EFFECTS OF CHITOSAN GLUTAMATE AND SODIUM TAURODEOXYCHOLATE ON PERMEATION OF 5-AMINOSALICYLIC ACID THROUGH PORCINE COLON

By

Uraiwan Pongpiriyajit

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Program of Pharmaceutical Sciences

Graduate School

SILPAKORN UNIVERSITY

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ผลของไคโตซานกลูทาเมตและโซเดียมทอโรดีออกซีโคเลตต่อการซึมผ่านของยากรด 5 – อะมิโนซาลิซัยลิก ผ่านลำไส้ใหญ่สุกร

โดย
นางสาวอุไรวรรณ พงศ์พิริยะจิต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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The Graduate School, Silpakorn University has approved and accredited the thesis title of "Effects of chitosan glutamate and sodium taurodeoxycholate on permeation of 5-aminosalicylic acid through porcine colon" submitted by Miss Uraiwan Pongpiriyajit as a partial fulfillment of the requirements for the degree of Program of Science in Pharmaceutical Sciences.

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URAIWAN PONGPIRIYAJIT : EFFECTS OF CHITOSAN GLUTAMATE AND SODIUM TAURODEOXYCHOLATE ON PERMEATION OF 5-AMINOSALICYLIC ACID THROUGH PORCINE COLON. THESIS ADVISOR : ASSOC. PROF. JURAIRAT NUNTHANID, Ph.D. 94 pp.

The objective of this research was to study effects of the polymer, chitosan glutamate (CSG), and the bile salt, sodium taurodeoxycholate (NaTDC), on *in vitro* permeation of 5-aminosalicylic acid (5-ASA), a poor absorption drug, through porcine colon. CSG and NaTDC at various concentrations of 0.01, 0.5 and 2.0 %w/v were used in the permeation study. The drug permeation was performed through freshly isolated porcine colon epithelium mounted in side by side diffusion cells. The temperature was controlled at 37$\pm$0.5 ^{o}C. The samples were collected every 30 minutes until 4 hours and the amount of drug was analyzed by high performance liquid chromatography (HPLC). The cell integrity of isolated colon epithelium was determined by measurement of transepithelial electrical resistance (TEER). The tissue viability was investigated by MTT assay and the leakage of the epithelium was also evaluated by permeation test of phenol red. The results indicated that the permeation of the drug through porcine colon was zero order kinetics. NaTDC significantly enhanced the permeability of 5-ASA. The drug permeability coefficient (P_{app}) was increased from 1.033$\pm$0.169 to 1.596$\pm$0.0.364, 1.992$\pm$0.126 and 2.352$\pm$0.414 (x 10^{-5}cm/s) when using NaTDC at the concentration of 0.01, 0.5 and of 2.0 %w/v, respectively. The results indicated that the both of CSG and NaTDC could open tight junctions of the epithelium without any sign of epithelial damage. However, the CSG could not enhance the drug permeation since there was interaction between CSG and 5-ASA.

Program of Pharmaceutical Sciences Graduate School, Silpakorn University Academic Year 2010
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Thesis Advisor's signature ……..…...……...…….

51361210 : สาขาวิชาวิทยาการทางเภสัชศาสตร์
คำสำคัญ : ไคโตซานกลูทาเมต/ โซเดียมทอโรดีออกซีโคเลต/ การซึมผ่านยา/ 5 – อะมิโนซาลิซัยลิก แอซิด/ ลำไส้ใหญ่สุกร

อุไรวรรณ พงศ์พิริยะจิต : ผลของไคโตซานกลูทาเมตและโซเดียมทอโรดีออกซีโคเลตต่อการซึมผ่านของยากรด 5 – อะมิโนซาลิซัยลิก ผ่านลำไส้ใหญ่สุกร. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ภญ.รศ.ดร.จุไรรัตน์ นันทานิช. 94 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของพอลิเมอร์ ได้แก่ ไคโตซานกลูทาเมต (CSG) และเกลือน้ำดี ได้แก่ โซเดียมทอโรดีออกซีโคเลต (NaTDC) ต่อการซึมผ่านของยากรด 5 – อะมิโนซาลิซัยลิก (5-ASA) ซึ่งเป็นยาที่มีการซึมผ่านต่ำ ผ่านลำไส้ใหญ่สุกร แบบภายนอกร่างกาย โดยใช้ CSG และ NaTDC ความเข้มข้นที่แตกต่างกัน คือร้อยละ 0.01 0.5 และ 2.0 โดยน้ำหนักต่อปริมาตร จากนั้นทดสอบการซึมผ่านของยา ผ่านผนังลำไส้ใหญ่สุกร ด้วยอุปกรณ์ side by side diffusion cells โดยควบคุมอุณหภูมิที่ 37$\pm$0.5 องศาเซลเซียส เก็บตัวอย่างทุก 30 นาที จนครบ 4 ชั่วโมง จากนั้นวิเคราะห์หาปริมาณยาด้วยเครื่องโครมาโทกราฟีแบบของเหลวสมรรถนะสูง ทั้งนี้ได้ทำการทดสอบความสมบูรณ์ในการจัดเรียงตัวของเซลล์เยื่อบุผนังลำไส้โดยการวัดค่าความต้านทานทางไฟฟ้า รวมถึงทดสอบความมีชีวิตของเซลล์เยื่อบุผนังลำไส้ด้วยเทคนิคการหาปริมาณสารเอ็มทีทีและทดสอบการรั่วของเซลล์เยื่อบุผนังลำไส้ ด้วยการทดสอบการซึมผ่านของสารฟีนอลเรด ผลการทดลองพบว่าการซึมผ่านของยาผ่านลำไส้ใหญ่สุกรเป็นแบบจลนพลศาสตร์อันดับศูนย์ โดย NaTDC สามารถเพิ่มการซึมผ่านยา 5-ASA ได้อย่างมีนัยสำคัญ การเพิ่มความเข้มข้นของ NaTDC มีผลทำให้ค่าสัมประสิทธิ์สภาพให้ซึมผ่านได้ (P_{app}) ของยา 5-ASA เพิ่มขึ้น จาก 1.033$\pm$0.169 เป็น 1.596$\pm$0.364 1.992$\pm$0.126 และ 2.352$\pm$0.414 (x 10^{-5}cm/s) เมื่อใช้ NaTDC ที่ความเข้มข้นร้อยละ 0.01 0.5 และ 2 โดยน้ำหนักต่อปริมาตร ตามลำดับ จากผลการทดลองพบว่าทั้ง CSG และ NaTDC สามารถเปิดช่องว่างระหว่างเซลล์ (tight junctions) ของเยื่อบุผนังลำไส้ใหญ่ได้ โดยไม่มีอันตรายต่อเซลล์เยื่อบุผนังลำไส้ อย่างไรก็ตาม พบว่า CSG ไม่สามารถเพิ่มการซึมผ่านของยา เนื่องจาก CSG เกิดอันตรกิริยากับยา 5-ASA

สาขาวิชาวิทยาการทางเภสัชศาสตร์ บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร ปีการศึกษา 2553
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CHAPTER 1

INTRODUCTION

1. Statement and significance of the research problem

In many recent years, oral drug delivery to human colon has been developed to protect some drugs which are not stable such as peptides, proteins, oligonucleotide drugs and vaccines from their susceptibility to degradation by acidic pH and proteolytic enzymes in the environment of the upper gastrointestinal (GI) tract (Sinha and Kumria 2001 : 557-564; Pichayakorn, Kusonwiriyawong, and Ritthidej 2003 : 91-108). Colonic drug delivery has gained increased importance not only just for its potential for the delivery of proteins and therapeutic peptides but also for the delivery of the drugs in the treatment of local diseases associated with colon such as Chron's diseases, ulcerative colitis, colorectal cancer and amebiasis. The therapeutic agents have to cross epithelial cells with important mechanisms of drug permeation through transcellular and paracellular pathways. The transcellular route is a suitable transport for lipophilic drugs. It depends on physicochemical properties of drugs such as molecular weight, charge and lipophilicity (Lipinski et al. 2001 : 235-249). The paracellular pathway is the main route of transport for hydrophilic drugs through the intercellular spaces. This pathway is controlled by tight junctions (TJs) which are a multiple unit structure and normally regulate the trafficking of compounds. They

form a regulated barrier in the space between cells, restricting the movement of compounds across epithelial cells (Salama, Eddington and Fasano 2006 : 15-28). For the assessment of intestinal drug permeability, many different *in vitro* methods are available such as the use of excised tissues, epithelial cell culture models, artificial membranes and these techniques have been recently reviewed (Tukker 2000 : 51-72). The methods based on drug transport across intestinal epithelial monolayer, Caco-2 cells culture, are at present most utilized. The cells are derived from human colonic adenocarcinoma cells (Merwe et al. 2004 : 225-235). In fact, they show features similar to human small intestine giving rise to good correlations with the fraction absorbed in humans. However, some important drawbacks, such as time consuming, high cost per assay, possibility of microbial contamination and wide inter laboratory variations, are considered the weakness of this model as a screening tool. Moreover, Caco-2 cells are generally lacking mucus layer which covers the intestinal epithelium resulting in the effect on drug permeability. Therefore, alternative procedures to the use of excised tissues are currently pursued (Castella et al. 2006 : 1543-1553). The examples of excised tissues used in *in vitro* models for colon permeation study are rat colon and porcine colon (Ma et al. 1995 : 12-20; Shah and Khan 2004 : 36-40; Aschenbach 2002 : 115-122). Peroral delivery of hydrophilic drugs is one of the greatest challenges in biopharmaceutical researches which usually present low bioavailability. One of the causes is their poor intestinal permeation through the paracellular pathway (Cano-Cebrian et al 2005 : 9-22). To improve this problem, several enhancer compounds such as surfactants, fatty acids, cyclodextrins and

polymers have been used to increase drugs permeability across the intestinal mucosa layer to bloodstream or lymphatic system.

Many types of polymers such as chitosan (CS), pectin, carbomer are used as absorption enhancers. CS, a polysaccharide derived from chitin, is currently attracting much attention due to its ability to mediate the increase of transepithelial drug permeation both *in vivo* (Illum et al. 2001 : 391-400) and *in vitro* (Dodane, Khan and Merwin 1999 : 21-32) without causing cells toxicity. Illum et al. demonstrated the ability of chitosan glutamate (CSG) to enhance the transport of insulin across the nasal mucosa of sheep and rats (Illum et al. 1994 : 1186-1189). The efficacy of CS as a nasal absorption enhancer was confirmed using salmon calcitonin in a rat model (Sinswat and Tengamnuay 2003 : 15-22). CS was found to cause a reduction in integrity of Caco-2 cells monolayer of up to 83%. A corresponding increase in horseradish peroxidase permeability across the monolayer of up to 18 times greater than the control was also observed. CS-mediated TJ disruption is caused by a translocation of tight junction proteins from the membrane to the cytoskeleton (Smith, Dornish and Wood 2004 : 43-49). In addition, cytotoxicity of CS salts was also studied and the ranking from low to high toxicity were as follows: CS hydrochloride>CS lactate>CS glutamate> CS aspartate, respectively (Opanasopit et al. 2007 : 447-455).

Bile salts are one type of surfactants that can effectively increase the drug permeability. The main mechanisms of bile salts involve in the increases of membrane fluidization (Song, Chung and Shim 2005 : 298-308). Consequently, the spaces between the cells are increased resulting in the improvement of drug

permeation. In addition, bile salts also increase the stability of proteins and peptide drugs. The drugs were protected from digestive enzymes by forming complexes with the drugs as liposomes (Song, Chung and Shim 2002 : 27-37). In the previous studies, sodium taurodeoxycholate (NaTDC) could significantly increase permeability of salmon calcitonin in Caco-2 cells up to 10.81-fold (Song, Chung and Shim 2005: 298-308) and also was reported to reduce the viscosity of mucus layer (Martin, Marriott and Kellaway 1978 : 103-107).

2. Objectives of this research

2.1 To evaluate the effects of a polymer and a bile salt on *in vitro* drug permeation through porcine colon. CSG and NaTDC were chosen according to the effective increase of drug permeability and their low toxicity. 5-ASA, a poor absorption drug was used as a model drug.

2.2 The effects of concentration of both enhancers on the drug permeation were also studied.

3. The research hypothesis

1) CSG and NaTDC can increase permeation of 5-ASA through porcine colon.

2) The concentrations of both enhancers are a factor affecting the permeation of 5-ASA.

CHAPTER 2

LITERATURE REVIEWS

Colon drug delivery

To date, oral delivery is the preferred route of drug administration according to the oral route proffers patients less pain, higher possibility of compliance, and greater convenience. Despite these advantages, it is not amenable to the administration of some drugs due to their high susceptibility to digestive enzymes in the gastro-intestinal tract, poor absorption, and their limited ability to transport across the intestinal epithelial barrier. As a result, new tactics of drug delivery have been developed to overcome obstacles encountered by oral delivery (Sinha and Kumria 2001). Among these tactics, colon-specific drug delivery systems have been well recognized and extensively studied. The colon is viewed as the promoted absorption site for oral administration of protein and peptide drugs, because of the reasonably low proteolytic enzyme activities in the colon. It has been demonstrated that insulin, calcitonin and vasopressin can be absorbed in this segment (Saffran et al. 1991: 267-278; Antonin et al. 1992 : 627-631). However, the poor permeability across colon epithelium results in low bioavailability of drugs.

The drug absorption in the GI tract primarily occurs in stomach, small intestine (duodenum, jejunum and ileum) and less in the large intestine. Each

of these segments has unique anatomical, biochemical and physiological properties that dictate parameters of digestion and solute absorption (Table 1).

Table 1 Biological and physiological parameters of the human intestinal tract

Gastro-intestinal segment	Approx. Surface area	Approx. length	Approx. Residence time	Approx. pH	Prominent catabolic activity
Oral cavity	100 cm^2	-	Seconds to minutes	6.5	Polysaccharides
Esophagus	200 cm^2	23-25	Seconds	-	-
Stomach	3.5 m^2	variable	1.5 h	1-2	Protease, lipase
Duodenum	1.9 m^2	0.35 m	0.5-0.75 h	4-5.5	Polysaccharides, oligosaccharide, protease, peptidase,
Jejunum	184 m^2	2.8 m	1.5-2.0 h	5.5-7.0	Oligosaccharides, peptidase, lipases
Ileum	276 m^2	4.2 m	5-7 h	7.0-7.5	Oligosaccharide, peptidase, lipases,nucleases, nucleotidases
Colon and rectum	1.3 m^2	1.5 m	1-60 h	7.0-7.5	Broad spectrum of bacterial enzymes

Approx. = Approximate

Source: Ann L. Daugherty, and Randall J. Mrsny, "Transcellular uptake mechanisms of the intestinal epithelial barrier Part one," Pharmaceutical Science & Technology Today 2 (1999): 145.

The small intestine is the main and perfect site of drug absorption, due to its anatomical location and morphological features, such as its length and large surface area. Moreover, it features various carrier mediated transport systems for compounds which can also be helpful in absorbing drugs. Recent advances in controlled-release techniques, however, allow for delivery of drugs to the lower part of the gastro intestinal tract and thus, the general interest in colonic drug absorption has been raised (Rouge, Buri and Doelker 1996 : 117-139). A summary of the approaches different systems are being developed for the purpose of site-specific drug delivery to the colon. These include ; prodrugs, microflora-activated systems (Sinha and Kumria 2001), and pH- and time-dependent systems (Leopold 1999 : 197-204; MacNeil and Stevens 1990).

The small intestinal epithelium is considered to be relatively "leaky" having low epithelial resistance and high permeability. Compared with the small intestine, colonic epithelium is considered to be a much "tighter" epithelium, having higher epithelial resistance and low paracellular permeability (Powell 1987:1267-1305; Ma et al. 1991: G669-G676). The colonic "pore" diameter has been previously calculated to be 6 Å; thus, colonic epithelium has been presumed to be impermeable to medium- or large-sized passively absorbed hydrophilic probes. The commonly used permeability probes polyethylene glycol 400 (5.3 Å), mannitol (6.7 Å), lactulose (9.5 Å), and Cr-ethylene diamine tetraacetic acid (10.8 Å) have lower molecular weights and smaller crosssectional diameters (<11 Å) (Hollander, Ricketts and Boyd 1988: 35a-38a), but because their size is larger than

the presumed "pore" size of the colon, they were not thought to permeate the colon to any appreciable extent.

Pathways of drug permeation

Epithelial cells barrier

The intestinal epithelial barrier is a boundary between the organism and the luminal environment. It plays a role by allowing the passage of compounds but preventing the passage of pathogens. The maintenance of its homeostasis is of very importance for the survival of the living being. The epithelial barrier is formed by a monolayer of the epithelium cells under constant renewal and maintained together via various cell-to-cell and cell-to-matrix interactions. Emerging proofs suggest that under physiological conditions, the intestinal epithelial barrier's functions are actively regulated by its cellular microenvironment (Blikslager et al. 2007 : 545-564). The epithelial cell forms have the capacity to allow selective passage of compounds. The epithelial cells have to be polarized structure (Lodish et al. 2000).

The colon epithelium comprises a monolayer of multiple cell types. Enterocytes, the absorptive cells, are the main focus in drug delivery issues. In addition to enterocytes, there are undifferentiated crypt cells, and mucus-secreting goblet cells. The morphology of colon tissue is shown in Figure 1.

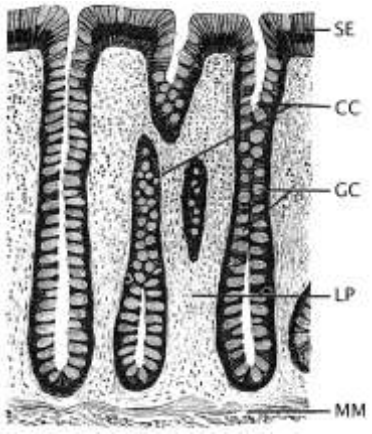


Figure 1 Morphology of normal colon tissue. Labels show surface epithelium (SE), colon crypts (CC), goblet cells (GC), lamina propria (LP), and muscularis mucosa (MM).

Source : Steven A. Frank, Dynamics of Cancer [Online], accessed 18 January 2011. Available from www.ncbi.nlm.nih.gov/books/NBK1562/

For drugs to be therapeutically effective, the therapeutic agents have to possess favorable characteristics to cross the biological barrier into the systemic circulation and reach the site of action. The different transport mechanisms are shown in Table 2.

Table 2 Transport mechanisms common to cellular barriers

Mechanism	Characteristics
I.Diffusion	Flux down an electrochemical gradient. Energy dependent.
a.Passive	Flux proportional to concentration gradient.
b.Facilitated	Rate independent of direction Carrier-mediated. Flux is saturable with increasing concentration.

Table 2 (continue)

Mechanism	Characteristics
	Competitive substrates. Flux may be asymmetrical.
II.Active carrier-mediated	Flux can be against an electrochemical gradient. Energy dependent directly or indirectly coupled. Substrate specificity, competition, saturation. Flux is asymmetrical.
III.Endocytosis	Invagination of the plasma membrane forming an internalized membrane vesicle. Usually energy dependent. Usually results in solute uptake into and use by a cell; however, transcytosis, in which the vesicle crosses the cell and fuses with the opposite plasma membrane domain, may occur. Flux against a gradient.
a.Receptor-mediated	Very substrate-specific. High affinity and saturable, but usually low capacity. Asymmetrical.
b.Adsorptive	No specific receptor involved; solute nonspecifically adsorbs to cell surface proteins or glycolipids. Can be saturable and show competition, often high capacity.
c.Fluid-phase	Soluble molecules are internalized with the vesicle volume. Nonspecific and nonsaturable.

Source: Anthony Adson et al., "Monolayers Quantitative approaches to delineate paracellular diffusion in cultured epithelial cell monolayers," Journal of Pharmaceutical Sciences 83 (1994): 1529-1536.

Drugs can across the intestinal epithelial barrier in a number of ways (Figure 2). The transport of drug molecules across epithelial cell barriers occurs by either of two general pathways: transcellular or paracellular route. The transcellular pathway associates the passage of drugs across the cells, while the paracellular pathway refers to the passage of drugs across intercellular spaces. The dominant rout for permeation of drug depends upon their physicochemical properties as well as the membrane. In general, lipophilic drugs cross the biological membrane via transcellular route while hydrophilic drugs cross the membrane via paracellular route (Salama et al. 2006 : 15-28).

