mL)	0.59	1.65	1.68	1.19	0.51	1.68	0.41	1.10	0.59	0.41-1.68
	tmax (hours)	8.00	12.00	12.00	6.00	12.00	12.00	8.00	10.00	2.58	6.0-12.0
	AUC(0-24h) (µg h/mL)	6.09	14.88	18.09	5.52	5.55	16.01	1.72	9.69	6.44	1.72-18.09
II	Cmax (µg/mL)	9.14	16.08	10.60	9.44	9.06	1.97	1.93	8.32	4.98	1.93-16.08
	tmax (hours)	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.00	0.17
	AUC(0-24h) (µg h/mL)	20.43	14.86	16.98	13.31	31.14	26.00	9.15	18.84	7.63	9.15-31.14

Table 5.5 Pharmacokinetic parameters of sulfapyridine following oral administration of sulfasalazine (group I) and sulfapyridine (group II) to rats (n=7).

Sulfapyridine											
Group	Parameter	Rats							Mean	SD	Range
		I	II	III	IV	V	VI	VII			
I	Cmax (µg/mL)	5.33	4.78	14.20	4.32	4.04	7.98	7.21	6.84	3.57	4.04-14.20
	tmax (hours)	24.00	3.00	6.00	12.00	8.00	8.00	8.00	9.86	6.79	3.0-24
	$AUC_{(0-24h)}$ (µg h/mL)	56.20	40.52	85.72	54.45	58.33	94.77	25.45	59.35	24.11	25.45-85.72
II	Cmax (µg/mL)	18.77	19.29	14.45	9.51	11.95	7.60	7.85	12.77	4.89	7.60-19.29
	tmax (hours)	4.00	0.75	4.00	4.00	0.17	0.17	1.50	2.08	1.85	0.17-4
	$AUC_{(0-24h)}$ (µg h/mL)	68.60	51.83	64.47	48.83	79.76	55.35	63.53	61.77	10.71	48.83-79.76

In group I (test), as it was expected considering that sulfasalazine and the diclofenac-β-cyclodextrin are both specific colonic prodrugs, a delay in the appearance of the respective drugs, diclofenac and sulfapyridine, in plasma was observed. The lag time between dosing of diclofenac-β-cyclodextrin and the appearance of diclofenac in plasma was 4 hours, and the maximum peak in plasma was reached after 10.00 ± 2.58 hours (t_{max}) of administration (C_{max} 1.10 ± 0.59 μg/mL). In case of sulfasalazine (group I), the maximum peak of sulfapyridine is reached at 9.86 ± 6.79 hours (t_{max}) with a C_{max} of 6.84 ± 3.57 μg/mL.

In contrast, in group II, no lag time was observed between oral intake of drugs and their appearance in blood. The maximum peak plasma concentrations of diclofenac occurred fairly rapidly after oral administration (C_{max} of 8.32 ± 4.98 μg/mL at 10 minutes post-administration), but also declined rapidly thereafter, maintaining low plasmatic levels until 12 hours post-dose. Also in the case of sulfapyridine, its appearance in plasma occurred immediately after administration. The maximum peak of sulfapyridine was obtained earlier at 2.08 ± 1.85 hours (T_{max}) following administration with a C_{max} of 12.77 ± 4.89 μg/mL. In this case, the appearance of sulfapyridine in plasma occurred immediately after administration.

The mean $AUC_{(0\text{-}24h)}$ values obtained with administration of each prodrug (group I) and the respective drug (group II) are 9.69 ± 6.44 μg h/mL and 18.84 ± 6.73 μg h/mL, respectively in the case of diclofenac, and 59.35 ± 24.11 μg h/mL and 61.77 ± 10.71 μg h/mL in the case of sulfapyridine.

In this study, *in vivo* proof of concept was obtained in rats for colonic target release of diclofenac-β-cyclodextrin. The pharmacokinetics profiles obtained are very different between prodrugs and the respective drugs.