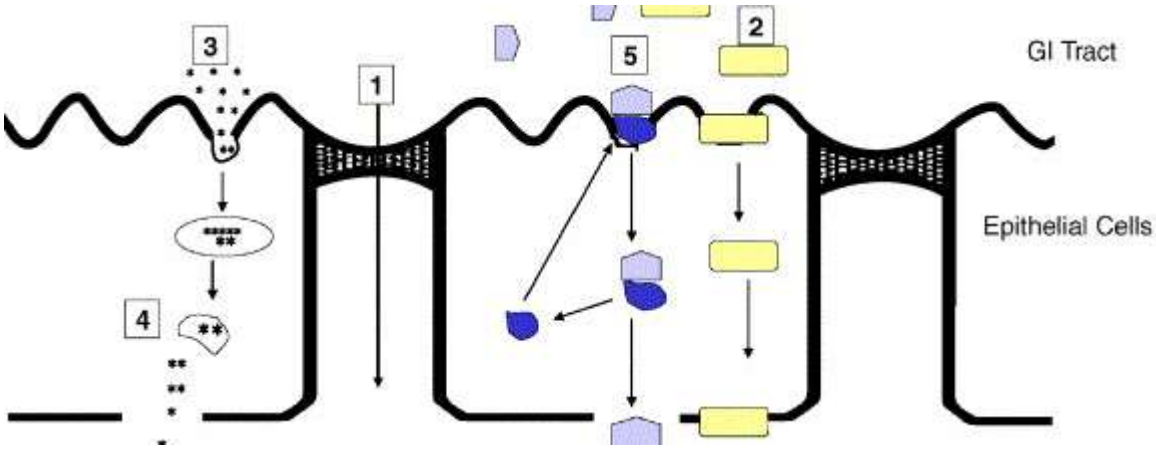


Figure 2 Schematic representation of the paracellular transport (1) and the transport of drugs or solutes (* *) across the epithelial cells of the GI tract into the systemic circulation. (2) transcellular passive dif-fusion, (3) transcellular endocytosis followed by exocytosis (4), (5) car-rier mediated transport processes between a specific carrier and a drug.

Source : N. N. Salama, N. D. Eddington, and A. Fasano, "Tight junction modulation and its relationship to drug delivery," Advanced Drug Delivery Reviews 58 (2006) : 17.

1.Transcellular route

Passive diffusion across intestinal epithelial cells occurs through non -specific permeability pathways. Transcellular diffusion is the movement of compounds across the intestinal barrier driven by concentration gradient from intestinal lumen to blood supply (Figure 3). The small amphipathic drugs move efficiently through the transcellular route by partitioning into and out of lipid bilayers so the partition coefficient of drug between an organic phase and an aqueous phase is used to predict passive diffusion. Small drug molecules that are too hydrophilic cannot enter the lipid bilayer (Lipinski et al. 2001: 235-249). In order to improve drug absorption via the transcellular pathway, the physicochemi-cal features of the drug have to be manipulated (lipophilicity, pKa, conformation, H-bond characteristics, etc.) or the membrane characteristics have to be altered.

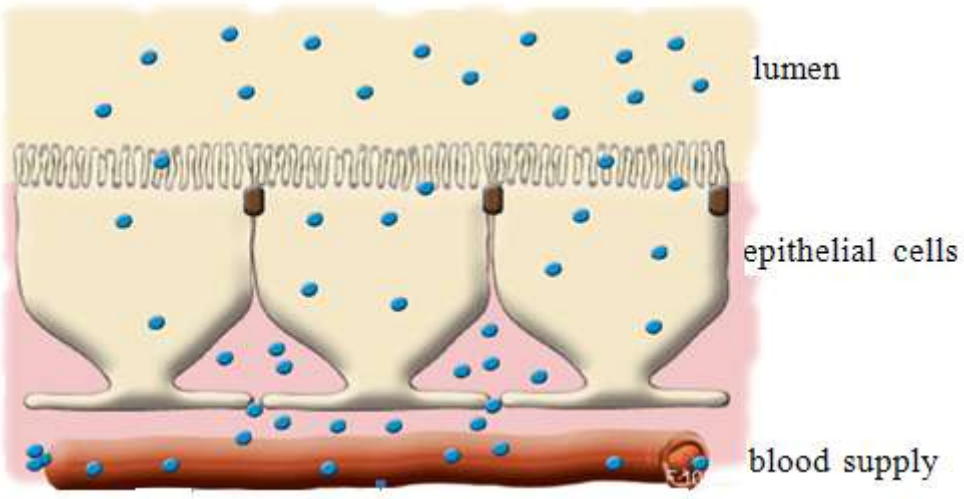


Figure 3 Transcellular absorption of compounds

Source: Luis Alejandro Bate, Absorption [Online], accessed 6 January 2011. Available from http://people.upei.ca/bate/html/absorption.html

2. Paracellular route

The paracellular route can be defined as the aqueous pathway along the intercellular space between adjacent cells, which is restricted by tight junctions (TJs) at the most apical part of the cells in Figure 4.

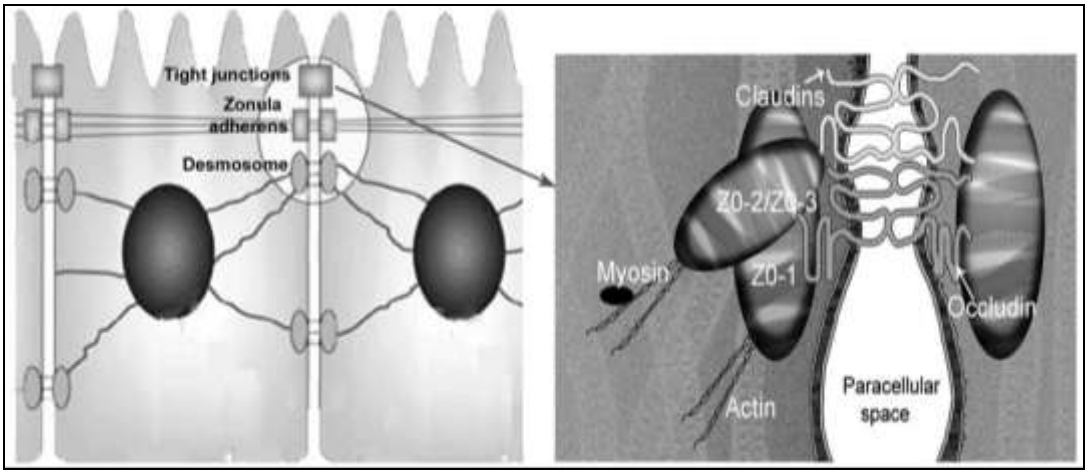


Figure 4 Left: The functional complex located in the apical part of the adjacent enterocytes is formed by TJs, zonula adherens and macula adherens or desmosomes. Right: schematic representation of the protein interactions at TJs.

Source: Maria Jose Cano-Cebrian et al., "Intestinal Absorption Enhancement Via the Paracellular Route by Fatty Acids, Chitosans and Others: A Target for Drug Delivery," Current Drug Delivery 2 (2005):10.

Components of TJs

The TJs are a multiple unit structure composed of multiprotein complex that affiliates with the underlying apical actomyosin ring. TJ proteins identified include transmembrane proteins; occludin and claudin, and TJ associated pro-

teins/ cytoplasmic plaque proteins; ZO-1, ZO-2, and ZO-3 and other proteins. Transmembrane proteins: occludin, a 65-kDa phosphor protein was first identified in different species. The findings reported to date are consistent with a functional role for occludin in defining the barrier. Claudins are a family of proteins. They appear to represent the major structural components of TJs strands, since they show an intrinsic ability to polymerize into linear fibrils, whereas occludin forms only short strand fragments. A role for claudins in barrier formation is thus evidenced. Recent studies have proposed that claudins are the pore-forming structures in TJs thus strongly supporting the idea that claudins confer specific selectivity to paracellular transport. Furthermore, a dense cytoplasmic network of proteins has been described at the TJs. These are referred to as TJ associated proteins, and are designated ZO-1, ZO-2 and ZO-3. These proteins interact among each other and also serve as a link between occludin and the actin filaments of the cytoskeleton. The association of TJs with the apical perijunctional actomyosin ring seems to regulate global TJ permeability. It is also known that the barrier assembly and permeability characteristics of TJs are influenced by many cellular signaling mechanisms though these remain largely undefined. In conclusion, despite rapid progress in the knowledge of TJ structure and molecular physiology, their function in the context of paracellular permeability is still far from fully clear (Cano-Cebrain et al. 2005 : 9-22).

Paracellular route is usually the main route of absorption for hydrophilic, proteins, peptide drugs, etc. The paracellular pathway is governed by the TJs. The modulation of the TJ by absorption enhancers for paracellular drug trans-

port enhancement therefore drug delivery improvement has been hampered for many years by lack of inclusive understanding of the structure and function of the TJs. TJs or Zonula Occludens constitute the major rate-limiting barrier towards the paracellular transport for permeation by ions and larger solutes (Madara 1998 : 143-159). The dimensions of the paracellular space lie between 10–50 Å, suggesting that solutes with a molecular radius exceeding 15 Å (~3.5 kDa) will be excluded from this uptake route (Rubas et al. 1996). TJs are dynamic structures, which normally regulate the trafficking of compounds ($\leq$ 15 Å) and relatively large amounts of fluids between the intestinal lumen and the submucosa (Hollander 1992 : 721-726). TJs have two primary functions in epithelia and endothelia. They form a regulated barrier in the paracellular space, restricting the movement of molecules as small as ions across epithelial cell and they act as a boundary within the plasma membrane itself, separating the compositionally unique apical and basolateral cell surface domains (Stevenson 1998 : 89-109). TJs play a role in the transduction of signals across cell membranes and in regulating links to the cytoskeleton of the cells (Dunina-Barkovskaya 1998 : 555-589).

Determination of membrane permeability

An improved understanding of drug membrane transport across various regions of the intestinal tract is vital to the development of new and effective pharmaceutical products. New candidate drugs and novel dosage forms are a lot tested in various animal models in preclinical development. There is a need, therefore, to thoroughly understand the permeability models applied and to ensure

that they predict outcomes even more accurately than models currently applied. Estimates of intestinal permeability are often based on multiple, parallel transport processes, such as passive transcellular diffusion, carrier-mediated absorption and carrier-mediated efflux and may also vary along the intestine (Brayden 2003 : 976-978). The intestinal permeability is the tendency of a compound to move across the epithelial barrier of the intestine. Permeability values from cells culture or animal experimental models offer lawful alternatives to human intestinal permeability (Salphati et al. 2001 : 1007-1013). There are many *in vitro* methods for determining intestinal permeability for compounds such as diffusion studies through cell cultured monolayers such as Caco-2 cells or intestinal segments from various animals i.e. rat, frog and rabbit and uptake studies in brush-border membrane vesicles prepared from intestinal segments of various animals.

The methods based on drug transport across intestinal epithelial monolayer, Caco-2 cell culture, are at present most utilized. The cells are derived from human colonic adenocarcinoma cells (Merwe et al. 2004 : 225-235). In fact, they show features similar to human small intestine giving rise to good correlations with the fraction absorbed in humans. However, some important drawbacks, such as time consuming, high cost per assay, possibility of microbial contamination and wide inter laboratory variations, are considered the weakness of this model as a screening tool. Moreover, Caco-2 cells are generally lacking mucus layer which covers the intestinal epithelium resulting in the effect on drug permeability. Therefore, alternative procedures to the use of excised tissues are currently pursued (Castella et al. 2006 : 1543-1553). The examples of excised

tissues used in *in vitro* models for colon permeation study are rat colon and porcine colon (Ma et al. 1995 : 12-20; Shah and Khan 2004 : 36-40). Aschenbach et al. investigated the intestinal mechanisms that possibly prevent systemic intoxication by luminal histamine across porcine colonic epithelia. It is concluded that histamine permeation across the porcine proximal colon is restricted by low permeability and sequential catabolism by histamine N-methyltransferase and diamine oxidase (Aschenbach, Ahrens and Garz 2002 : 627-631). Fetih et al. also investigated the effects of various absorption enhancers (sodium glycocholate (NaGC), *n*-dodecyl-β-D-maltopyranoside (LM), sodium salicylate (NaSal) and sodium caprate (NaCap) on permeation of 5(6)-carboxyfluorescein (CF) by an in situ closed loop method. It was found that the absorption enhancing effects of these enhancers for intestinal absorption were greater in the colon than those in the jejunum and the ileum. Especially, among these enhancers tested in this study, LM showed much greater absorption enhancing effect in the colon than in the jejunum and the ileum. However, whole animal pharmacokinetic or toxicokinetic studies also provide intensively useful information with deference to the generally exhibition to a chemical. The complexity of the *in vivo* models due to the con-founding processes of metabolism, distribution, gastric emptying, protein binding, and food effects (Cook and Shenoy 2003 : 125-133).

The therapeutic problem

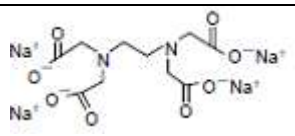
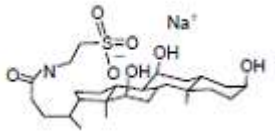
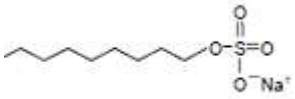
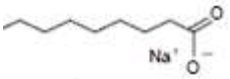
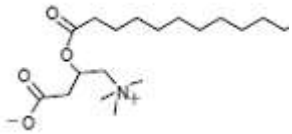
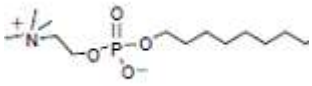
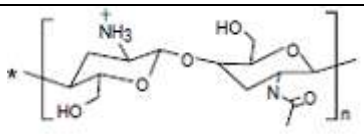
Several therapeutic agents are hydrophilic and cannot be delivered through the oral route because the hydrophilic drugs are poor absorption across the

epithelium barrier. For example, the hydrophilic antibiotic cefoxitin has an oral bioavailability of 5% in animals owing to poor intestinal permeability (Sutton et al. 1993 : 1516-1520) and is currently only marketed as an intravenous formulation. Enalaprilat, an angiotensin-converting enzyme inhibitor, is also poorly absorbed and marketed as an intravenous formulation. Enalaprilat was chemically modified to produce a more lipophilic prodrug, enalapril, which is well absorbed (MacFadyen et al. 1993 : 274-282). The enalapril modification illustrates one way to increase the absorption of hydrophilic drugs across the intestinal epithelium: convert them into a more lipophilic prodrug, thus increasing transcellular flux of the drug. Another approach involves redesigning the drug so that it is a substrate for a carrier. The controlled and reversible opening of the TJs represents a way to increase the absorption of hydrophilic drugs across the intestinal epithelium. This approach is attractive because it could be applied to many different hydrophilic drugs.

Enhancing drug permeation

Many studies focused on the investigation of absorption enhancers with compounds acting across one or more mechanisms, including influence on the thermodynamic activity of the drug in solution, alteration of the molecular structure of the cell membrane ranging from temporary membrane pore formation to complete membrane destruction, loosening of the TJs between epithelial cells, inhibition of the protease activity present in the mucosa, and alteration of the characteristics of the mucus in ways which reduce its diffusion barrier properties.

Table 3 Paracellular permeability enhancers in Caco-2 cell monolayers

Class	Specific example	Tentative mechanism
Ca^{2+} chelators	 Ethylene diamine tetra-acetate sodium	Extracellular chelation of Ca^{2+}, disrupt cell-to-cell contact (cadherin) and associated intracellular events
Bile salts	 Sodium taurocholate	Membrane perturbation, alteration of intracellular events: increase Ca^{2+}, actin disbandment
Anionic surfactants	 Sodium dodecyl sulfate	Membrane perturbation, increase Ca^{2+}, ATP depletion
Medium chain fatty acids	 Sodium caprate	Alteration of several intracellular events: increase Ca^{2+}, decrease ATP, phospholipase C mediation
Fatty acid esters	 Palmitoyl carnitine	Membrane perturbation, ATP depletion; no effect on Ca^{2+} or actin
Phosphate esters	 Dodecylphosphocholine	Modulation of tigh junctions via phospholipase C inhibition increased manitol flux 10-fold
Cationic polymers	 Chitosan	Mucus adhesion; increased apical membrane permeability; actin depolymerization; change in ZO-1 localization

Source: P. D. Ward, T. K. Tippin, and D. R. Thakker, "Enhancing paracellular permeability by modulating epithelial TJs," Pharmaceutical Science & Technology Today 3 (2000): 352.

(Donovan, Flynn and Amidon 1990 : 808-815). A variety compounds used asparacellular enhancer is shown in Table 3. Numerous classes of compounds with diverse chemical properties, including detergents, surfactants, bile salts, Ca^{2+} chelating agents, fatty acids, medium chain glycerides, acyl carnitine, alkanoyl cholines, mucoadhesive polymers, and phospholipids have been reported to enhance the intestinal absorption of small drug molecules and large polypeptide drugs (LeCluyse and Sutton 1997 : 163-183).

Bile salts

Bile salts are normally present in the gastrointestinal contents, which suggest that under some conditions they might not be toxic. Bile salts can change intestinal absorption in possibilities: they improved transport of the drug to the mucosal absorptive surface, and they increased permeability of the epithelial cells membrane (Kakemi et al. 1970 : 275-280). The relative of these effects could vary depending on the drug. Bile salts have been shown to increase intestinal absorption of larger, poorly permeable molecules. For example, sodium cholate and deoxycholate increased the colonic absorption of heparin in rats and baboons (Ziv et al. 1983 : 773-776). Deoxycholate and chenodeoxycholate (0.5 mM perfusion) were reported to increase the intestinal permeation of horseradish peroxidase with molecular weight of 40,000 Da, without causing histological damage to the membrane (Teichberg et al. 1983 : G122–G132). Bile salts, sodium cholate, sodium taurocholate and sodium taurodeoxycholate, which are ionic surfactants, show also a concentration-dependent effect on epithelial permeability and morphology in Caco-2 cells and human nasal epithelial cells, respectively. A dose

dependent reduction of cell viability was also observed at higher than critical micelle concentration, but bile salts exerted less toxicity, than nonionic surfactants Tween 80 and Poloxamer F68 (Lin et al. 2007 : 135-157). Neubert (1989 : 743-747) reported that NaTDC could increase the permeability by forming lipophilic ion-pair complexes with various organic cations across biological membranes. The permeation enhancer property of NaTDC was agreed with its effect on salmon calcitonin in Caco-2 cells (Song, Chung and Shim 2005). An increase in the passive diffusion of drug molecules across biological membranes, as well as membrane fluidizing by bile salts, would be expected in their presence.

Cationic polymers

Cationic polymers, like poly-L-lysines, polyethyleneimine and chitosan, are able to induce reversible opening of TJs in epithelial cell models. Chitosan is obtained by alkaline deacetylation of chitin, which is abundant polysaccharide next to cellulose. Chitin is the component of protective cuticles of crustaceans such as crabs, shrimps. Chitosan comprises of copolymers of glucosamine and *N*-acetyl-glucosamine (Singla and Chawla 2001 : 1047-1067). Chitosan has one primary amino and two free hydroxyl groups for each C_6 building unit in Figure 5. Due to the easy availability of free amino groups in chitosan, it carries a positive charge and thus in turn reacts with many negatively charged compounds.

Chitosan is a weak base and is insoluble in water and organic solvents but it is soluble in dilute aqueous acidic solution (pH<6.5), which can convert the glucosamine units into a soluble form $R\text{-}NH_3^+$ (Chandy and Sharma 1990 : 1-24). It precipitates in alkaline solution (Demarger-André and Domard 1994 :177-184).

Chitosan is available in the form of dry flakes, solution and powder. It has an average molecular weight ranging between 3800 and 2,000,000 Da and is from 66 to 95% deacetylated (Kas 1997 : 689-711). Particle size, density, viscosity, degree

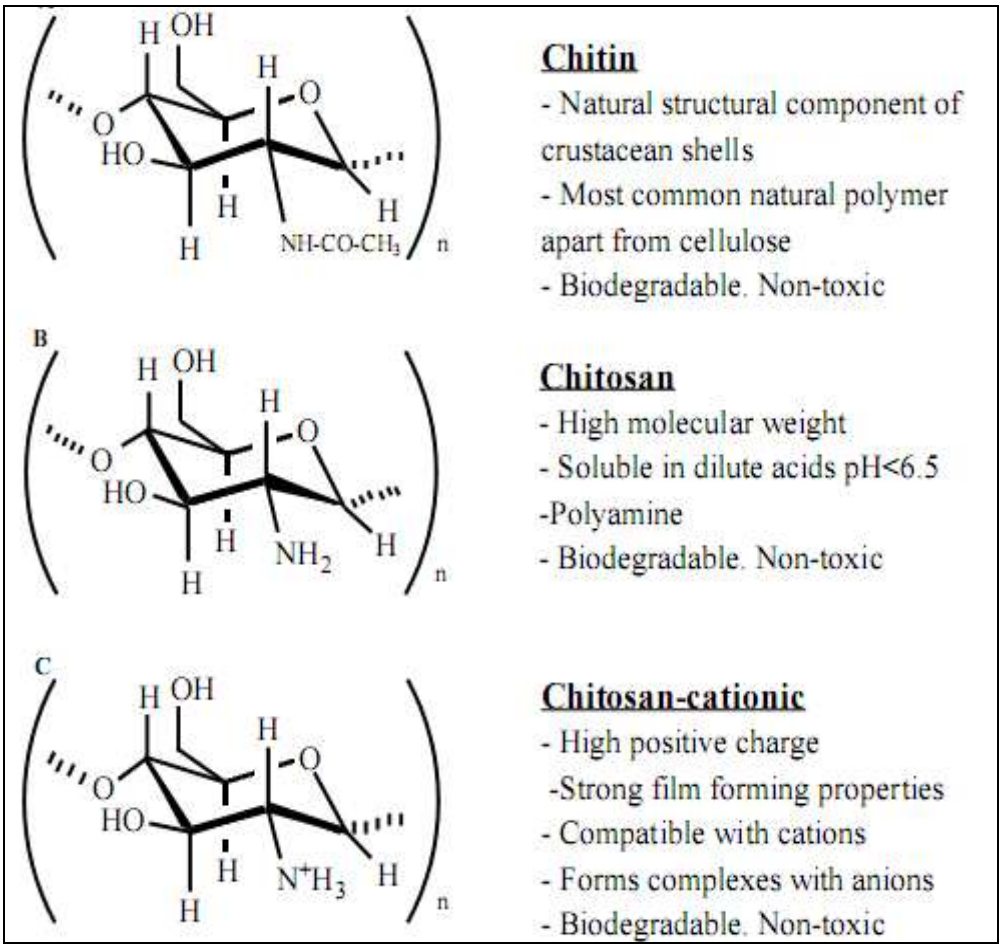


Figure 5 Chemical structures of chitosan and chiosan cationic.

Source: Maria Jose Cano-Cebrian et al., "Intestinal Absorption Enhancement via the Paracellular Route by Fatty Acids, Chitosans and Others: A Target for Drug Delivery," Current Drug Delivery 2 (2005) : 10.

of deacetylation, and molecular weight are important characteristics of chitosan which influence the properties of pharmaceutical formulations. Chitosan is a catio-

nic polymer favorable for interpenetration of polymer and glycoprotein chains into mucous (Felt et al. 1998 : 185-193). The positive charge of chitosan polymer gets to strong electrostatic interaction with mucus on the mucosal surface (Illum et al. 1994 : 1186-1189). Chitosan and its derivatives are low-toxic biocompatible polymeric absorption enhancers because it is metabolized by lysozyme. Because of their mucoadhesive as well as absorption enhancer properties, these cationic polysaccharides have been extensively studied as excipients for drug delivery across nasal, dermal, pulmonary epithelial barriers, and gastrointestinal. Chitosan also shows good bioadhesive characteristics and can reduce the rate of clearance of drug from the nasal cavity thereby increasing the bioavailability of drugs incorporated in it (Soane et al. 1999 : 55-65). The chitosan induced reversible increase in TJ permeability involves the redistribution of occludin, ZO-1 and the actin-cytoskeleton in Caco-2 cells. They are not absorbed and systemic side effects are thus excluded. In addition, prolonged localization in the mucosa is ensured, which in turn would prolong the promoting effect. Chitosan and its derivatives are interest as excipients and drug carriers in the pharmaceutical field and they are promising candidates to enhance drug delivery in clinical setting. Many publications have dealt with the absorption enhancing effects of chitosan and its derivatives in epithelia. (Mária A. Deli 2009 : 892-910; Cano-Cebrian et al. 2005 : 9-22)

Schipper et al. (1996 : 1686-1692) investigated the effect of chitosan structural characteristics (molecular weight and degree of deacetylation) on their absorption enhancing properties *in vitro* (Caco-2 cell monolayers), using chitosan

hydrochloride salts at pH 5.5. It was found that the capacity of chitosan to improve mannitol transport is dependent on molecular weight and the degree of deacetylation; accordingly, while chitosans with a high degree of deacetylation were efficient as permeation enhancers at low and high MW, those with low degrees of deacetylation were efficient only at high molecular weights. Most subsequently published articles in this field coincide that > 80% deacetylation affords the greatest promoter effect on cells in culture. Shipper et al. (1997 : 923-929) also investigated the mechanism underlying absorption enhancement at molecular level, using two chitosans with different chemical compositions and molecular weights. Even though the chitosans reportedly induced absorption enhancement with different kinetics and displayed different toxicities, their mechanisms of action were found to be very similar. The polymers were able to bind tightly to the epithelium and to induce redistribution of cytoskeletal F-actin and the TJ protein ZO-1, this being followed by enhanced transport via the paracellular pathway. The authors also concluded that the binding and promoting effects of chitosan are mediated by their positive charges. This was further confirmed by Dodane et al (1999 : 21-32) using chitosan hydrochloride with a degree of deacetylation of 80%. Involvement of TJs was visualized by confocal laser scanning microscopy using occludin and ZO-1 proteins. They also observed a slight perturbation of the plasma membrane, indicating an increased intracellular uptake. According to the authors, this observation suggests an action of chitosan upon intracellular uptake. A transient effect of chitosan on the cell barrier was proposed, since reversible effects on the permeability and structure of Caco-2 cell

were detected. Two chitosan salts, chitosan hydrochloride and chitosan glutamate, were evaluated for their ability to enhance the permeation of [^{14}C] mannitol across Caco-2 cell monolayers at two pH values, 6.2 and 7.4. At low pH both chitosans showed a pronounced effect on the permeability of the marker, leading to 25- (glutamate salt) and 36- fold hydrochloride salt) enhancement. However, at pH 7.4 both chitosans failed to increase the permeability, due to solubility problems. The authors concluded that there is a need for chitosan derivatives with increased solubility for use as absorption enhancers in more basic environments such as in the large intestine and colon (Kotze et al. 1998 : 35-46).

CHAPTER 3

MATERIALS AND METHODS

1. Materials

1.Acetic acid (Lot. E15W69, CAS NO. 64-19-7, J.T. Baker, Thailand

2.Acetonitrile HPLC (Batch No. 09 07 0012, LAB SCAN, Thailand)

3. Chitosan M.W. 460 kDa, 85 % degree of deacetylation (Seafresh Lab co., Ltd., Thailand)

4. Hydrochloric acid 37% (CAS 7647-01-0, QRec, Pinang)

5. Hydrogen peroxide 30% (Ultrex®, Canada)

6. KBr (MERCK, Germany)

7. L-Glutamic acid (B/NO. AF609071, Ajax Finechem, New - Zealand)

8. Methanol HPLC grade (Batch No. 09 07 0085, LAB SCAN, Thailand)

9.Phenol red (CAS 143-74-8, Sigma Aldrich, New Zealand)

10.Sodium chloride (UNIVAR®Ajax Finechem)

11.Sodium taurodeoxycholate (Sigma-Aldrich, New Zealand)

12. Sterile Water for Irrigation (General Hospital Products Public)

13. Taurodeoxycholic acid sodium (CAS 207737-97-1, Sigma Aldrich, New Zealand)

14.Tris (hydroxymethyl) aminomethane (Pacific Science, Thailand)

15. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide ; M-TT (St. Louis, Sigma Aldrich, USA)

16.5-Aminosalicylic acid (EC NO. 2019191, Fluka Chemika, Swizer-land)

2. Equipments

17. Cellulose acetate filter 0.45 μm (Sartorius AG. 37070, Germany)

18. Centrifuge (Universal 320R, Labquip, , Thailand)

19. Column (Eclipse XDB-C18-5 μm, 150x4.6 mm, Agilent, USA)

20. Differential scanning calorimeter (DSC) (model Sapphire, Perkin-Elmer,USA)

21. Fourier transform infrared spectrophotometer (FTIR) (Magna-IR system 750, Nicolet Biomedical Inc., USA)

22. Epithelial volt/ohmmeter (Millipore ERS, United States)

23. High performance liquid chromatography (HPLC) (UV 2070 plus, Jasco, Japan)

24. Hot air oven

25. Laser scattering particle size distributibution analyzer (LA-950, Ho-riba, Japan)

26. Light microscope (CX41RF, Olympus, Japan)

27. Magnetic stirrer (Model: NTB.07024524, Becthai)

28. Micropipette 20-100 μl, 100-1000 μl (masterpette, Bio-Active Co.,

Ltd)

29.pH Meter (SevenEasy pH, Mettler Toledo InLab®413, Switzer-land)

30.Scanning electron microscope (SEM) (model MX 2000, Cam Scan, England)

31.Side-by-side diffusion cells (3 mL, 0.951 cm^2, Ussing style chamber, Japan)

32.Spray dryer (SD-06 Brand, Lab-Plant Ltd./UK)

33.UV Spectrophotometer (Lamda 2, Perkin Elmer, USA)

34.6-well cell culture cluster (Costar®; Corning Invorporated, USA)

35.96-well cell culture cluster (Costar®; Corning Invorporated, USA)

3. Methods

3.1 Test System Validation

Test system was study to confirm that the isolated porcine colon epithelium can be used for *in vitro* drug permeation test during 4 h period without epithelium cell damage.

3.1.1 Preparation of isolated colon epithelium

Porcine colons were obtained immediately after slaughter in the general slaughterhouse (Nakhon Pathom, Thailand). The segment of 10 cm length next to the ileocecal valve around 20 cm of the proximal colon was collected (Figure 6). The colon was rinsed and placed in ice-cold physiological saline (0.9% w/v NaCl solution).

Afterwards, the colonic segment was cut along the mesenteric border. Epithelium layer was separated from the muscular and serosal layers using a slide strip technique (Traynor and Grady 1996 : C848-C858). The mucosal or epithelium layer was cut into small pieces around 3.5x3.5 cm^2.

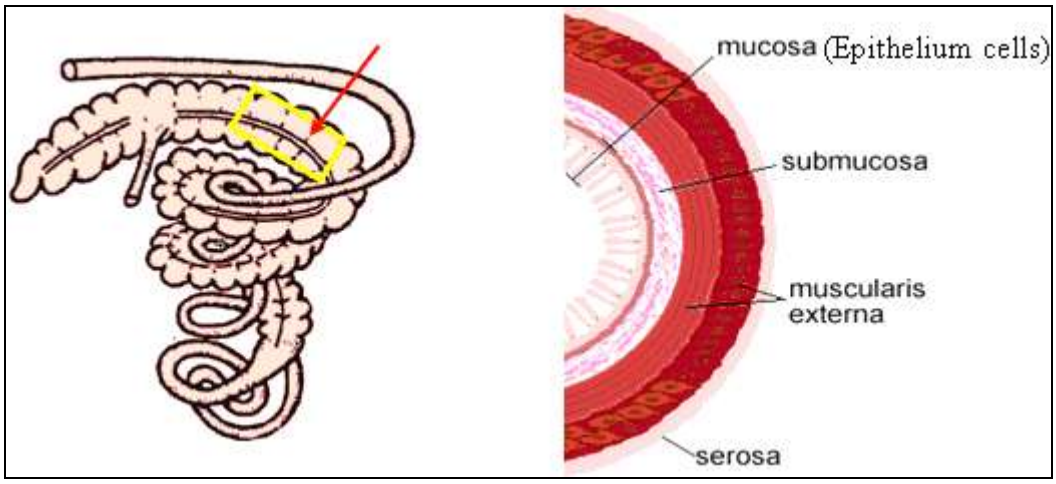


Figure 6 Porcine colon and its cross-section structure

Source : ประไพพรรณ สิทธิกูล, การผลิตสุกร [ออนไลน์], เข้าถึงเมื่อวันที่ 21มกราคม 2554. เข้าถึงได้จาก http://courseware.rmutl.ac.th/courses/110/unit000.html

: Donna Myers, Colon Anatomy [Online], accessed 20 January 2011. Available from http://coloncancer.about.com

3.1.2 Measurement of the transepithelial electrical resistance (TEER)

The tightness of the intercellular junctional complex can be characterized as a leaky to moderately leaky epithelium by measuring the transepithelial electrical resistance (TEER) that exists across the cell monolayers because of the restriction of movement of ions imposed by the TJs. It was shown

conclusively in the 1970s that the permeability of ions through a simple epithelium (gall bladder from Necturus) was primarily paracellular, through the TJs and the lateral space between the cells, rather than across the cell membrane itself (Fromter and Diamond 1972 : 9-13). TEER value of a leaky epithelium was in rang of 10–50 Ω cm^2 indicating the high permeability of the membrane, whereas a tight epithelium has a high TEER value of >1000 Ω cm^2 indicating the low permeability of the membrane (Ward, Tippin and Thakker 2000 : 346-358). The measurement of the TEER values was also used to predict the paracellular transport of hydrophilic molecules (Merwe et al. 2004 : 225-235).

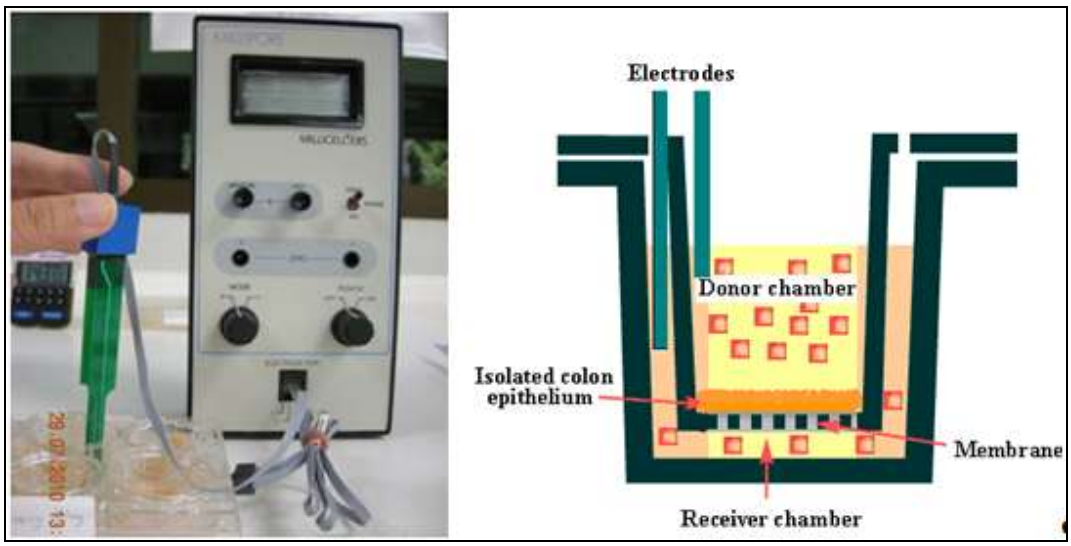


Figure 7 Epithelial volt/ohmmeter (Millipore_ERS, United States) connected to a pair of chopstick electrodes and 6-well plates diagram**.**

The integrity of the isolated colon epithelium was determined by measurement of TEER using the epithelial volt/ohmmeter (Millipore_ERS, United States) connected to a pair of chopstick electrodes (Borchard et al. 1996 : 131-138). Each of the isolated colon epithelium was placed into individual wells

of 6-well plates (Costar®; Corning Invorporated, USA) as shown in Figure 7. The isolated colon epithelium was mounted between donor and receiver chamber. 2.5 mL of pH 5.5 Tris- HCl buffer was added into donor chamber (mucosal side) and 2.5 mL of pH 7.4 Tris-HCl buffer was added into receiver chamber (serosal side). TEER of the isolated colon epithelium was measured at 1, 2 and 4 h. TEER of the blank individual wells was also measured. The experiment was performed in triplicate. The TEER values of colon epithelium were reported after subtraction with the TEER value of the blank which was $201 \pm 1.73\ \Omega.\ cm^2$.

3.1.3 Tissue viability assay

The MTT assay was evaluated from mitochondrial dehydrogenase activity in living cells. MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide, is a yellow, water-soluble compound that is enzymetically reduced to dark purple and insoluble formazan by viable cells. The protocol used in this study was adapted from the earlier study conducted with excised cornea (Imbert and Cullander 1999 : 39-50).

The isolated colon epithelium was mounted between donor and receiver chamber into individual wells of 6-well plates. 2.5 mL of pH 5.5 Tris-HCl buffer was added into donor chamber (mucosal side) and 2.5 mL of pH 7.4 Tris-HCl buffer was added into receiver chamber (serosal side). At various time interval, 0, 2 and 4 h, the isolated colon epithelium were taken and cut into small pieces around 1 mm^2 by surgical scissors and then being weighed. The isolated colon epithelium untreated with 30% H_2O_2 for 30 min was used as a positive control and that treated with 30% H_2O_2 was used as a negative control. The cut

sample was placed into individual wells of 96-well plates (Costar®; Corning Incorporated, USA). MTT solution, 250 µL was added to each well, and the plate was incubated for 2 h at 37±1°C in a shaking chamber. After incubation, the remaining MTT solution was removed and rinsed twice with 500 µL of pH 7.4 Tris-HCl. The solution of 99% ethanol and 1% conc. hydrochloric acid, 500 µL was added to each well to extract formazan from the cells. The absorbance (A) of the solution was measured at 550 nm and the TR index were calculated (TR index = A/ mg of tissue). The experiment was performed at least in triplicate.

3.2 *In vitro* permeation study

3.2.1 Effects of CSG on permeation of 5-ASA

3.2.1.1 Preparation and characterization of spray dried chitosan glutamate (CSG)

- Preparation of CSG

Chitosan (CS) flakes (M.W. 460 kDa, 85 % degree of deacetylation) was dissolved in an aqueous solution of glutamic acid (at molar ratio of CS: glutamic acid 1: 0.8) to make a 0.5 % w/v CS acidic solution under stirrer for 12 h. The solution was filtered and spray dried by using a spray dryer (Model: SD-06 Brand, Lab Plant Ltd, England) as shown in Figure 8. The spray drying condition was as follows: inlet temperature of 130±1 °C, outlet temperature of 80±5 °C, flow rate 5 mL/min and the obtained CSG powders was collected and kept in a desiccator prior to use.

Figure 8 Spray dryer (Model: SD-06 Brand, Lab Plant Ltd, England)

- Characterization of CSG

Morphology study : The morphology of CSG was observed under a scanning electron microscope (SEM, model MX 2000, Cam Scan, Cambridge, England). The samples were attached to the slab surfaces with double sided adhesive tapes and then coated with gold to thickness about 30 nm under vacuum to make the sample conductive. Scanning electron photo-micrographs were taken at magnification of 1000x.

Particle size : The particle size of CSG was deter-mined by using laser scattering particle size distribution analyzer (LA-950, Horiba, Japan)

Thermal behavior : DSC thermograms of CSG, CS and glutamic acid were measured using a scanning calorimeter (model Sapphire, Perkin-Elmer, USA). The samples of 2-3 mg were accurately weighed into solid aluminum pans with covers. The measurements were performed over 20-300 °C at a heating rate of 10 °C/min under nitrogen purge. TGA thermogram of CSG was measured using a thermogravimetric analyzer (model TGA7, Perkin-Elmer, USA). The sample amount of 5-10 mg was weighed into an aluminium pan. The measurements were conducted over 20-300 °C at a heating rate of 10 °C/min under nitrogen purge.

Fourier Transform Infrared (FTIR) Spectroscopy :

Transmission infrared spectra of CS and CSG were measured using a Fourier transform infrared spectroscope (model Magna-IR system 750, Nicolet Biomedical Inc., Madison, WI, USA). The CS and CSG were measured by KBr method. The IR spectra were recorded in the region of 4000 – 400 cm^{-1}. The spectrum was an average of 32 scans with 4 cm^{-1} resolution.

3.2.1.2 Permeation study

Since the pH of colon is in range of 5.5-7.0 (Haeberlin and Friend 1992 : 1-44). pH 5.5 Tris-HCl buffer was chosen to prevent the precipitation of the CSG in the donor chamber. However, the pH of solution was drop to around 4.6 when dissolving the drug in the buffer solution.

The isolated colon epithelium was mounted between two half-cells of a side by side diffusion chamber (3 mL, 0.951 cm^2, Ussing style chamber, Japan) in Figure 9.