Following administration of diclofenac, and based on previous reported studies, its plasmatic profile (Figure 5.3) is related to the low half-life time of diclofenac, and with the irregular absorption of this poorly soluble molecule (Zhang, Zheng et al., 2011). Otherwise, following the oral administration of sulfapyridine suspension, it was expected that it dissolved mostly in stomach according to its pKa value of 8.4. However, its plasmatic profile indicates a slow absorption, which may be due to the low solubility of this molecule in water < 0.1g/100mL.

When administered in the form of prodrugs, both drugs suffered a decrease in the C_{max}, as compared to the free drug. However, this decrease was shown to be sharper in case of

diclofenac. These results showed a decrease of 87% in the maximum plasma diclofenac concentration (*t*-test, $p < 0.01$**),** and a significant increase in the mean time required to reach the maximum level (*t*-test, $p < 0.01$**)** following diclofenac-β-cyclodextrin administration compared with diclofenac sodium administration. There was 53% decrease in the maximum plasma sulfapyridine concentration (*t*-test, $p < 0.05$) and a significant increase in the mean time required to reach the maximum level (*t*-test, $p < 0.05$) following sulfasalazine administration relative to sulfapyridine administration.

Although the plasmatic t_{max} of drugs after administration of prodrugs is not significantly different for sulfapyridine and diclofenac (*t*-test, $p > 0.05$), the lag time for the appearance of diclofenac in blood was longer than that for sulfapyridine. This fast appearance of sulfapyridine was in agreement with results reported previously that had shown that appearance of sulfapyridine in plasma was rapid (~5 min) following direct instillation of sulfasalazine in the caecum in the fasted state in humans (Kellow, Borody et al., 1986). It could be argued that these results are also in accordance with *in vitro* ones that had shown the slow rate degradation of diclofenac-β-cyclodextrin when compared with sulfasalazine (Chapter III and IV).

Relative to the $AUC_{0\text{-}24h}$, there was no significant change in bioavailability for sulfapyridine (*t*-test, $p > 0.05$) between groups I and II, though the same was not observed for diclofenac. In the case of diclofenac, after administration of the diclofenac-β-cyclodextrin the bioavailability was about half of the bioavailability when the free drug was administrated (*t*-test $p < 0.05$).

Different explanations can be given for the disparity of $AUC_{0\text{-}24h}$ between both prodrugs (Figure 5.4), including the slow rate of degradation of diclofenac-β-cyclodextrin as compared with sulfasalazine at the level of the large intestine in rats, as shown in previous *in vitro* studies (Chapter III and IV). This slow degradation can be associated with the small volume of watery environment necessary to guarantee the dissolution of the diclofenac-β-cyclodextrin and release of diclofenac to the lumen of the colon for subsequent absorption (Gleiter, Antonin et al., 1985; Schiller, Fröhlich et al., 2005). The quantity of free water available in gut contents is otherwise too small compared with the quantity present in the *in vitro* media used to perform stability experiments.