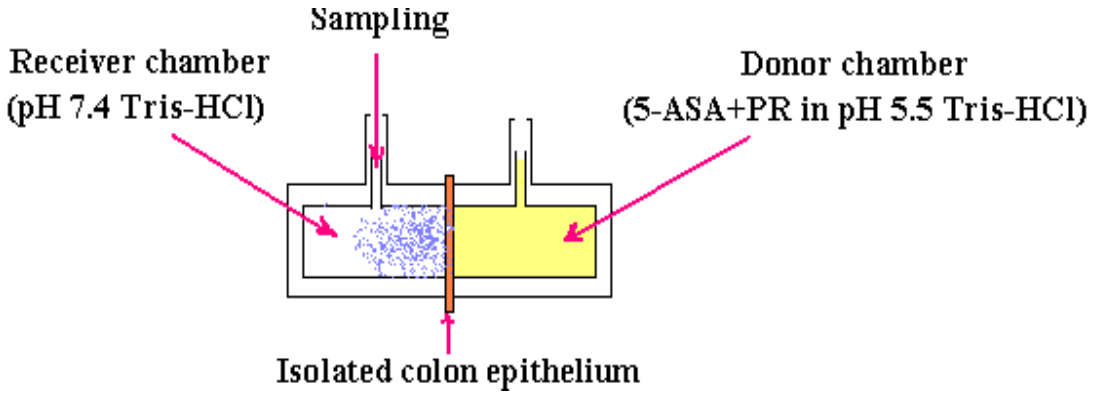


Figure 9 Model of side by side diffusion cells

The solution containing 5 mM 5-ASA, 0.1 mg/mL phenol red and CSG at various concentrations (0.01, 0.5 and 2.0 %w/v), was added into the donor chamber (mucosal side) and 3 mL of pH 7.4 Tris-HCl buffer was added into the receiver chamber (serosal side) with continuous air flow. The temperature of donor and receiver chambers was maintained at 37± 0.5 °C. Each 1 mL of the sample was collected from the receiver chamber at 30, 60, 90, 120, 150, 180, 210 and 240 min and the equal volume of fresh pH 7.4 Tris-HCl was added for replacement. The permeation of pure 5-ASA through isolated colon epithelium was also investigated as a control. The experiment was done for at least triplicate ($n \geq 3$).

Note : Phenol red (Phenolsulfonphthalein; PR), a water-soluble dye, is a well known poorly absorbed compound from the rat stomach and small intestine. It is generally used as a non absorption marker in *in vitro*, *in situ* and *in vivo* absorption experiments (Jovov et al. 1991 : 196-203). 0.1 mg/mL of PR was also added into the donor chamber every permeation tests to investigate the leakage of the isolated colon epithelium during the experiment.

3.2.1.3 Analysis Study

- Analysis of 5-ASA by high performance liquid chromatography (HPLC) method

The samples at various time intervals were filtered and centrifuged at 11,000 rpm for 10 min. The supernatant was diluted to 1: 2 with pH 7.4 Tris-HCl and sonicated for 30 min to remove air bubbles. The amount of 5-ASA permeation through isolated colon epithelium was analysed by using a high perfor-mance liquid chromatography (HPLC, UV 2070 plus, Jasco, Japan). The sample was injected into HPLC under the conditions in Table 4. In each permea-tion experiment, the permeation profile of drug was obtained by plotting the amount permeated (µmol) versus time (h), and then the slope (mol/s) was taken as the permeability rate from the linear regression equation (as shown in appendix : Figure 18-29). The apparent permeability coefficient (P_{app}) was calculated afterward.

Table 4 Analysis conditions of 5-ASA using HPLC

Column	Eclipse XDB-C18 5 µm, 150 mm x 4.6 mm column, Japan
Mobile phase	Acetonitrile : water = 22:78 v/v and 0.5% acetic acid
Flow rate	0.6 mL/min.
Detector	UV detector at 300 nm.
Injection volume	20 µL

- Analysis of phenol red by UV-VIS spectroscopy

The amount of phenol red in the receiver chamber

at various time intervals was analyzed by using a UV-VIS Spectrophotometer (Lamda 2, Perkin Elmer, USA) and the absorbance of the samples was determined at maximum wavelength of 559 nm. In each permeation experiment, the permea-tion profile of PR was obtained by plotting the amount permeated (µmol) versus time (h), and then the slope (mol/s) was taken as the permeability rate from the linear regression equation (Appendix : Figure 30-41). Then, the P_{app} was calculated follow the equation (1).

- Calculation of the apparent permeability coefficients (P_{app})

The apparent permeability coefficients (P_{app}) were determined according to the following equation : (1)

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0} \qquad \text{..... (1)}$$

where P_{app} is the apparent permeability coefficient (cm/s), dQ/dt is the permeability rate of the drug (mol/s; dQ/dt was obtained from the slope in linear regression equation of permeation profiles), *A* is the diffusion area of the Ussing-type chamber (0.951 cm^2), C_0 is the initial concentration of the drug in the donor chamber (5 x 10^{-6} mol/cm^3). These *in vitro* experiments were assessed during sink conditions.

The results of P_{app} were reported as means ($\pm$SD). The statistical difference between treatment groups was evaluated using analysis of variance (ANOVA) and the identification of significances at *p* values 0.05 was carried out with LSD's post hoc test.

3.2.1.4 Measurement of the transepithelial electrical resistance (TEER)

Before and after the permeation test, the integrity of all the isolated colon epithelium was investigated as mentioned before in section 3.1.2.

3.2.1.5 Tissue viability assay

After the permeation test and measurement of TEER values, the tissue viability of isolated colon epithelium was investigated as mentioned before in section 3.1.3.

3.3 Effects of sodium taurodeoxycholate (NaTDC) on permeation of 5-ASA

The effects of bile salt, NaTDC was studied by adding various concentration of NaTDC (0.01, 0.5 and 2.0 %w/v) into the donor chamber and drug permeation test and the processes of analysis studies were investigated under the same conditions as in section 3.2.1.2-3.2.1.3. The measurement of the TEER and tissue viability assay was investigated as mentioned before in section 3.1.2-3.1.3.

CHAPTER 4

RESULTS AND DISCUSSION

1. Test System Validation

TR index values of the isolated colon epithelium at 0, 2 and 4 h were calculated as shown in Table 5. The TR index was decreased from 0.046±0.004 to 0.044±0.003 and 0.039±0.004 at 0, 2 and 4 h, respectively. According to Hood's criteria, TR index above 0.025 is from viable samples (Imbert and Cullander 1997 : 666-674). The results indicated that the viability of the isolated colon epithelium slightly decreased as the time increased, suggesting the few cell damaged. It was concluded that the isolated porcine colon epithelium can be used for *in vitro* drug permeation test during 4 h period.

Table 5 TR index values of the isolated colon epithelium in Tris-HCl buffer at various times

Time (h)	TR Index (means ± S.D., n = 3)
0	0.046±0.004
2	0.044±0.003
4	0.039±0.004

Note : TR index value of the isolated colon epithelium treated with H_2O_2 (negative control) was 0.004±0.000

The TEER values of the isolated colon epithelium (after subtraction with that of the blank) at 0, 2 and 4 h are shown in Table 6. They were in range of 56-59 $\Omega.cm^2$ and not significantly different during 4 h period of the experiment. The result exhibited well aligned cell integrity of the epithelium cells (Illum 1998 : 1326-1331).

Table 6 TEER values of the isolated colon epithelium in Tris-HCl buffer at various times

Time (h)	TEER ($\Omega.cm^2$) (means ± S.D., n = 3)
0	56±1.2
2	59±0.6
4	56±3.5

* $p<0.05$ (t-test)

2. *In vitro* permeation study

2.1 Effects of CSG on permeation of 5-ASA

2.1.1 Characterization of spray dried chitosan glutamate (CSG)

- Morphology Study

The morphology of chitosan glutamate (CSG) was investigated by means of SEM analysis in Figure 10. The particles appeared as spherical shape with smooth surfaces.

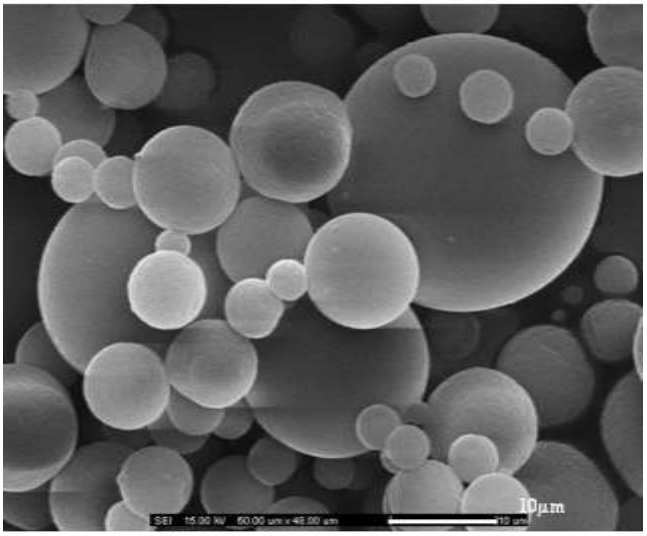


Figure 10 Scanning electron photomicrograph of CSG

- Particle size of CSG

The mean particle size of CSG was 29.24±0.28 µm in diameter.

- Thermal behavior

DSC thermograms of CS, CSG and glutamic acid are illustrated in Figure 11. In the DSC thermogram of CS, the broad exothermic peak due to water loss around 20-100 °C and the endothermic decomposition peak at onset around 233 °C were observed. The DSC thermogram of glutamic acid showed the sharp melting peak at 208.2 °C. In the DSC thermogram of CSG, the broad endothermic peak due to water loss around 20-130 °C, and the melting peak at onset around 130 °C were observed. After spray drying, the moisture content of CSG was higher while the decomposition temperature was lower than those of original CS.

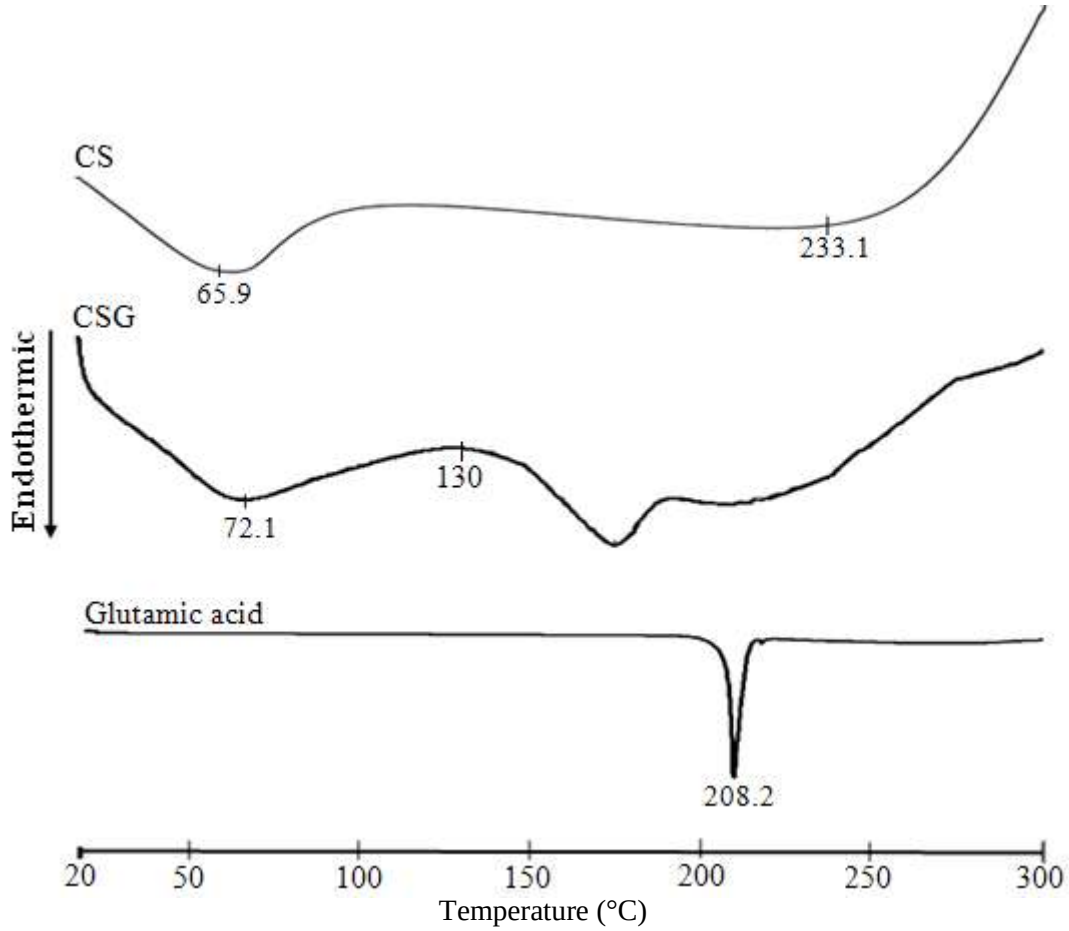


Figure 11 DSC thermograms of CSG, CS and glutamic acid

The TGA thermogram of CSG is illustrated in Figure 12. The two steps of weight loss were observed. The change in weight loss at around 25-123 °C was assigned to the dehydration of water and the change at temperature around 123 °C was assigned to the decomposition of the polymer. The result was in good agreement with that of the DSC thermogram. The less thermal stability of CSG was similar to that of chitosan acid salt complexes, chitosan terepthalic acid salt complex (Singh and Dutta 2009 : 384-392) and chitosan acetate (Nunthanid et al. 2004 : 15-26).

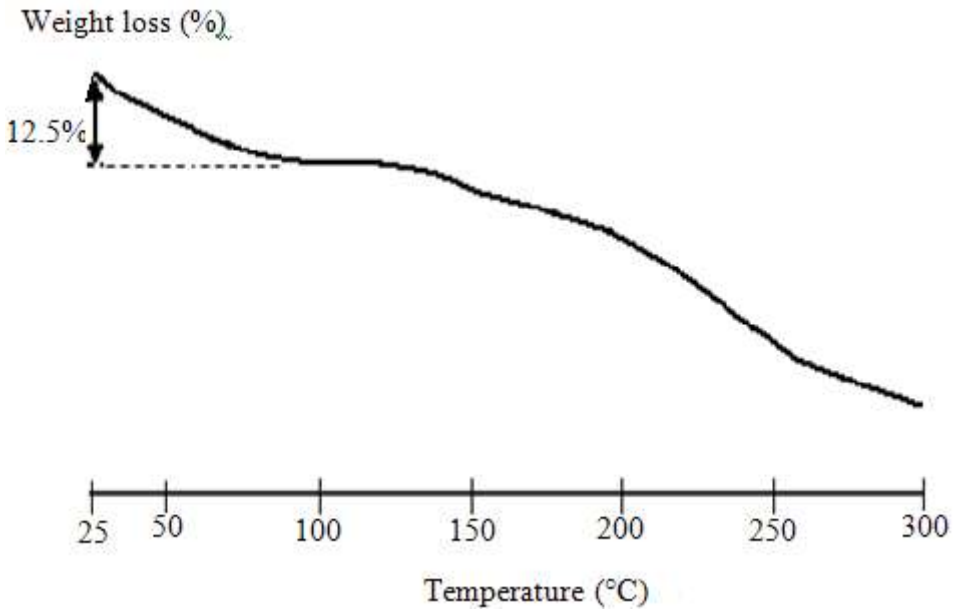


Figure 12 TGA thermogram of CSG

Fourier transform infrared spectroscopy (FTIR)

The transmission infrared spectra of CS and CSG are illustrated in Figure 13. The spectra of CS and CSG exhibited the broad peaks in the range of 3460-3400 cm^{-1} assigned to OH stretching indicating intermolecular hydrogen bonding of CS and CSG molecules. The peak also overlapped in the same region of NH stretching.

The spectrum of CS exhibited the C=O stretching (amide I) peak near 1651 cm^{-1} representing the structure of *N*-acetylglucosamine as well as the NH_2 stretching peak at 1593 cm^{-1} representing the glucosamine functional group. In the spectrum of CSG, the strong peak at 1561 cm^{-1} and the peak near 1400 cm^{-1} region attributed to an asymmetric and a symmetric carboxylate anion stretching respectively were observed. (Silverstein, Bassler and Morrill 1991). This indicated that the spray drying of CS using glutamic acid as a dissolving

vehicle could yield a spray-dried chitosan as glutamate salt. The C=O stretching peak at 1631 cm^{-1} representing the structure of –COOH groups of glutamate was also observed.

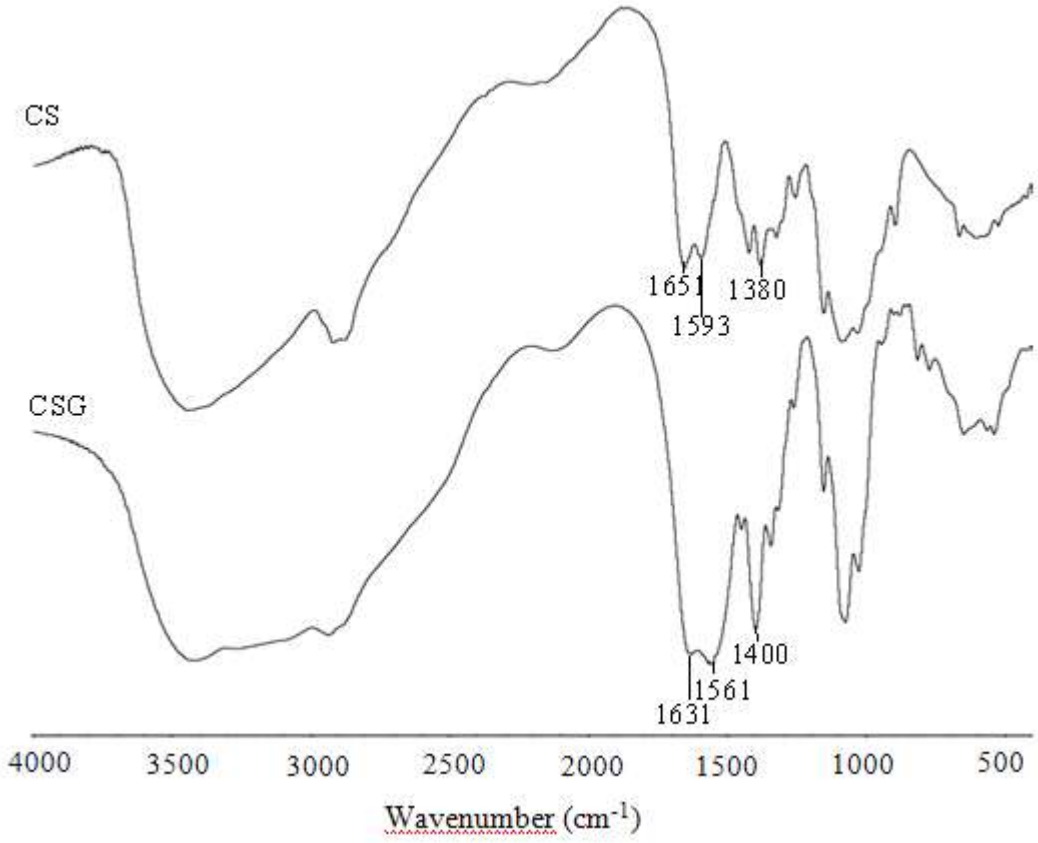


Figure 13 Transmission infrared spectra of CG and CSG

2.1.2 Permeation study

The effect of CSG on the TR index of the isolated colon epithelium in the permeation test during 4 h period was calculated as shown in Table 7. They were in range of 0.034-0.045. After the permeation test (with or without CSG), most of the TR index of the isolated colon epithelium was slightly decreased. It was suggested that the cells were hardly damaged but overall of the

epithelium cells were still viable and CSG showed low cytotoxicity effect on the epithelium cells during the permeation test.

Table 7 TR index values of the isolated colon epithelium with and without various concentrations of CSG after the permeation test (4 h).

Samples	TR Index (means ± S.D., $n \geq 3$)
5-ASA+ PR (control)	0.035±0.002
5-ASA+ PR +CSG 0.01%w/v	0.042±0.005
5-ASA+ PR +CSG 0.5%w/v	0.034±0.002
5-ASA+ PR +CSG 2.0%w/v	0.045±0.004

Note : TR index value of the isolated colon epithelium untreated with 30% H_2O_2 for 30 min (positive control) was 0.046±0.004

TR index value of the isolated colon epithelium treated with H_2O_2 (negative control) was 0.004±0.000

TEER values of the isolated colon epithelium before and after the drug permeation test are shown in Table 8. The initial TEER values of the epithelium were in range of 109-116 $\Omega.cm^2$. After the test, they were significantly decreased ($p<0.05$) as the concentration of CSG was increased. The decrease of ΔTEER when adding CSG at all concentrations was significantly different from that of the control suggesting the effect of CSG on loosening the tight junctions (TJs) of the epithelium cells. In many previous studies, CS was able to bind tightly to the cell membrane of Caco-2 and to induce redistribution of cytoskeletal F-actin

and the TJ protein ZO-1, this being followed by enhanced transport via the paracellular pathway (Shipper et al. 1997 : 923-929). Illium et al. (1994 : 1186-1189) also concluded that the cationic material chitosan was shown to greatly enhance the absorption of insulin across the nasal mucosa of rat and sheep. This was further confirmed by Dodane et al. (1999 : 21-32) using chitosan hydrochloride with a degree of deacetylation of 80% for enhancement of manitol across Caco-2 cells. Chitosan glutamate was also reported to enhance paracellular transport of [^{14}C]-mannitol across Caco-2 cells monolayer (Borchard et al. 1996 : 131-138; Kotz´e et al. 1998 : 35-46).

Table 8 TEER values of the isolated colon epithelium with and without various concentrations of CSG before and after the permeation test.

Samples	TEER ($\Omega.cm^2$) means ± S.D., $n \geq 3$		
	Before	After	ΔTEER
5-ASA+ PR (control)	109±8	98±7*	-11±6
5-ASA+ PR +CSG 0.01%w/v	116±13	80±9*	-36±7**
5-ASA+ PR +CSG 0.50%w/v	115±9	67±6*	-48±11**
5-ASA+ PR +CSG 2.00%w/v	113±17	48±11*	-65±12**

* $p<0.05$ (t-test) and ** $p<0.05$ (LSD's post hoc test)

The permeation of 5-ASA with and without CSG through porcine colon was zero order kinetics (Appendix: Figure 18-29). The apparent permeability coefficients (P_{app}) of the drug and PR were calculated as shown in

Table 9. It was found that the permeability of the drug was decreased as the concentration of CSG was increased. The P_{app} of 5-ASA was significantly decreased from 10.33±1.69 to 8.38±0.07, 8.68±0.22 and 5.41±0.40 ($x10^{-6}$) cm/s (p<0.05) when adding CSG at concentration of 0.01, 0.5 and 2.0 %w/v, respectively. The results of the drug permeation were not in agreement with the decreasing of the TEER values.

Table 9 P_{app} of 5-ASA and PR in the permeation test through the isolated colon epithelium with and without various concentrations of CSG

Samples	$P_{app} \times 10^{-6}$ (cm/s), means ± S.D., $n \geq 3$	
	5-ASA	PR
5-ASA+ PR (control)	10.33±1.69	0.32±0.15
5-ASA+ PR +CSG 0.01%w/v	8.38±0.07*	0.32±0.22
5-ASA+ PR + CSG 0.5%w/v	8.68±0.22*	0.37±0.04
5-ASA+ PR + CSG 2.0%w/v	5.41±0.40*	0.57±0.21*

* p<0.05 (LSD's post hoc test)

The decrease permeation of the drug was due to the drug–polymer interaction between $-COO^-$ groups of 5-ASA and $-NH_3^+$ groups of CS and hence the drug permeation was decreased. This was confirmed by the result from FTIR spectra in Figure 14. CSG was added into the solution of 5-ASA and then dried over night at 50 ºC. The IR spectra of the dried powder, 5-ASA and physical mixture of 5-ASA and CSG were measured. It was found that the carbonyl stretching peak at 1651 cm^{-1} representing the –COOH groups of 5-ASA

disappeared and a new peak at 1553 cm^{-1} and 1403 cm^{-1} attributed to an asymmetric and a symmetric carboxylate anion stretching were observed, respectively. In addition, the new peak at 1630 cm^{-1} assigned to NH_3^+ bending was also observed. The interaction between CS and salicylic acid was also reported by Puttipipatkhachorn et al. (2001:143-153).

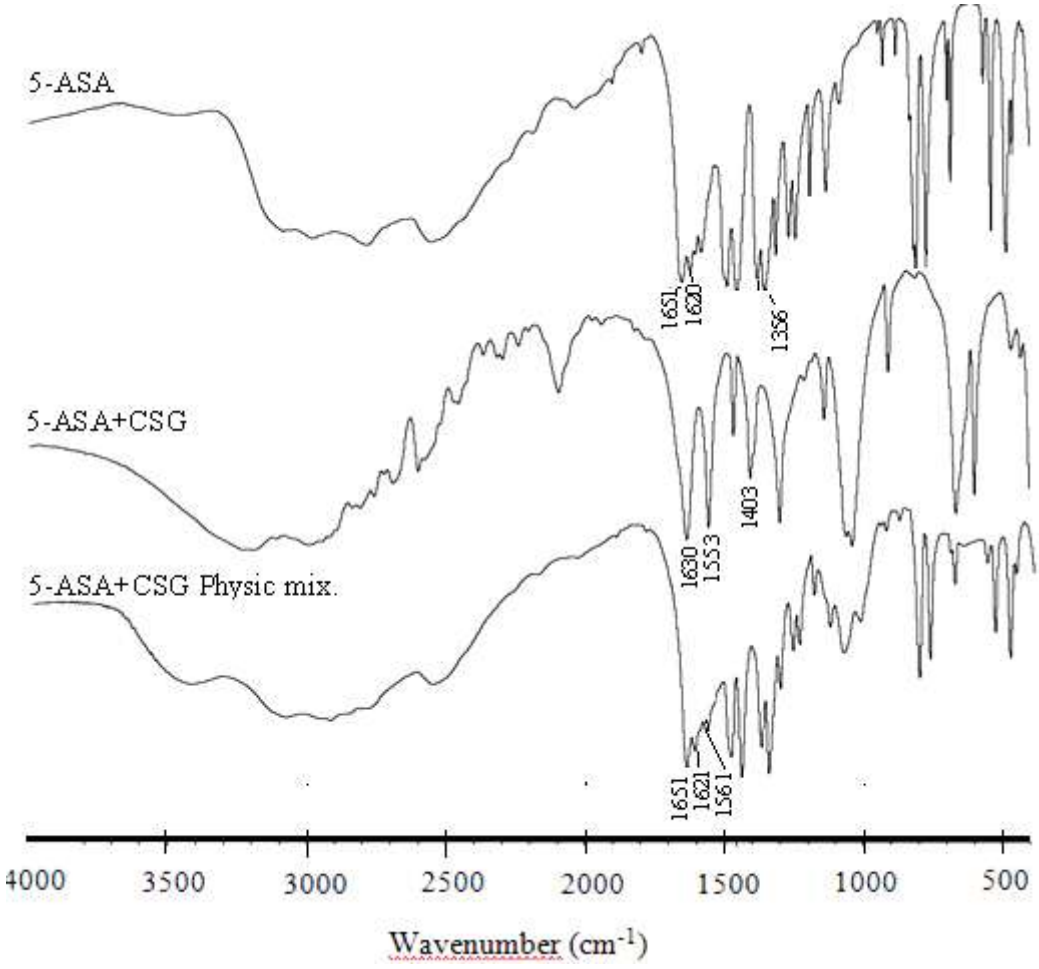


Figure 14 Transmission infrared spectra of 5-ASA, 5-ASA+CSG and 5-ASA+CSG (physical mixture)

The P_{app} of PR in Table 9 was very low and the cumulative amount of the permeated PR was less than 0.1% during the experiment. When adding CSG at concentration of 2.0 %w/v, the P_{app} of PR was significantly increased from 0.32 ± 0.15 to 0.57 ± 0.21 ($x10^{-6}$) cm/s ($p<0.05$). The result was consistent with the effect of CSG on TEER.

To prevent the drug-polymer interaction, the solution of CSG at various concentrations was added into the donor chamber at first 30 min. After removing the solution of CSG, the solution of 5-ASA was replaced into the donor chamber and the processes were continued as mentioned before.

The TR index of the isolated colon epithelium untreated and treated with various concentrations of CSG at first 30 min was calculated as shown in Table 10. They were in range of 0.035-0.048 suggesting the low cytotoxicity effect of CSG on the epithelium cells during the drug permeation test.

The effect of CSG on loosening TJs of the treated epithelium at first 30 min was also observed according to the TEER values in Table 11. ΔTEER of the treated epithelium were significantly different from that of the control. Furthermore, the decrease of TEER values at concentration of 2.0%w/v was the lowest. It was due to the binding between CSG at high concentration and mucin of the epithelium which acts as a barrier for the efficacy of CS to loosening TJs of the cells.

Table 10 TR index values of the isolated colon epithelium untreated and treated with various concentrations of CSG at first 30 min after the permeation test (4 h).

Samples	TR index (means ± S.D., $n \geq 3$)
5-ASA+ PR (control)	0.035±0.002
5-ASA+ PR +CSG 0.01%w/v 30 min	0.048±0.003
5-ASA+ PR +CSG 0.5%w/v 30 min	0.041±0.002
5-ASA+ PR +CSG 2.0%w/v 30 min	0.037±0.002

Note : TR index value of the isolated colon epithelium untreated with 30% H_2O_2 for 30 min (positive control) was 0.046±0.004

TR index value of the isolated colon epithelium treated with H_2O_2 (negative control) was 0.004±0.000

Table 11 TEER values of the isolated colon epithelium untreated and treated with various concentrations of CSG at first 30 min before and after the permeation test

Samples	TEER ($\Omega.cm^2$) means ± S.D., $n \geq 3$		
	Before	After	ΔTEER
5-ASA+ PR (control)	109±8	98±7*	-11±6
5-ASA+ PR +CSG 0.01%w/v 30 min	121±15	48±27*	-73±14**
5-ASA+ PR +CSG 0.50%w/v 30 min	129±10	62±8*	-67±12**
5-ASA+ PR + CSG 2.00%w/v 30 min	125±22	91±14*	-34±12**

* $p<0.05$ (t-test)

The P_{app} of the drug through isolated colon epithelium untreated and treated with various concentrations of CSG at first 30 min was calculated as shown in Table 12. The result showed that the P_{app} of 5-ASA was not significantly increased (p<0.05) when adding CSG at various concentrations. It was due to the interaction between the drug and some CSG that still remained in the donor chambers and hence the enhancement of drug permeation was not observed.

The P_{app} of PR was also calculated as shown in Table 12. When adding CSG at concentration of 0.01, 0.5 and 2.0 %w/v, the P_{app} of PR was significantly increased from 0.32±0.15 to 0.51±0.14 and 0.58±0.17 ($\times 10^{-6}$) cm/s (p<0.05), respectively. The result was consistent with the effect of CSG on TEER values in Table 11 since there was no interaction between PR and CS.

Table 12 P_{app} of 5-ASA and PR in the permeation test through the isolated colon epithelium untreated and treated with various concentrations of CSG at first 30 min

Samples	$P_{app} \times 10^{-6}$ (cm/s), means ± S.D., $n \geq 3$	
	5-ASA	PR
5-ASA+ PR (control)	10.33±1.69	0.32±0.15
5-ASA+ PR + CSG 0.01%w/v 30min	10.53±0.83	0.51±0.14*
5-ASA+ PR + CSG 0.5%w/v 30min	11.99±2.03	0.58±0.17*
5-ASA+ PR + CSG 2.0%w/v 30min	11.49±1.42	0.48±0.17

* p<0.05 (LSD's post hoc test)

2.2 Effect of NaTDC on permeation of 5-ASA

The effect of sodium taurodeoxycholate (NaTDC) on the TR index of the isolated colon epithelium in the permeation test was calculated as shown in Table 13. They were in range of 0.032-0.045 suggesting the low cytotoxicity effect of NaTDC on the epithelium cells during the drug permeation test.

Table 13 TR index values of the isolated colon epithelium with and without various concentrations of NaTDC after the permeation test (4 h).

Samples	TR Index means ± S.D., $n \geq 3$
5-ASA+ PR (control)	0.035±0.002
5-ASA+ PR +NaTDC 0.01%w/v	0.045±0.003
5-ASA+ PR +NaTDC 0.5%w/v	0.035±0.003
5-ASA+ PR +NaTDC 2.0%w/v	0.032±0.003

Note : TR index value of the isolated colon epithelium untreated with 30% H_2O_2 for 30 min (positive control) was 0.046±0.004

TR index value of the isolated colon epithelium treated with H_2O_2 (negative control) was 0.004±0.000

The TEER values of the isolated colon epithelium before and after the drug permeation test are shown in Table 14. The initial TEER values of the isolated colon epithelium were in range of 104-111 $\Omega.cm^2$. After the test, the decrease of the ΔTEER value when adding the bile salt at all concentrations was significantly difference (p<0.05) from that of the control. The TEER value was

decreased as the concentration of NaTDC was increased suggesting the effect of NaTDC on opening the TJs of the epithelium cells.

Table 14 TEER values of the isolated colon epithelium with and without various concentrations of NaTDC at first 30 min after the permeation test.

Samples	TEER ($\Omega.cm^2$) means ± S.D., $n \geq 3$		
	Before	After	ΔTEER
5-ASA+ PR (control)	109±8	98±7*	-11±6
5-ASA+ PR +NaTDC 0.01%w/v	107±11	16±4*	-92±12**
5-ASA+ PR +NaTDC 0.50%w/v	111±16	17±7*	-93±19**
5-ASA+ PR +NaTDC 2.00%w/v	104±2	1±6*	-105±3**

* $p<0.05$ (t-test) and ** $p<0.05$ (LSD's post hoc test)

The effect of NaTDC on *in vitro* permeation of 5-ASA through isolated colon epithelium was evaluated. It was found that the P_{app} increased as the concentration of NaTDC was increased as shown in Table 15. The P_{app} of 5-ASA was significantly increased from 10.33±1.69 to 15.96±3.64, 19.92±1.26 and 23.52±4.14 ($x10^{-6}$) cm/s ($p<0.05$) when adding NaTDC at concentration of 0.01, 0.5 and 2.0 % w/v, respectively. The permeation enhancement of NaTDC was similar to its effect on the permeation of salmon calcitonin through Caco-2 cells. The main mechanism of NaTDC involve in the increases of membrane fluidizing (Song, Chung and Shim 2005 : 298-308). Consequently, the space between the cells was increased resulting in the improvement of drug permeation (Song, Chung and Shim 2002 : 27-37) .

In Table 15, the P_{app} of PR of the control was very low indicating no leakage of the colon epithelium but they were significantly increased from 0.32±0.15 to 1.34±0.14, 1.51±0.18 and 1.68±0.19 ($\times 10^{-6}$) cm/s (p<0.05) when adding NaTDC at concentration of 0.01, 0.5 and 2.0 %w/v, respectively. The result was in good agreement with the P_{app} of the drug and the TEER values.

Table 15 P_{app} of 5-ASA and PR in the permeation test through the isolated colon epithelium with and without various concentrations of NaTDC

Samples	$P_{app} \times 10^{-6}$ (cm/s), means ± S.D., $n \geq 3$	
	5-ASA	PR
5-ASA+ PR (control)	10.33±1.69	0.32±0.15
5-ASA+ PR + NaTDC 0.01%w/v	15.96±3.64*	1.34 ±0.14*
5-ASA+ PR + NaTDC 0.5%w/v	19.92±1.26*	1.51±0.18*
5-ASA+ PR + NaTDC 2.0%w/v	23.52±4.14*	1.68±0.19*

* p<0.05 (LSD's post hoc test)

CHAPTER 5

CONCLUSIONS

This research aimed to study the effects of the polymer, chitosan glutamate (CSG), and the bile salt, sodium taurodeoxycholate (NaTDC), on *in vitro* permeation of 5–aminosalicylic acid (5-ASA). This study used the isolated porcine colon epithelium as the membrane. Validation methods using MTT assay and TEER measurement showed that CSG and NaTDC were not toxic to epithelium cell. This result indicated that isolated porcine colon epithelium can be used as *in vitro* model to drug permeation test up to 4 h.

Since there was some interaction between CSG and the acidic drug, the enhancement of the drug permeability by CSG was inconclusive. Even though, there was the effect of CSG on loosening tight junctions (TJs) of the epithelium according to the decreasing of the TEER values.

The results indicated that only NaTDC could significantly enhance the drug permeability ($p<0.05$) and the apparent permeability coefficients (P_{app}) of the drug increased as the concentration of NaTDC was increased. The ability of NaTDC in improvement of the poor absorbable drugs or hydrophilic molecules across colon epithelium was also confirmed by the permeability of PR and TEER values. The drug permeability was enhanced due to the increasing of membrane fluidization of the epithelium. Consequently, the space between the cells was

increased resulting in the improvement of drug permeation. Taken together NaTDC can increase 5-ASA permeation across isolated colon epithelium by loosening TJ.

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APPENDIX

Preparation of 0.1M Tris-HCl pH 5.5

Preparation of 0.1M pH 5.5 Tris-HCl by 1.2114 g of Tris was dissolved in 50 ml distilled water in a 100 ml volumetric flask. The solution was adjusted pH 5.5 with hydrochloric acid and then adjusted volume with distilled water to 100 ml for make a 0.1 molar solution.

Preparation of 0.1M Tris-HCl pH 7.4

Preparation of 0.1M pH 7.4 Tris-HCl by 1.2114 g of Tris was dissolved in 50 ml distilled water in a 100 ml volumetric flask. The solution was adjusted pH 7.4 with hydrochloric acid and then adjusted volume with distilled water to 100 ml for make a 0.1 molar solution.

Preparation of 5 mM 5-aminosalicylic acid (5-ASA)

Preparation of 5 mM 5-ASA solution by 76.50 mg of 5-aminosalicylic acid was dissolved in 0.1M pH 5.5 Tris-HCl in a 100 mL volumetric flask The solution was adjusted volume with distilled water to make 5 mM solution**.**

Preparation of Phenol red solution

10 mg of phenol red was dissolved in 0.1M pH 5.5 Tris-HCl in a 100 ml to make 0.1 mg/mL solution**.**

Preparation of MTT solution

25 mg of MTT was dissolved in 5 ml freshly-prepared 0.1M pH 7.4 Tris

HCl**.** Any undissolved crystals were removed by filtration through a 0.45 µm cellulose acetate filter.

Preparation of mobile phase

The mobile phase was prepared by using acetonitrile: water 22:78 v/v and 0.5% acetic acid. It was mixed well, filtered through a cellulose filter paper with a pore size 0.45 µm and sonicated for 30 min.

Preparation of 5-aminosalicylic acid stock solution

100 mg of 5-ASA was dissolved in 100 mL of 0.1M pH 7.4 Tris-HCl in a 100 mL volumetric flask and diluted to volume with 0.1M pH 7.4 Tris-HCl to prepare a 5-ASA stock solution having a concentration of 1 mg/mL.

Calibration curve of 5-aminosalicylic acid

A series of standard solution was prepared to give the final concentrations ranging from 1 – 7 µg/mL by serial dilution using 0.1M pH 7.4Tris HCl. The standard solution was measured by HPLC method at wavelength of 300 nm. The calibration curve was obtained by plotting the peak area ratio versus the concentration of 5-ASA. The straight line of best fit was obtained by using regression analysis program.

Preparation of phenol red stock solution

100 mg of phenol red was dissolved in 100 mL of 0.1M pH 7.4 Tris HCl

in a 100 mL volumetric flask and diluted to volume with 0.1M pH 7.4 Tris HCl to prepare a phenol red stock solution having a concentration of 1 mg/mL.

Calibration curve of phenol red

A series of standard solution was prepared to give the final concentrations ranging from 1 – 10 µg/mL by serial dilution using 0.1M Tris HCl pH 7.4. The standard solution was measured for the absorbance UV-VIS spectrophotometry at wavelength of 559 nm. The calibration curve was obtained by plotting the absorbance of phenol red versus the concentration of phenol red. The straight line of best fit was obtained by using linear regression analysis program.