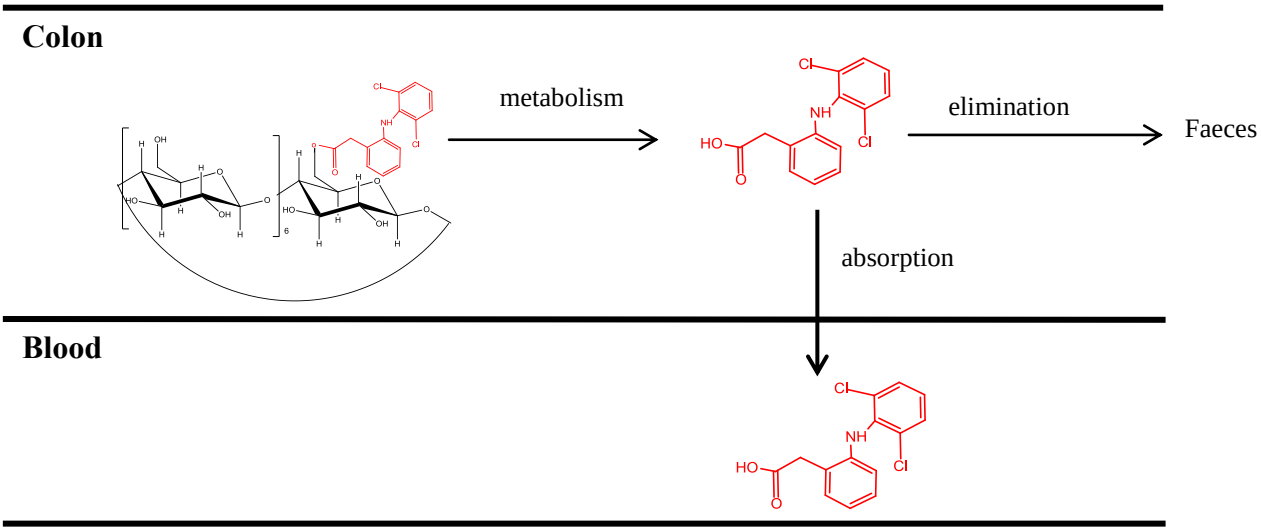


Figure 5.4 Schematic representation of the hipothesis that can explain the diminuished of plasmatic bioavailability of diclofenac when this is administered in the form of cyclodextrin prodrug.

Otherwise, it appears that the residence time of the diclofenac-β-cyclodextrin and/ or diclofenac in colonic contents of rats is insufficient for the diclofenac-β-cyclodextrin to be completely metabolized and/or for the drug released to move to the lumen and to be absorbed.

Rats have a large caecum, though the colon is neither sacculated nor long, unlike in humans. (Kararli, 1995). Indeed, humans have a poorly define caecal region, which is continuous with the colon. Additionally, the human colon is sacculated and consists of ascending, transverse and sigmoidal segments. Based on these anatomic differences between rats and humans, a different bioavailability of diclofenac released from the conjugate is expected between both species. As reported, the absorption in humans is much faster, and also to a greater extent than in rats due to these anatomic differences (Desesso and Jacobson, 2001).

In order to clarify the pharmacokinetic parameters, a longer duration of sampling would be necessary to correctly estimate the extrapolated area under the curve (Yáñez, Remsberg et al., 2011). According to the literature, as long as the conjugate is completely degraded, diclofenac will be well absorbed (Gleiter, Antonin et al., 1985; Billa, Yuen et al., 2000; Schiller, Fröhlich et al., 2005; Tannergren, Bergendal et al., 2008). Thus, as long as the diclofenac-β-cyclodextrin is completely degraded, the amount of diclofenac released could be absorbed in its totality.

5.6 Conclusion

These results confirm the *in vivo* ability of this diclofenac-β-cyclodextrin to target and release diclofenac in the colon. The lag time of appearance in blood of diclofenac with a delay in the t_{max} as observed for sulfapyridine demonstrated the colonic release of diclofenac from the diclofenac-β-cyclodextrin. However, results demonstrated that the bioavailability of diclofenac decreases by half when administered in its prodrug form. Contrarily, the bioavailability of sulfapyridine is not altered when administered in the form of sulfasalazine, as compared with the administration of free drug. These differences can be associated with slow metabolism of diclofenac-β-cyclodextrin when comparing with sulfasalazine, or can be related with limitations on the time necessary for complete absorption of diclofenac.

Overall, this study confirms the *in vivo* ability of this newly cyclodextrin prodrug to target and release diclofenac specifically in the colon.

CHAPTER VI

GENERAL DISCUSSION AND FUTURE WORK

6 GENERAL DISCUSSION AND FUTURE WORK

6.1 General Discussion

This dissertation describes the development of a new oral prodrug for colonic specific delivery of diclofenac. A diclofenac-β-cyclodextrin conjugate was successfully synthetised and characterised. Additionally, *in vitro* and *in vivo* experiments allowed a further understanding of its behaviour along the GI tract and to assess its potential to target and release diclofenac specifically into the colon.