Calculations of apparent permeability coefficient (P_{app})

For example

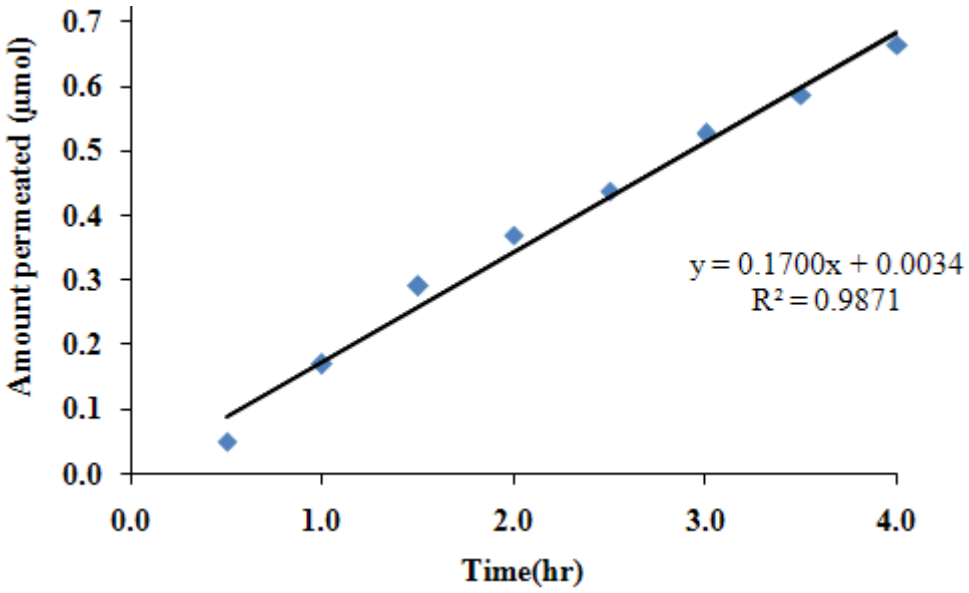

Figure 15 The linear regrestion line of amount permeated of 5-ASA

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{C_0 A}$$

y = 0.1700x+0.0034

$\Delta Q / \Delta t$ = Slope = 0.1700/3600 x 10^{-6} = 4.7 x 10^{-11} mol/s

A = ¶r^2 = (22/7) x $(0.55)^2$ = 0.951 cm^2

C_0 = 5 x 10^{-6} mol/ml

P_{app} = (4.7 x 10^{-11})/ (5 x 10^{-6} x 0.951)

= 9.88 x 10^{-6} cm/s

The calibration curve of phenol red

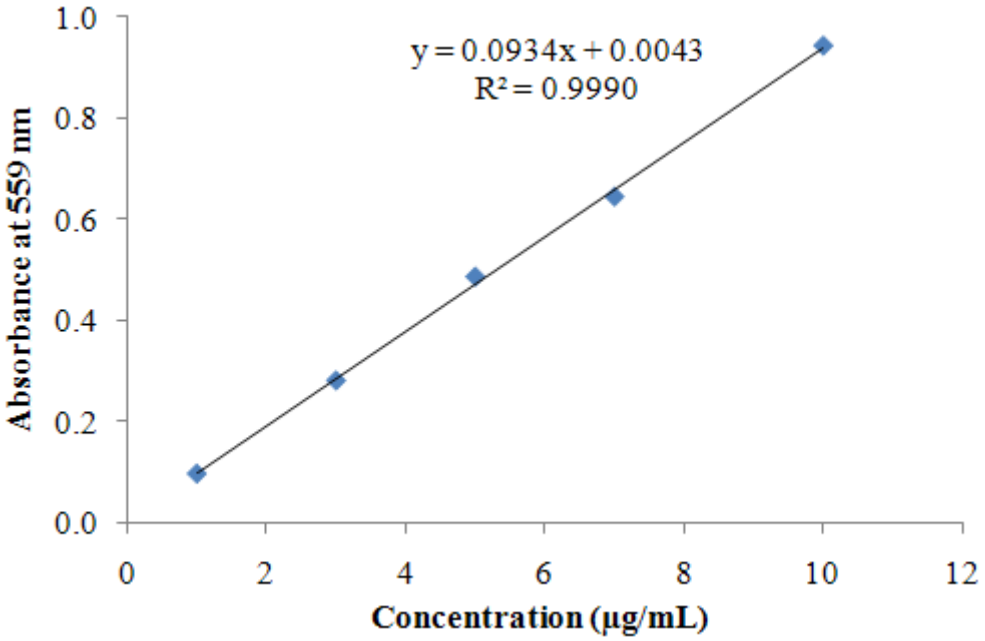

Figure 16 The calibration curve of phenol red

The calibration curve of 5-aminosalicylic acid

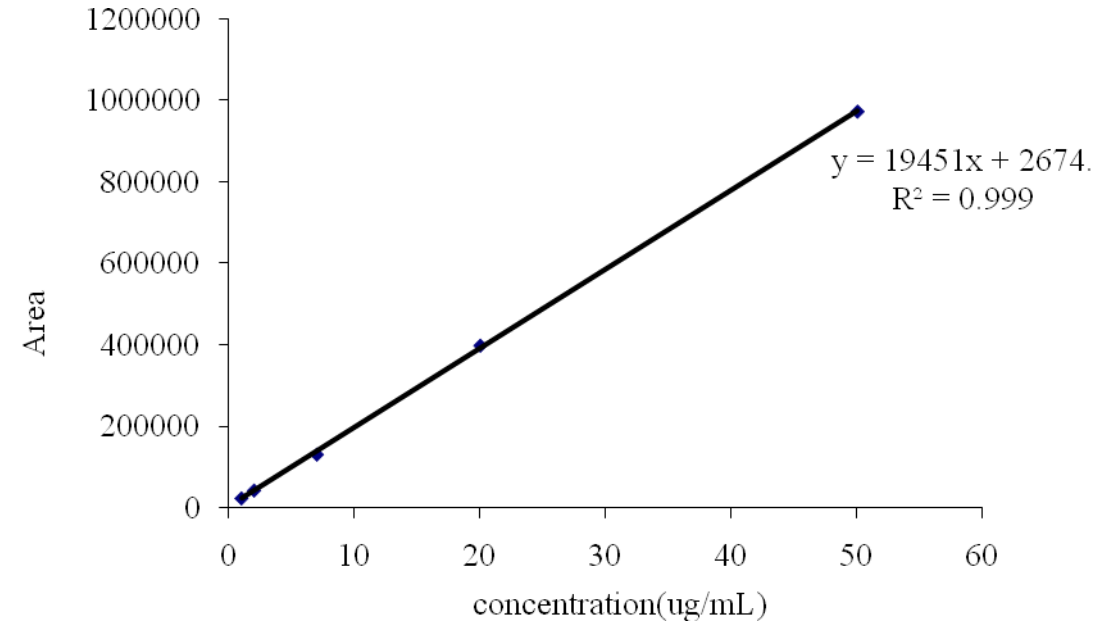

Figure 17 The calibration curve of 5-aminosalicylic acid

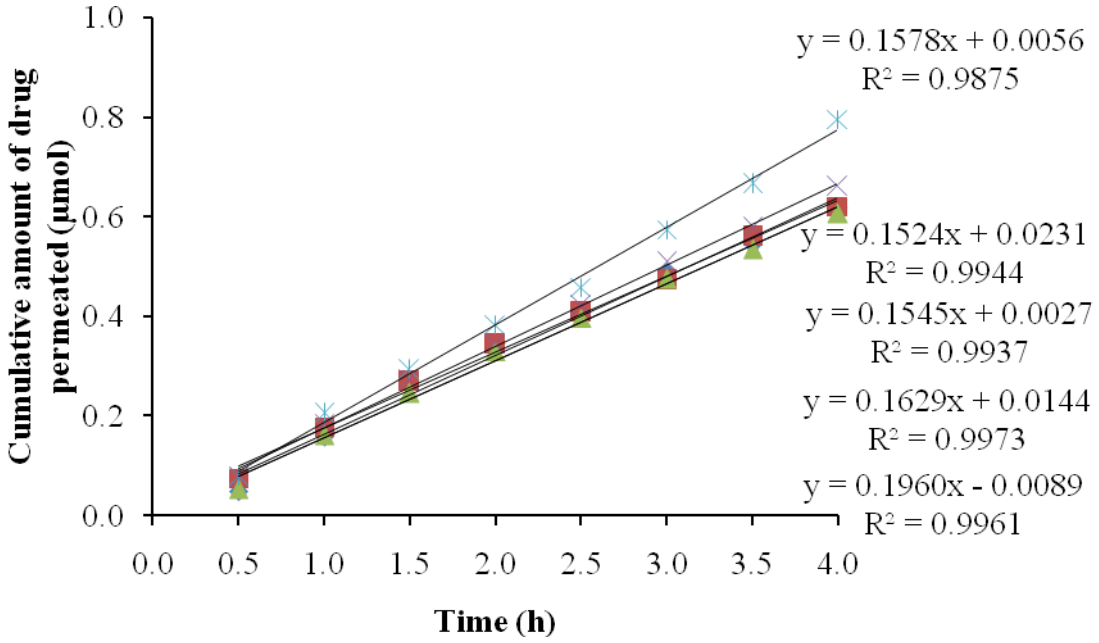

Figure 18 The linear regression line of amount permeated of 5-ASA without CSG through porcine colon at various time intervals (control)

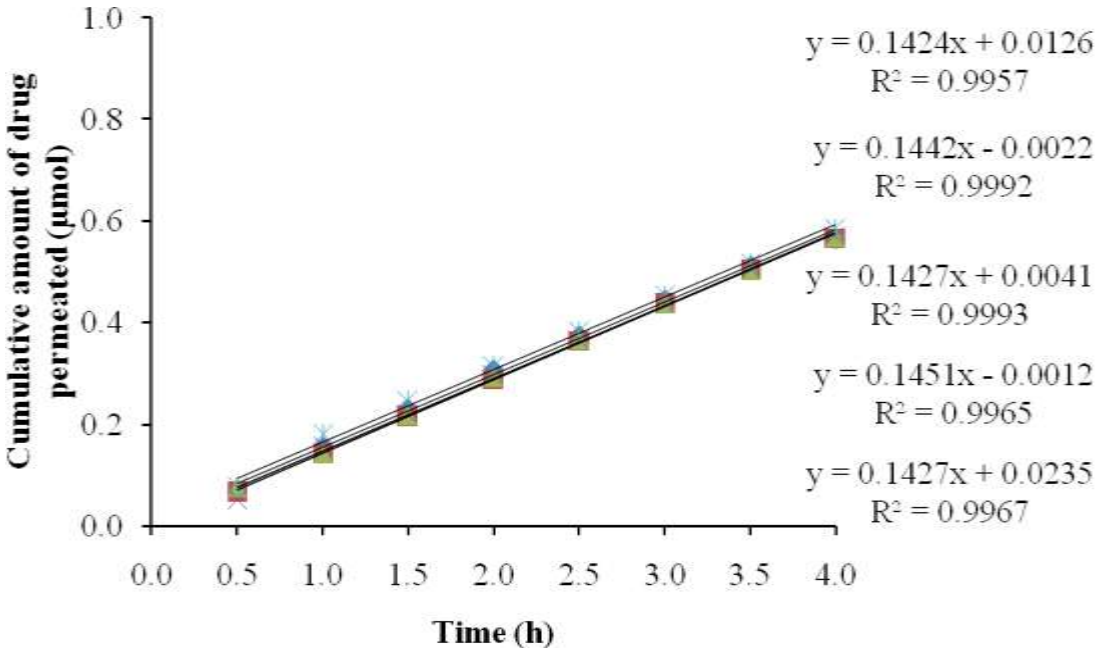

Figure 19 The linear regression line of amount permeated of 5-ASA with 0.01 %w/v CSG through porcine colon at various time intervals

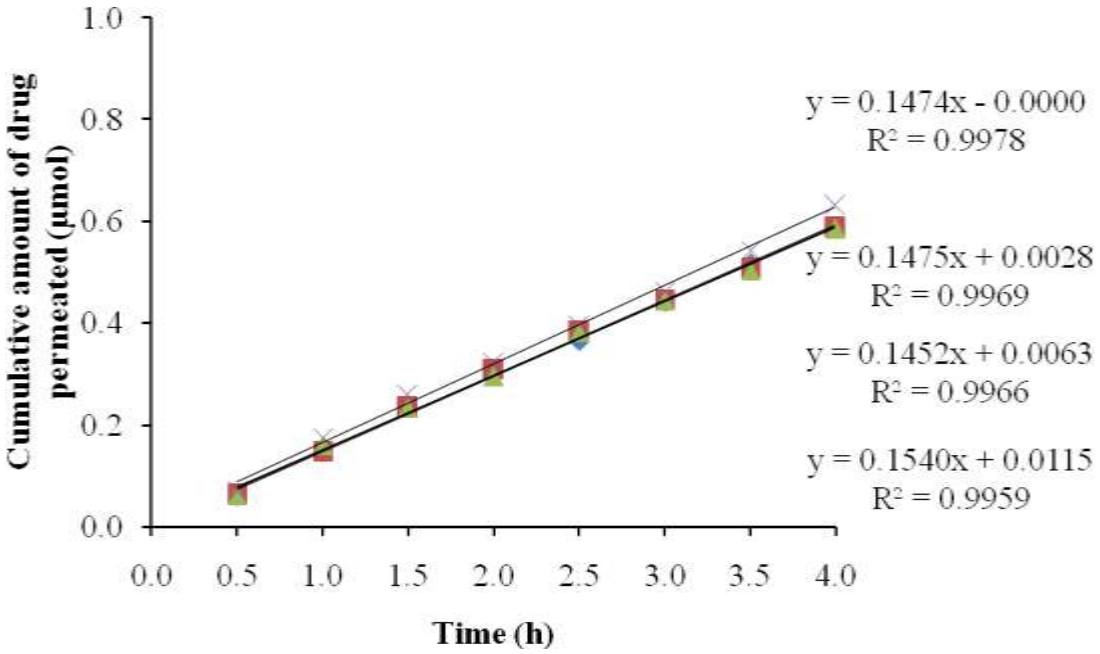

Figure 20 The linear regression line of amount permeated of 5-ASA with 0.5 %w/v CSG through porcine colon at various time intervals

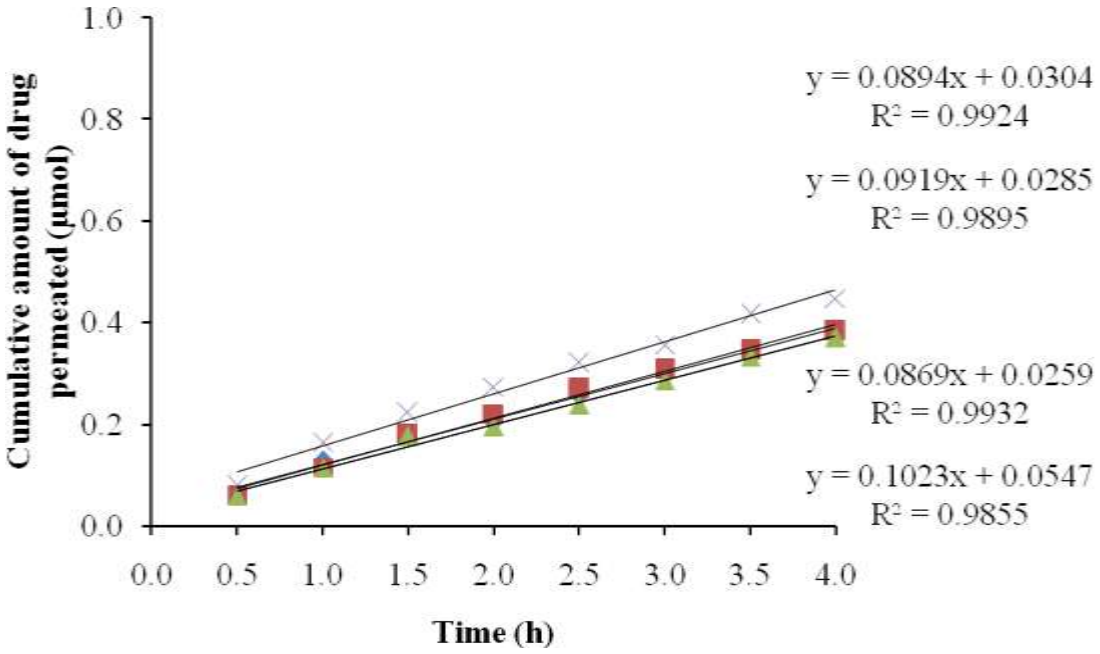

Figure 21 The linear regression line of amount permeated of 5-ASA with 2.0 %w/v CSG through porcine colon at various time intervals

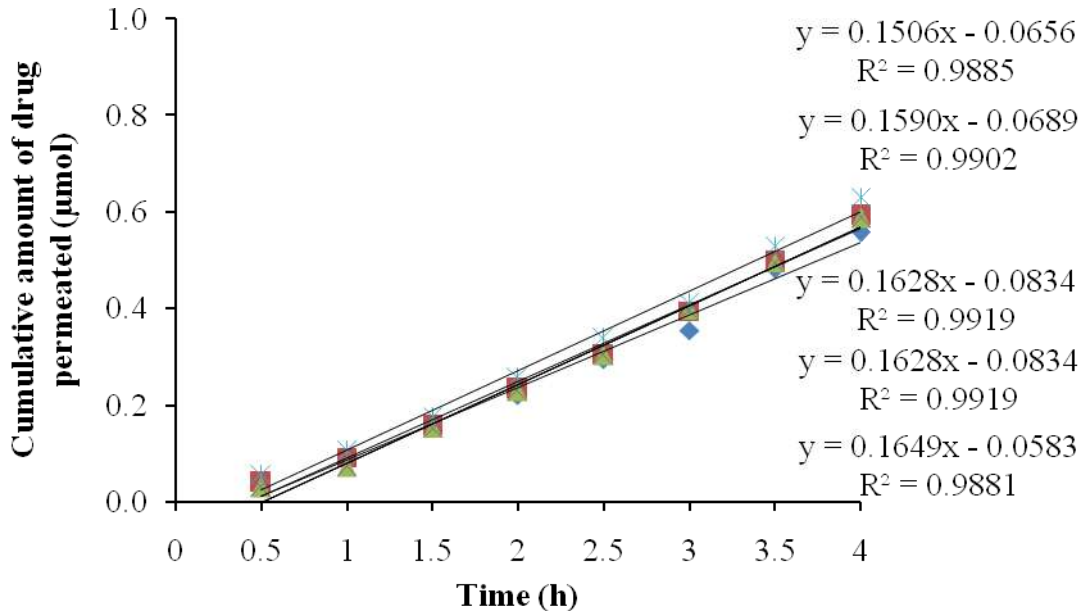

Figure 22 The linear regression line of amount permeated of 5-ASA untreated with CSG through porcine colon at various time intervals (control)

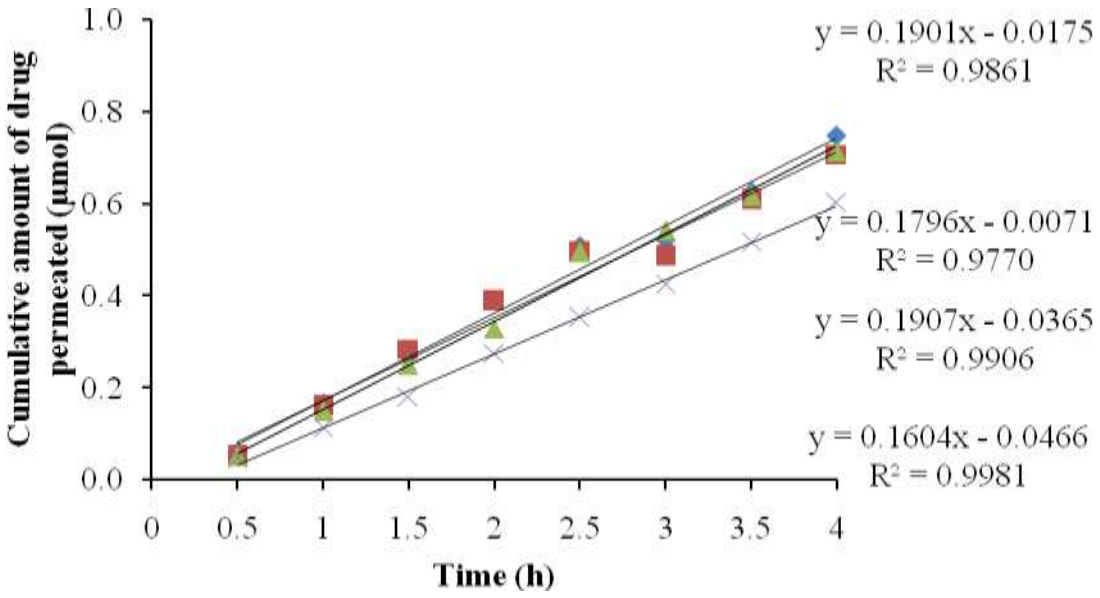

Figure 23 The linear regression line of amount permeated of 5-ASA treated with 0.01%w/v CSG first 30 min through porcine colon at various time intervals

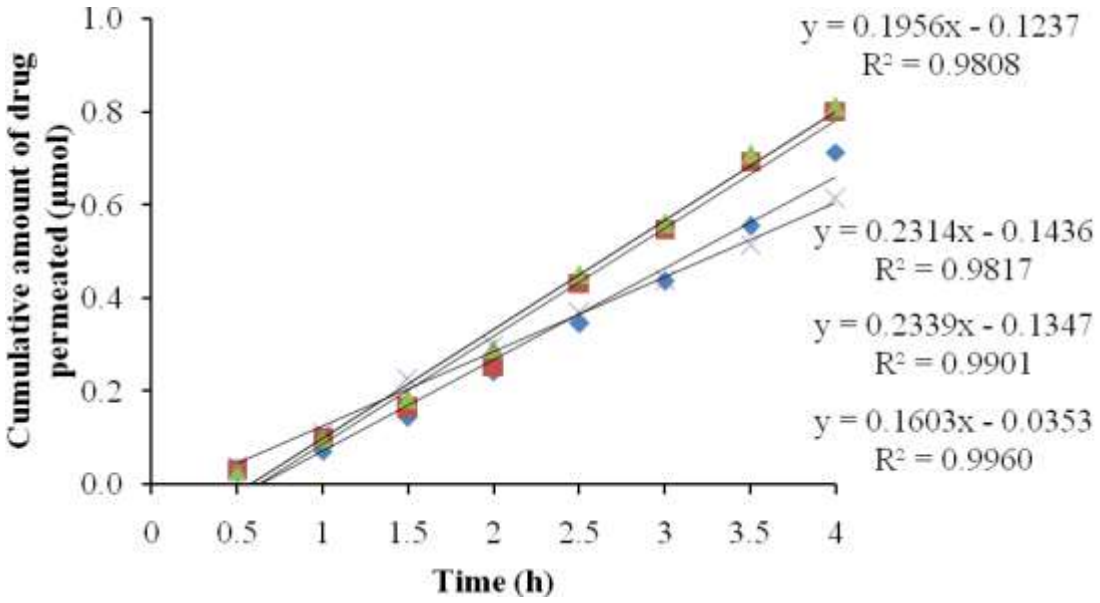

Figure 24 The linear regression line of amount permeated of 5-ASA treated with 0.5 %w/v CSG first 30 min through porcine colon at various time intervals

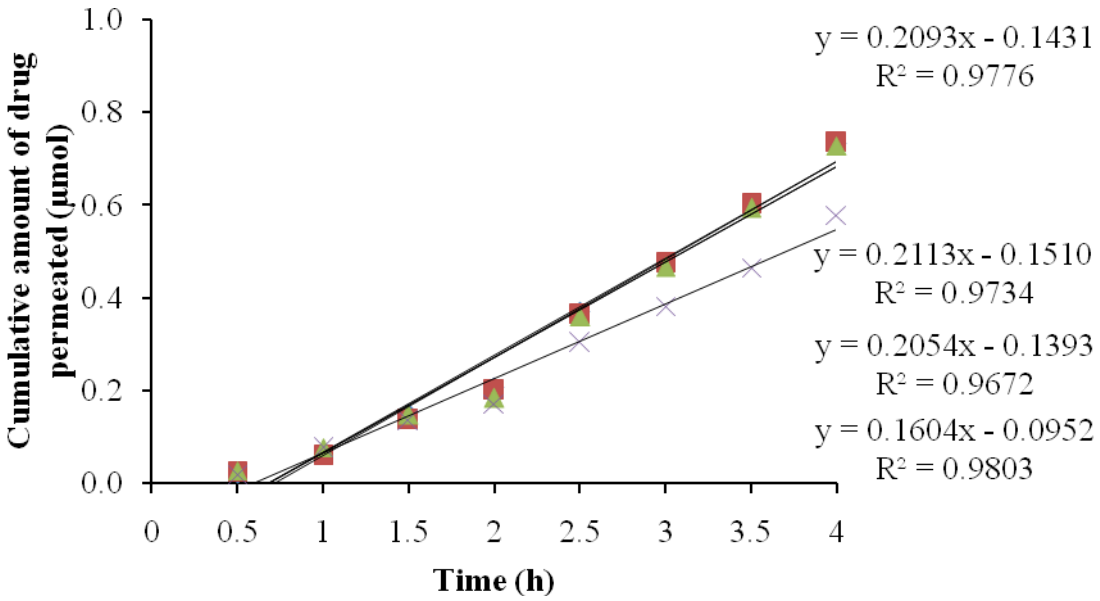

Figure 25 The linear regression line of amount permeated of 5-ASA treated with 2.0 %w/v CSG first 30 min through porcine colon at various time intervals

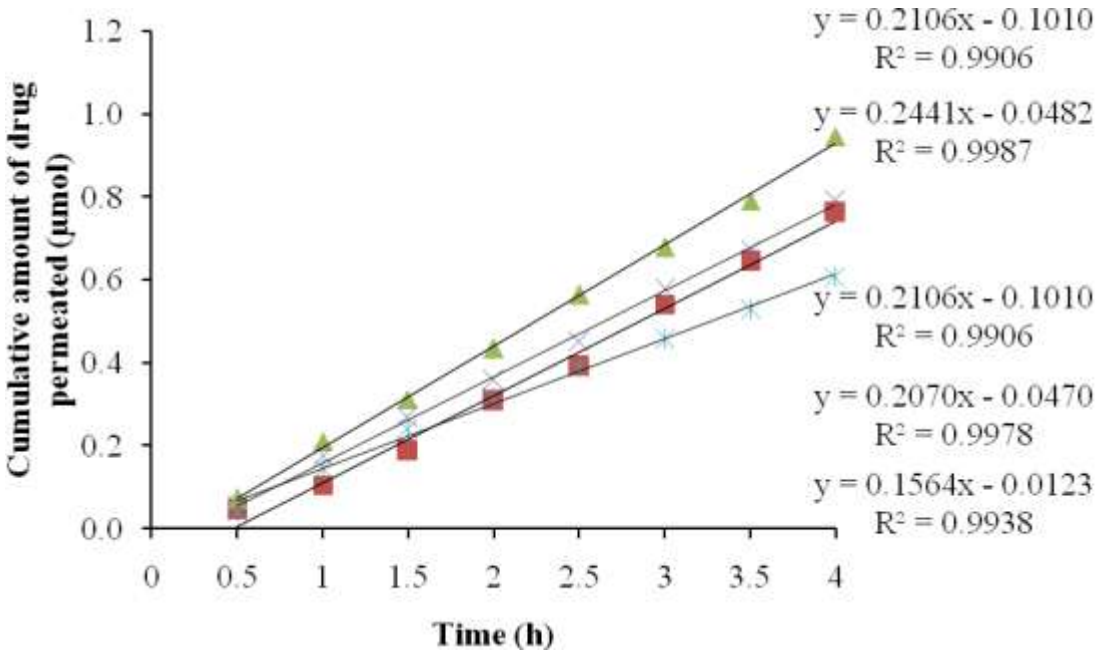

Figure 26 The linear regression line of amount permeated of 5-ASA without Na-TDC through porcine colon at various time intervals (control)

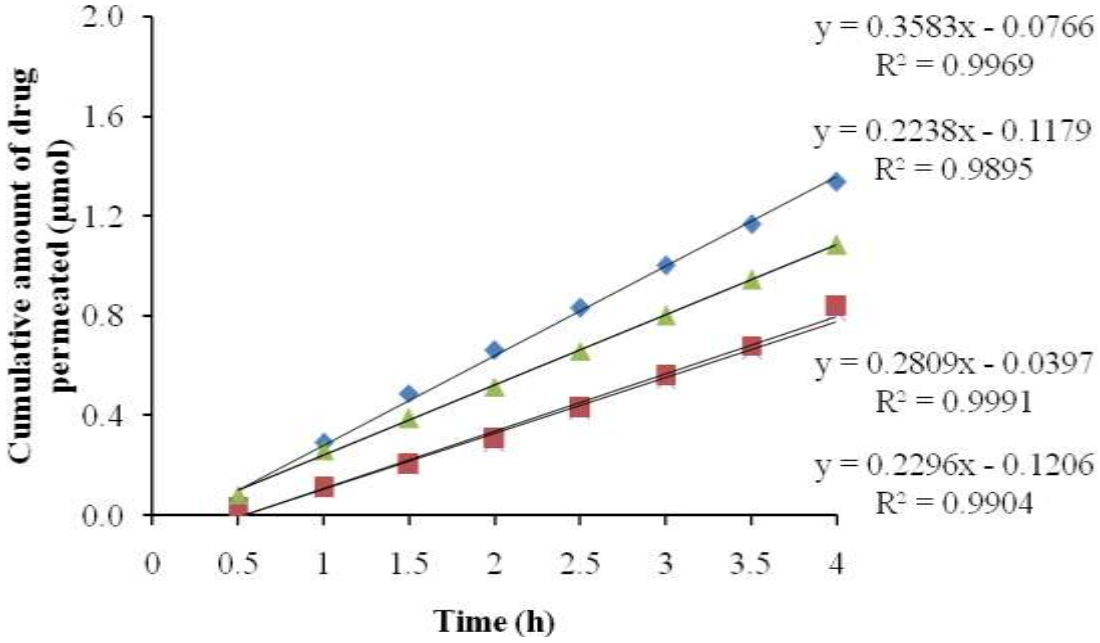

Figure 27 The linear regression line of amount permeated of 5-ASA with 0.01 %w/v of NaTDC through porcine colon at various time intervals

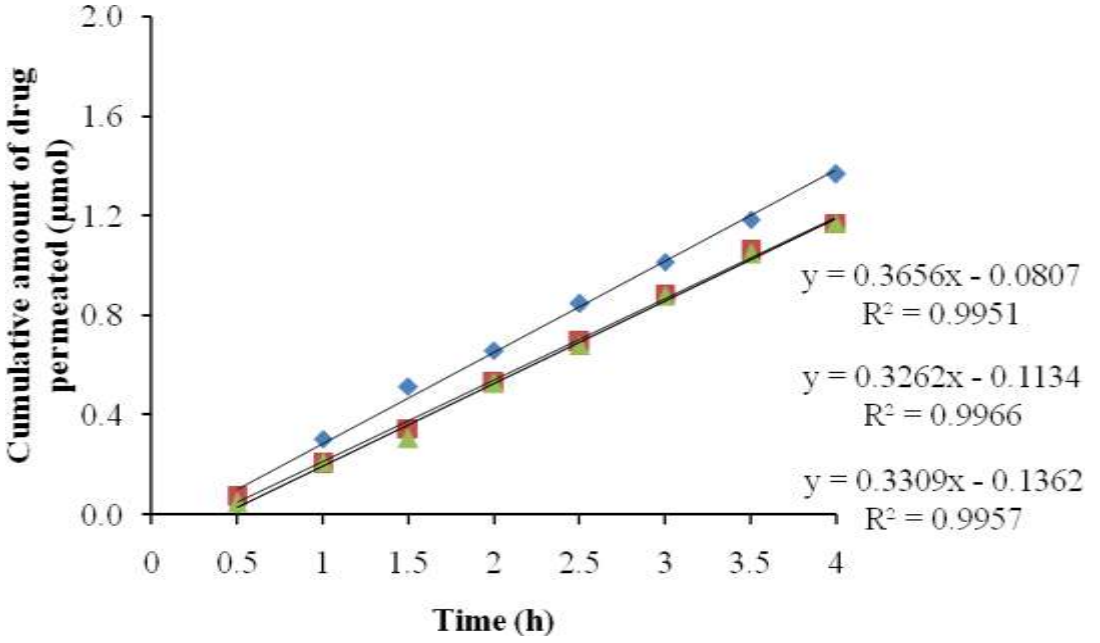

Figure 28 The linear regression line of amount permeated of 5-ASA with 0.5 %w/v of NaTDC through porcine colon at various time intervals

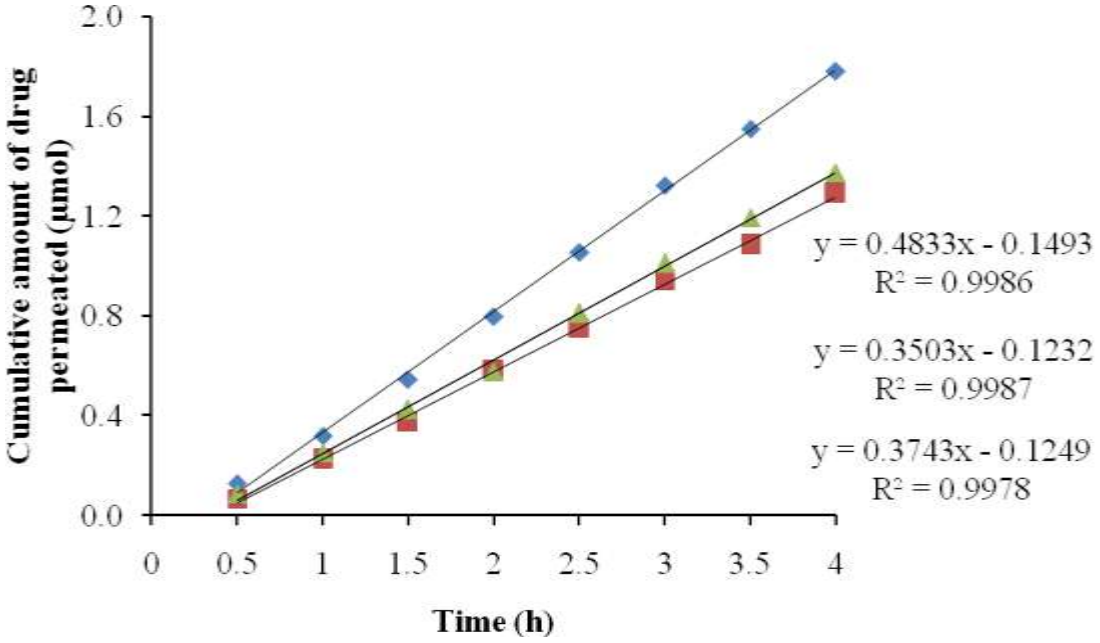

Figure 29 The linear regression line of amount permeated of 5-ASA with 2.0 %w/v of NaTDC through porcine colon at various time intervals

Table 16 P_{app} of 5-ASA in the permeation test through the isolated colon epithelium with and without various concentrations of CSG (n≥3)

P_{app} x 10^{-6} cm/s			
5-ASA+PR (n=5)	5-ASA+PR +0.01%CSG (n=5)	5-ASA+PR +0.5%CSG (n=4)	5-ASA+PR +2.0%CSG (n=4)
9.172	8.319	8.611	5.223
8.903	8.424	8.617	5.369
9.026	8.336	8.482	5.077
9.516	8.476	8.996	5.976
11.450	8.336		

Table 17 P_{app} of 5-ASA in the permeation test through the isolated colon epithelium untreated and treated with various concentrations of CSG at first 30 min (n≥3)

P_{app} x 10^{-6} cm/s			
5-ASA+PR (n=5)	5-ASA+PR +0.01%CSG 30 min (n=4)	5-ASA+PR +0.5%CSG 30 min (n=4)	5-ASA+PR +2.0%CSG 30 min (n=4)
8.798	11.105	11.427	12.227
9.288	10.492	13.518	12.344
9.510	11.140	13.664	11.999
9.510	9.370	9.364	9.370
9.633			

Table 18 P_{app} of 5-ASA in the permeation test through the isolated colon epithelium with and without various concentrations of NaTDC (n≥3)

P_{app} x 10^{-6} cm/s			
5-ASA+PR (n=5)	5-ASA+PR +0.01% NaTDC (n=4)	5-ASA+PR +0.5% NaTDC (n=3)	5-ASA+PR +2.0% NaTDC (n=3)
12.303	20.931	21.358	28.233
14.260	13.074	19.056	20.464
12.303	16.410	19.331	21.866
12.093	13.413		
9.137			

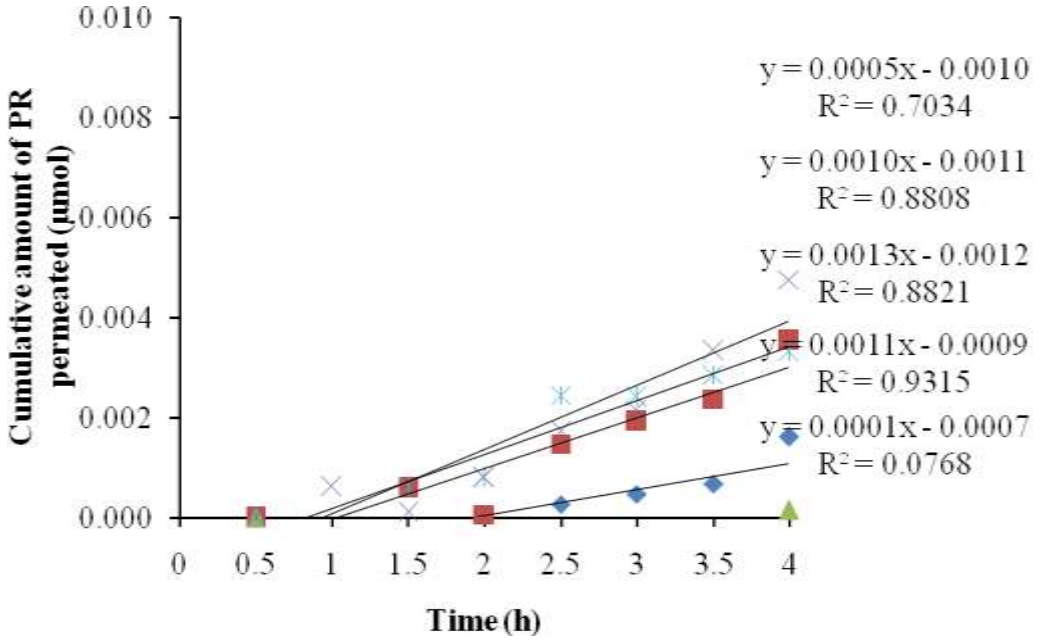

Figure 30 The linear regression line of amount permeated of PR without CSG through porcine colon at various time intervals (control)

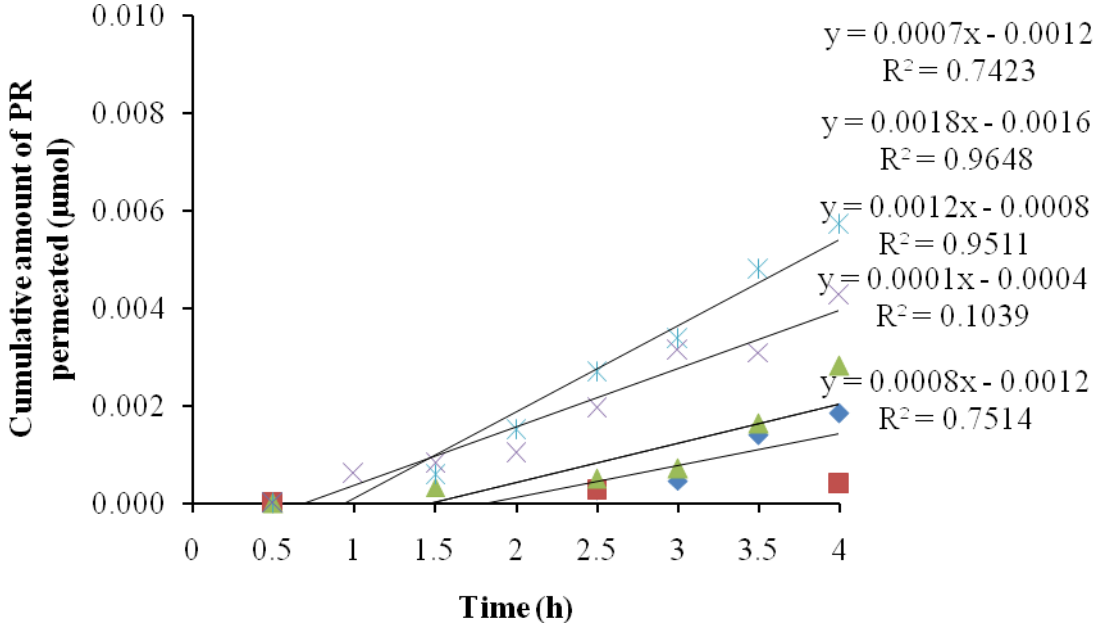

Figure 31 The linear regression line of amount permeated of PR with 0.01 %w/v CSG through porcine colon at various time intervals

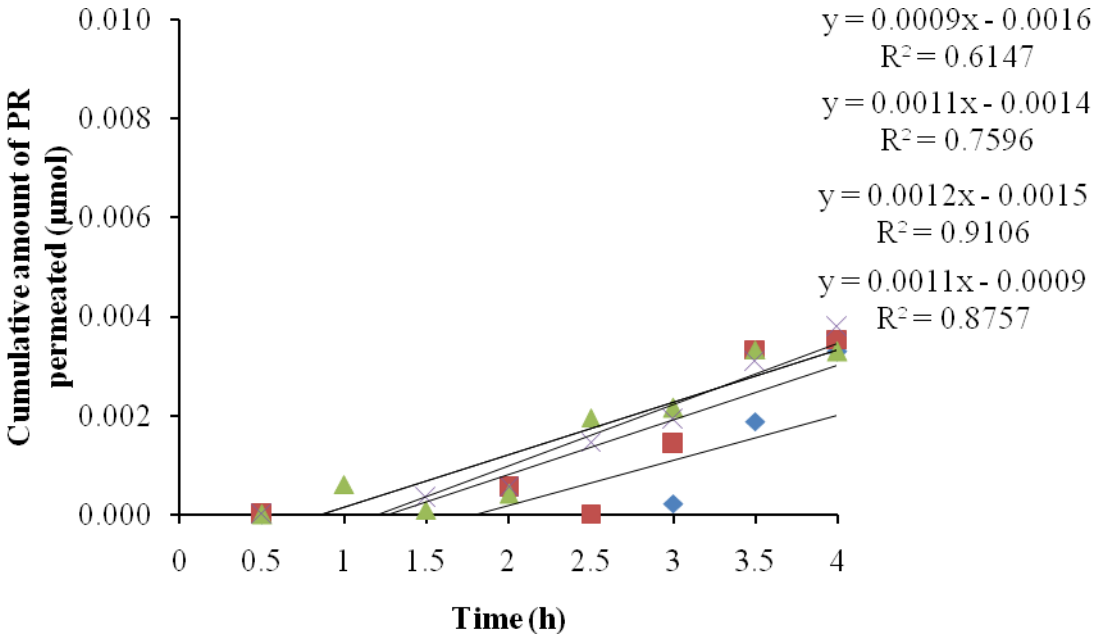

Figure 32 The linear regression line of amount permeated of PR with 0.5 %w/v CSG through porcine colon at various time intervals