The goal of developing a new system to target the colon exploited the idea of utilising cyclodextrins as a carrier for colonic target delivery. These oligosaccharides, mainly α- and β-cyclodextrins, remain intact in the upper GI tract and are enzymatically degraded exclusively at a colonic level. Thus, using cyclodextrin as a carrier, the idea of creating a prodrug for colon-specific delivery of diclofenac emerged.

This project started with the investigation of a method suitable for the synthesis of diclofenac-cyclodextrin conjugates. This became a big challenge due to the high amount of unsuccessful results faced whilst exploring conventional synthetic routes that in theory would be able to create an ester linkage between diclofenac and cyclodextrin. Beyond all the reactive hydroxyl groups of cyclodextrin that are available to react, diclofenac was shown to be very sensitive and easily suffered an intramolecular reaction, which limits its intermolecular reactivity. After, the study of different conventional approaches using different conditions, the nucleophilic route performed under microwave irradiation allowed for the successful and efficient production of the diclofenac-β-cyclodextrin conjugate. This process assured reproducible synthesis of the conjugate with the advantage of drastically decreasing the reaction time when compared to the conventional eating method. The novelty and innovation associated with this process of preparation of a diclofenac-cyclodextrin conjugate prompted the need for protecting the intellectual property of this work by a patent.

After overcoming the first challenge related to the synthesis process, the next step concerned carrying out studies which demonstrated the ability of a cyclodextrin to remain intact in the upper GI tract and carry a drug to the colon when both were covalently linked. Different

stability studies were designed and performed, namely using simulated gastrointestinal fluids and gastrointestinal fluids collected from different animals (rat, rabbit and pig). The prodrug demonstrated resistance to both chemical and enzymatic degradation in the upper GI tract. Otherwise, at the level of the lower intestine, the conjugate suffered degradation with simultaneous release of diclofenac. Parallel studies with sulfasalazine were carried out in order to compare its performance with diclofenac-β-cyclodextrin. Results demonstrated that sulfasalazine metabolism was always faster than the metabolism of the conjugate. This leads to the conclusion that the mechanism subjacent to sulfasalazine degradation is faster than the mechanism that allows for release of a drug from a cyclodextrin.

The slowest release of the drug from the cyclodextrin prodrug can be attributed to the fact that this conjugate requires the intervention of two different enzymes: amylases and esterases. Although we have an ester prodrug, this is not degraded by esterases in the upper GI tract. It seems that cyclodextrin creates a steric hinderance around the ester linkage, which prevents esterase to attack. Firstly, cyclodextrin has to be degraded, which just occurs at colonic level, and after that, enough space is generated allowing esterase to attack with consequent breakdown of the ester linkage, which coincides with the release of diclofenac.

Thereafter, studies were conducted in an attempt to understand the influence of feeding regimen on the physiological conditions and metabolism of colonic prodrugs. Studies have previously shown that different feeding regimens affect dramatically the physiological conditions in the gastro-intestinal tract. These observations have therefore an influence on the metabolism of pro-drugs and subsequently expected to interfere with the drug disposition. In fact, the metabolism of diclofenac-β-cyclodextrin was faster in fluids collected from fasted rats. The ideal colonic prodrug would present a rate of metabolism not affected by the changes on the microbiota activity; in other words, its metabolism would not be affected by the presence or absence of food.

This thesis ended with *in vivo* studies conducted to show how the conjugate behaves after oral administration in rats, using sulfapyridine as a marker of colonic absorption. These studies represented a proof of concept for the colonic target delivery of diclofenac-β-cyclodextrin, and release of diclofenac. This was evidenced by the lag time observed for diclofenac appearance in plasma after oral delivery of conjugate, similar to that observed for sulfapyridine after administration of sulfasalazine. Comparison of the plasmatic

bioavailability of diclofenac after oral administration of the prodrug and the bioavailability after oral intake of the own drug showed that the plasmatic bioavailability is reduced to half when diclofenac was administered in the conjugate form.

Therefore, these results led to the conclusion that diclofenac-β-cyclodextrin allows for release of diclofenac at a colonic level. Although diclofenac is well absorbed, some factors are present which prevent its complete absorption. The decrease of plasmatic concentration can be associated with excretion of the intact conjugate, and therefore its incomplete metabolism. Additionally, at colonic level, the viscous contents/mucous with small volumes of fluid create difficulty for diclofenac dissolution, and consequent difficulty in reaching the colonic mucous to be further absorbed. These hypotheses need to be explored in order to assess the reason for the different blood concentration between drug and prodrug.

Overall, this work provided a proof of concept of the ability of diclofenac-β-cyclodextrin to release diclofenac at a colonic level. Given the short half life time of diclofenac in plasma, this prodrug can be considered as an useful chronopharmacological therapeutic agent for the management of arthritis pain. Moreover, the concomitant formulation of this prodrug with free diclofenac and its oral administration assures sustained therapeutic blood levels of diclofenac, avoiding the repeated daily dosing of diclofenac.

It is expected that this anti-inflammatory molecule in the form of a colonic prodrug causes less mucosal irritation when compared with the free drug, due to its lower direct contact with the mucous. This can be expected due to its low affinity for the lipidic membranes, not only due to the large size of the molecule, but also due to the hydrophilic nature of the cyclodextrin surface.

Moreover, the cyclodextrin carrier brings health benefits related to its fermentation that produce short-chain fatty acids (SCFA) - important for the maintenance of the health and integrity of the colonic epithelium.

6.2 Future work

Whilst carrying out this Project, a number of ideas were identified and can be further investigated. Some ideas are the direct result of the conducted research described in this thesis that are related to development of the synthetic process for producing diclofenac-cyclodextrin

conjugates and investigation of its performance after oral intake as a colonic-specific prodrug. The last ideas are associated with the potential pharmacological advantages.

Potential topics to further develop are outlined below:

Although diclofenac-β-cyclodextrin was produced using a reliable method with a high degree of purification, more needs to be done in order to optimize its yield. The first idea that arises is the exploration of the possibility of purification of the intermediate tosylated cyclodextrin product. Additionally, would be interesting to explore one way to scale up the reaction and the product purification. Preliminary experiments using the Biotage system showed to be an efficient automatic method for purification.

Since dimethylformamide (DMF) is a protic solvent used to perform the nucleophilic reaction, its use should ideally be avoided on account of its toxicity. Thus, the use of other solvents less toxic than DMF on the performance of the nucleophilic reaction should be investigated further. Preliminary experiments with an aprotic solvent, PEG-200, proved to be a suitable solvent to produce the conjugate.

Since this new process of synthesis takes advantage of microwaves and allows the synthesis of the conjugate in a short period of time compared with the conventional approach, this process could be adapted for the synthesis of other cyclodextrin conjugates.

Improvement of the synthesis process of the α- and γ- cyclodextrin tosylated derivatives and access of the right conditions to synthetize the respective diclofenac conjugates.

Diclofenac was shown to be a highly reactive molecule by an intramolecular reaction mechanism, and which suffers modifications according to the pH change. HPLC analysis of the diclofenac released from the conjugate and also of the diclofenac after incubation in different buffer media revealed the appearance of extra peaks on the HPLC chromatogram. These extra peaks need to be accurately analysed by LC-MS in order to clarify its origin.

Further investigation into the stability of the conjugate in gastrointestinal fluids from other animal models such as monkeys, guinea pigs, or dogs using a greater number of subjects *per* species, may also be used. This will also allowed assessing which animal model gives a better prediction of the human behaviour. Also, the possible gender differences on the metabolism

of the conjugate should be investigated, as the studies presented here only included male species.

To better predict the behaviour of the conjugate in humans, it is also essential to assess its stability in human gastrointestinal fluids, which should involve a reasonable number of individuals (including male and female). Moreover, the metabolism can be accessed in fluids collected from individuals with colonic diseases in order to assess its performance.

Investigate the causes associated with the decrease in bioavailability of diclofenac when administered as the conjugate form in rats. Ideally, all faeces produced after oral intake of diclofenac-β-cyclodextrin should be collected, and the existence/absence of conjugate and/or diclofenac determined. Also, at the end of the study (after 24 hours of oral intake), gastrointestinal fluids should be collected and evaluated for the presence of diclofenac and/or conjugate. Also, the conjugate can be radiolabelled to easily follow its transit through the gastrointestinal tract after oral intake.

Evaluation of the acute and chronic ulcerogenicity of diclofenac-β-cyclodextrin versus diclofenac at the level of upper GI tract (stomach and small intestine), and also at the level of the lower GI tract (colon) using the rat as an animal model. Study design for the *in vivo* determination of anti-inflammatory activity of diclofenac-β-cyclodextrin versus diclofenac using the carrageenan induced rat paw edema model may additionally be carried out.

A deeper understanding of the pharmacological effect of this conjugate is necessary, including its potential to be used as a chronotherapeutic agent for rheumatic arthritis.

Assess other potential targets (colonic cancer).

Development of an appropriate formulation (capsule or tablets) of diclofenac-β-cyclodextrin.

APPENDIX

APPENDIX

Appendix A - Chapter I

- **Preparation of the buffer pH 1.2**

Sodium chloride (200 mg) was added to a 100 mL flask and dissolved in water followed by addition of 700 µL HCl (10 M) to adjust the pH to 1.2.

Table 1 Composition of the buffer 1.2.

Formula	Amount
Sodium chloride	200 mg
HCl 1M	8 mL

- **Preparation of acetate buffer pH 4.5**

Acetate buffer pH 4.5 was prepared according to to the USPXXIV as described in Table 2.

Table 2 Composition of the buffer phosphate pH 4.5 described in USP Indicated volumes are required to prepare 100 ml of buffer solution.

Formula	Amount
Sodium acetate trihydrate	299 mg
Acetic acid 2N	1.4 mL

- **Preparation of phosphate buffers**

Phosphate buffers pH 6.8 and pH 7.4 were prepared according to the USPXXIV by mixing the corresponding quantities of salt solutions as specified in Table 3.

Table 3 Composition of compendial phosphate buffers. Indicated volumes are required to prepare 200 ml of buffer solution.

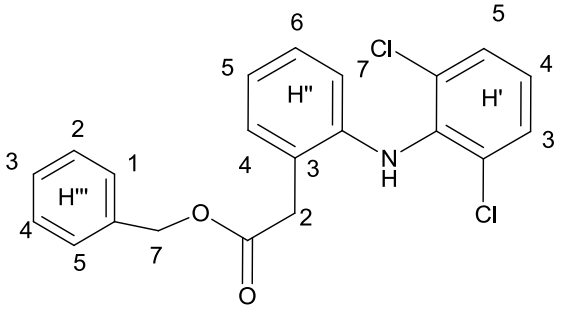
	Phosphate buffer pH 6.8	Phosphate buffer pH 7.4
0.2 M KH_2PO_4 (mL)	50	50
0.2M NaOH (mL)	22.4	39.1

Appendix B - Chapter II

Reaction of sodium diclofenac with benzyl chloride.

2.5g of sodium diclofenac (7.86 mmol) is dissolved in 10 mL of DMF and 50 mL of acetonitrile at 50 ^{0}C. 1g K_2CO_3 was added followed by 1 mL of benzyl bromide and the mixture reacted during 2 hours. Addition of hexane and eter in on attempted to precipitate the resulting product did not work. The resulting solution was evaporated and the product of reaction was left in the minimum quantity of solvent at air. After 3 days it was observed formation of crystals with high dimensions. Analysis of ^{1}H NMR shows that crystals correspond to the ester of diclofenac.

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 6.94 (m, 2H, H-5'',H-6''), 7.13 (dd, 1H, H-4'), 7.23 (d, 1H, H-4''), 7.33(d, 7H, H-3', H-5', H-1''', H-5''', H-2''', H-4''', H-3'''), 6.88 (s, NH-amine) 6.55 (d, H-7''), 3.86 (s, H-2'') 5.18 (s,H-7)



Appendice C - Chapter III

- *Preparation of simulated gastric fluid (SGF)*

Sodium chloride (200 mg) was added to a 100 mL flask and dissolved in water followed by addition of 700 µL HCl (10 M) to adjust the pH to 1.2. Pepsin (320 mg) was then added to the medium (see Table 1).

Table 1 Composition of the simulated gastric fluid according to USP formula.

FORMULA	AMOUNT
Sodium chloride	200 mg
HCl (10M)	700 µL
Pepsin	320 mg
Water	Up to 100 mL

- *Preparation of simulated intestinal fluid (SIF)*

The simulated small intestinal fluid was prepared through dissolution of 680 mg potassium phosphate monobasic in water. To this solution 770 µL of 0.2 M sodium hydroxide solution was added and the remaining water added to make the volume up to 100 mL. After adjusting the pH to 6.8 ± 0.1 with either 0.2 M of NaOH or 0.2 M hydrochloride acid, 1 g of pancreatin (from porcine pancreas, 3 USP units activity/g) was added and shaken gently.

Table 2 Composition of the simulated intestinal fluid according to USP formula.

FORMULA	AMOUNT
Potassium dihydrogen orthophosphate	680 mg
Sodium hydroxide 0.2 M	770 µL
Pancreatin (3USP units/g)	1 g
Water	Up to 100 mL

The composition of pancreatin enzyme as described by the USP and the composition of pancreatin enzyme obtained from Sigma-Aldrich used for the present studies are represented in the Table 3.

Table 3 Composition of the pancreatin.

E	ENZYM	AMOUNT	
		USP (units/mg)	**Sigma-Aldrich (x3 USP) (units/mg)**
	Amylase	25	75
	Lipase	2	6
	Tripsin	25	75

- **Preparation of HEPES/NaOH buffer**

The HEPES/NaOH buffer were prepared according to the described in Table 4.

Table 4 Composition of the buffer HEPES/NaOH buffer.

Formula : HEPES/NaOH	Quantity
HEPES 0.2M	25 mL
NaOH	2.04 mL
Water	Up to 100 mL volume

- **Preparation of 0.01M CaCl2/0.2M acetate buffer pH 5.5.**

The buffer acetate pH 5.5 was prepared according to theTable 5..

The solution of 0.01M of CaCl2 in acetate buffer was prepared by dissolving 1.47g of $CaCl_2.2H_2O$ in the acetate buffer pH 5.5.

Table 5 Compostion of the acetate buffer pH 5.5.

Formula: Acetate buffer 5.5, 0.2M	amount
Acetic acid 1M	2.02 mL
Sodium acetate 0.3M	59.92 mL
Water	Up to 100mL volume

Appendix D - Chapter IV

Determination of amylase activity using the EnzCheck Ultra amylase Assay Kit

α-Amylase activity in gastrointestinal fluids was determined by the EnzChek Ultra Amylase Assay Kit (E33651).

Materials

The EnzChekt Ultra Amylase Assay Kit (E33651) was purchased from Molecular Probes. The Kit contains:

Substract: DQ starch from corn BODIPY FL conjugate;

Reaction buffer: 0.05 M MOPS (3-(N-morpholino) propanesulfonic acid)), pH 6.9;

Sodium acetate 50mM, pH 4.0;

α-amylase from *Bacillus sp.* (Sigma A-6380) was purchased from Sigma Aldrich;

Gastrointestinal fluids (stomach, small intestinal, caecum, colon and faecal slurry prepared from colon fluids) obtained from pig, rat and rabbit.

Methods

A solution of 200 µg/mL subtract in acetate buffer was prepared according to the instructions described on the kit. A stock solution of α-amylase (400 mM/mL) was prepared in MOPS. Different standard solutions (0 - 40 mU/mL) were prepared from this stock solution in order to create an enzyme standard curve between 0 and 20 mU/mL.

For determination of amylase activity, it was use the supernatant obtained after centrifugation at 10 000 rpm during 10 minutes. Colon fluids were also prepared as faecal slurry (described in Chapter III). Each fluid was diluted with MOPS buffer (4 µL of fluid to 396 µL of MOPS).

Fluorescence of the digestion products from the substrate was measured by 96 -Plate reader at an excitation wavelength of 505 nm and an emission wavelength of 512 nm at room temperature.

Results and discussion

Figure 1 shows the activity of α-amylase measured in fluids from different sections of the gut of rat, rabbit and pig.

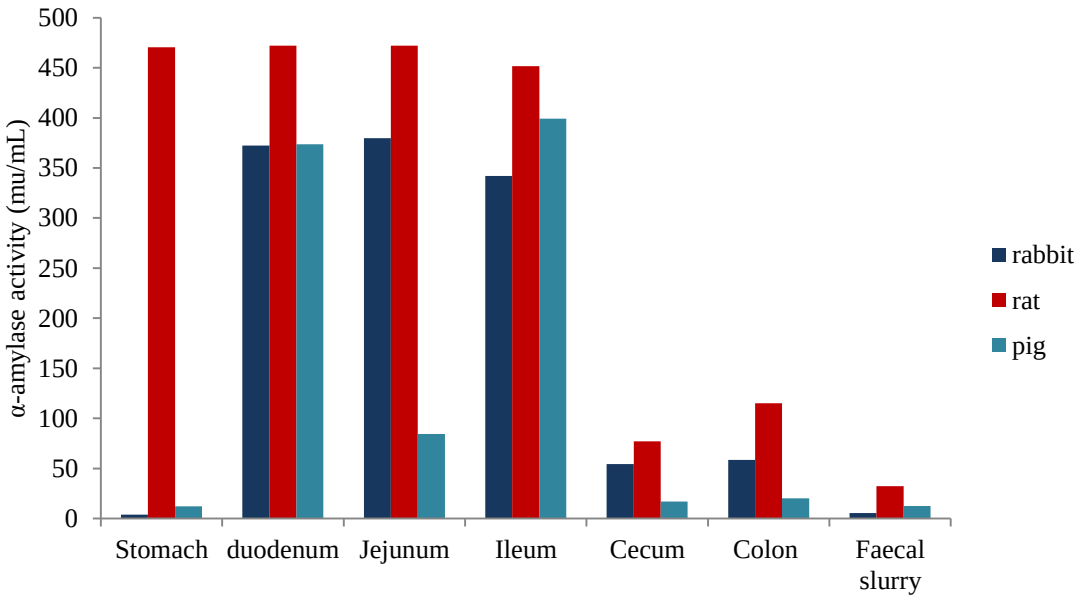


Figure 1 α-amylase activity (mU/ mL) in fluids from different sections of the gastrointestinal tract. The α-amylase was determined in the supernatant of fluids (stomach, duodenum, jejunum, ileum, caecum and colon) and in colon fluids prepared as faecal slurry.

The results show that the rat has the higher α-amylase activity in all the sections of the gastrointestinal tract when compared with the pig and the rabbit.

Analysing each section separately, it is observed that stomach of rats has much higher α-amylase activity than that observed in case of rabbit and pig.

The higher α- amylase activity is observed at the level of small intestine for all the animal models. At the level of the lower intestine the α-amylase activity is comparable between caecum and colon. When colon fluids were prepared as faecal slurry is observed a decrease in α-amylase activity, which can be associated with the dilution that occurs during the preparation of faecal slurry.

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Albert Einestein