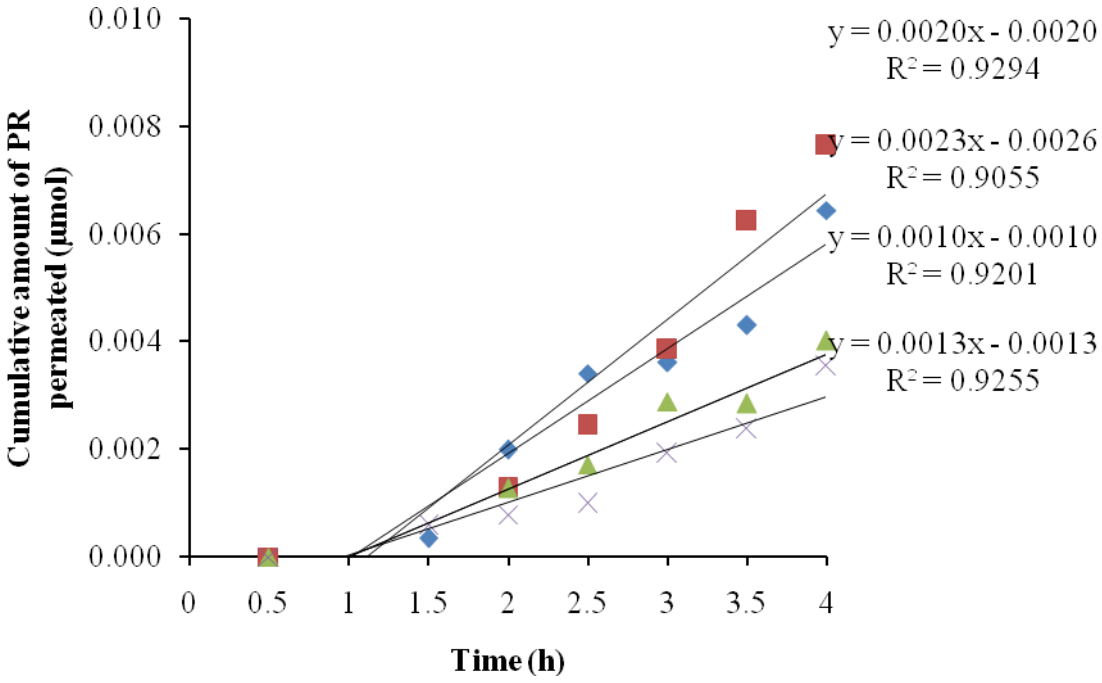

Figure 33 The linear regression line of amount permeated of PR with 2.0 %w/v CSG through porcine colon at various time intervals

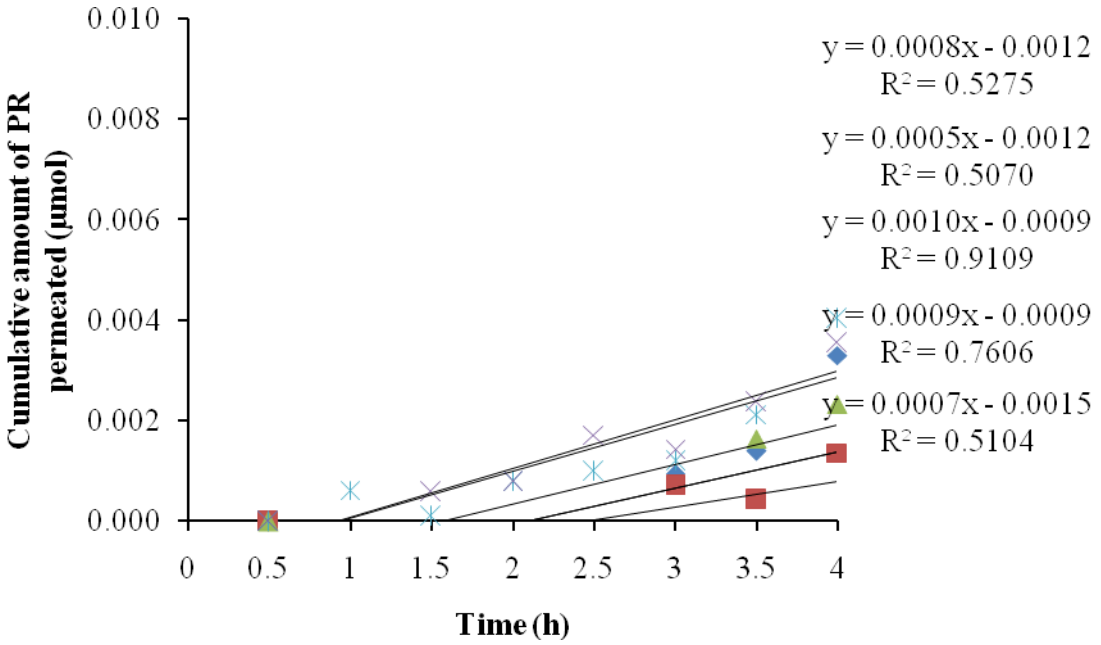

Figure 34 The linear regression line of amount permeated of PR untreated with CSG through porcine colon at various time intervals (control)

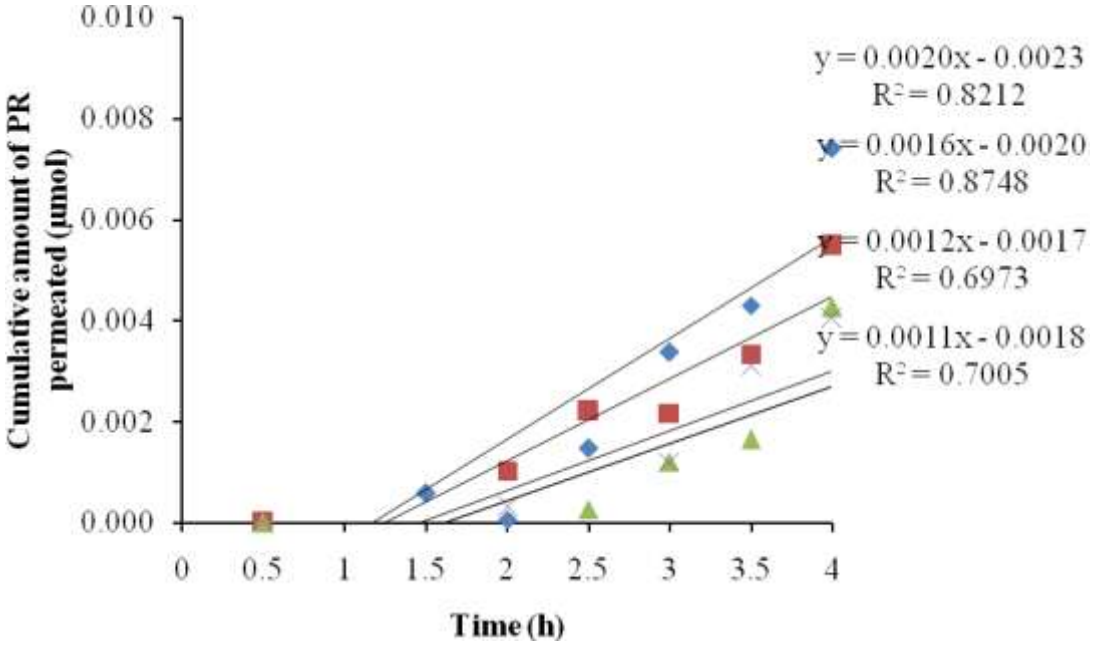

Figure 35 The linear regression line of amount permeated of PR treated with 0.01 %w/v CSG first 30 min through porcine colon at various time intervals

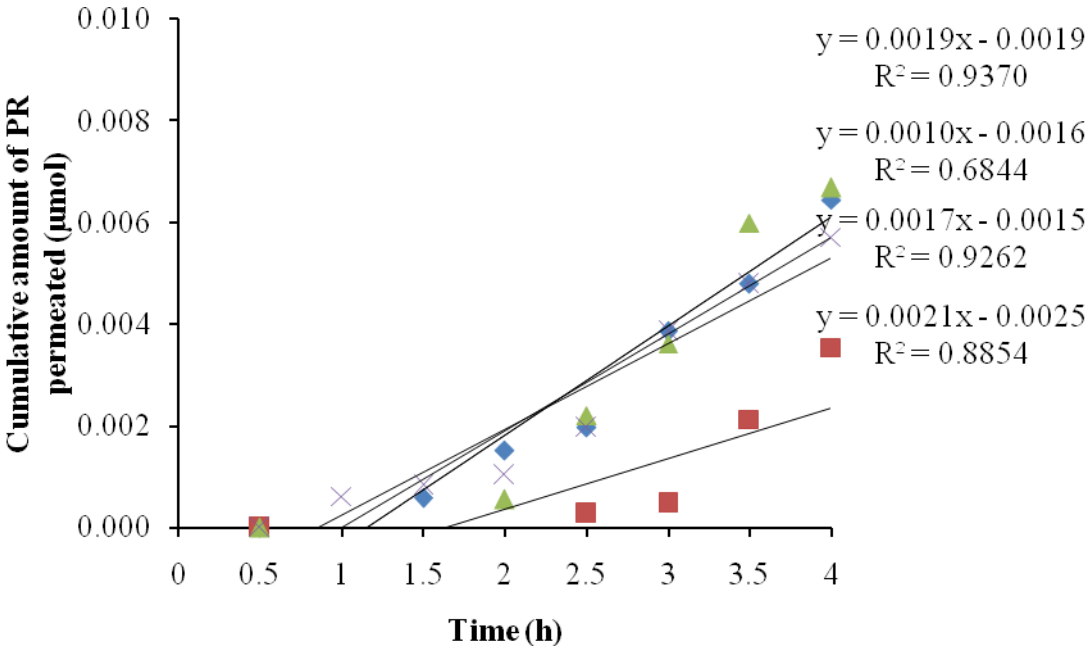

Figure 36 The linear regression line of amount permeated of PR treated with 0.5 %w/v CSG first 30 min through porcine colon at various time intervals

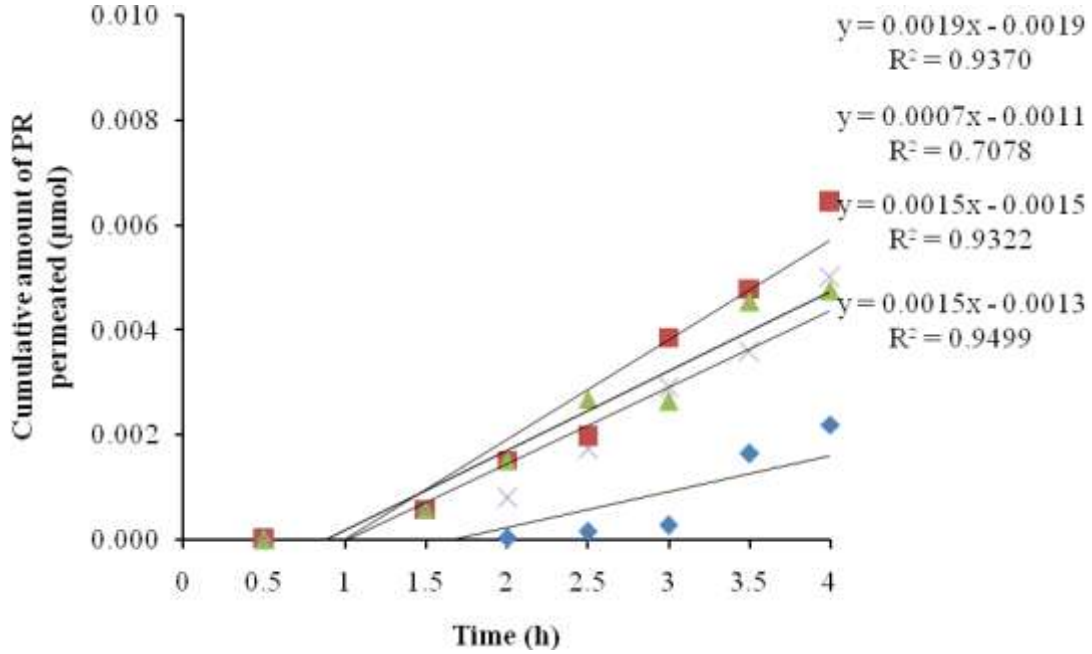

Figure 37 The linear regression line of amount permeated of PR treated with 2.0 %w/v CSG first 30 min through porcine colon at various time intervals

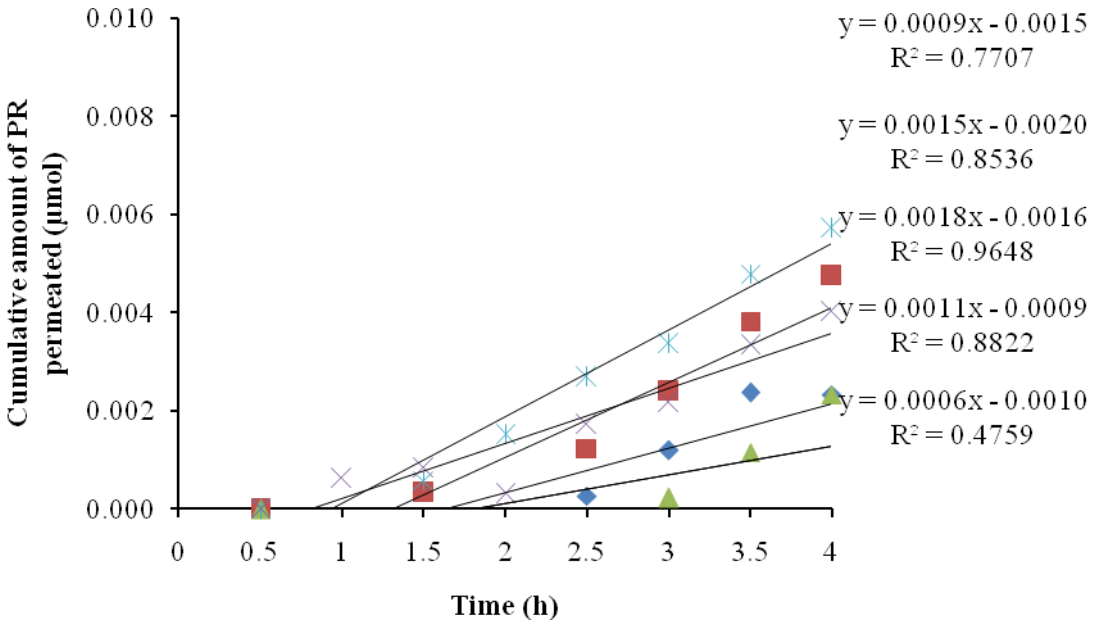

Figure 38 The linear regression line of amount permeated of PR without NaTDC through porcine colon at various time intervals (control)

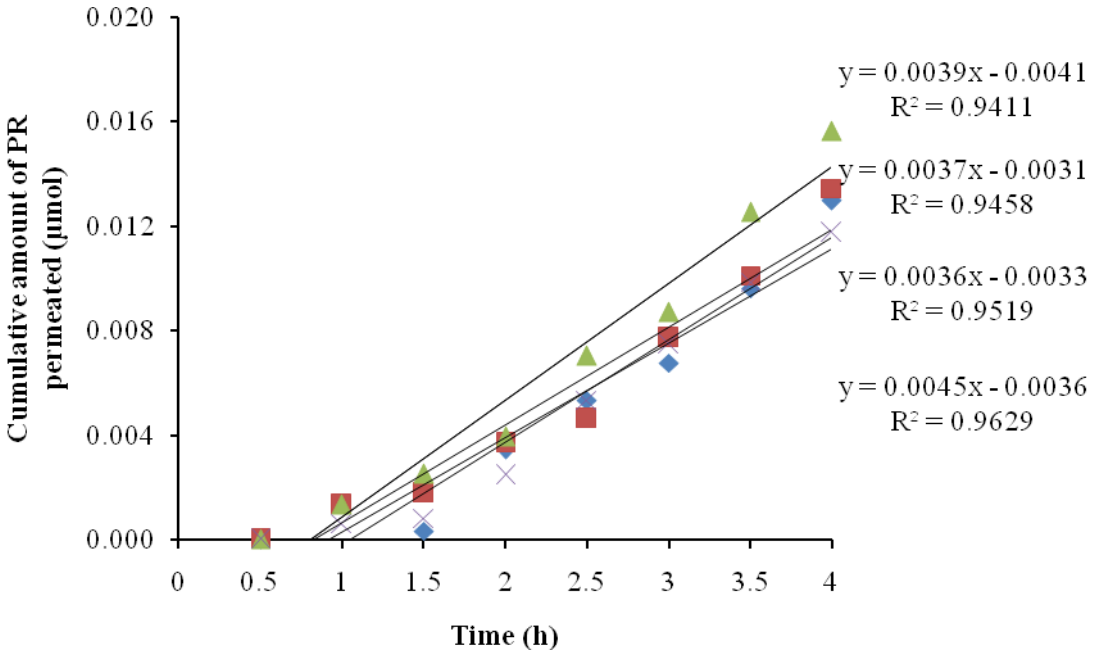

Figure 39 The linear regression line of amount permeated of PR with 0.01 %w/v of NaTDC through porcine colon at various time intervals

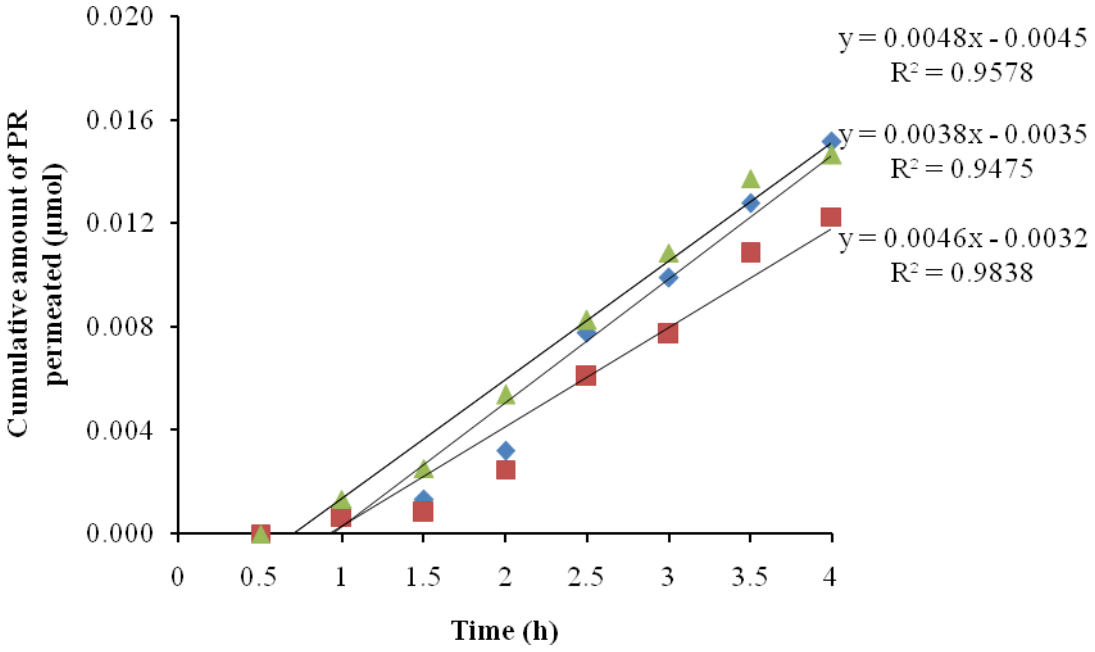

Figure 40 The linear regression line of amount permeated of PR with 0.5 %w/v of NaTDC through porcine colon at various time intervals

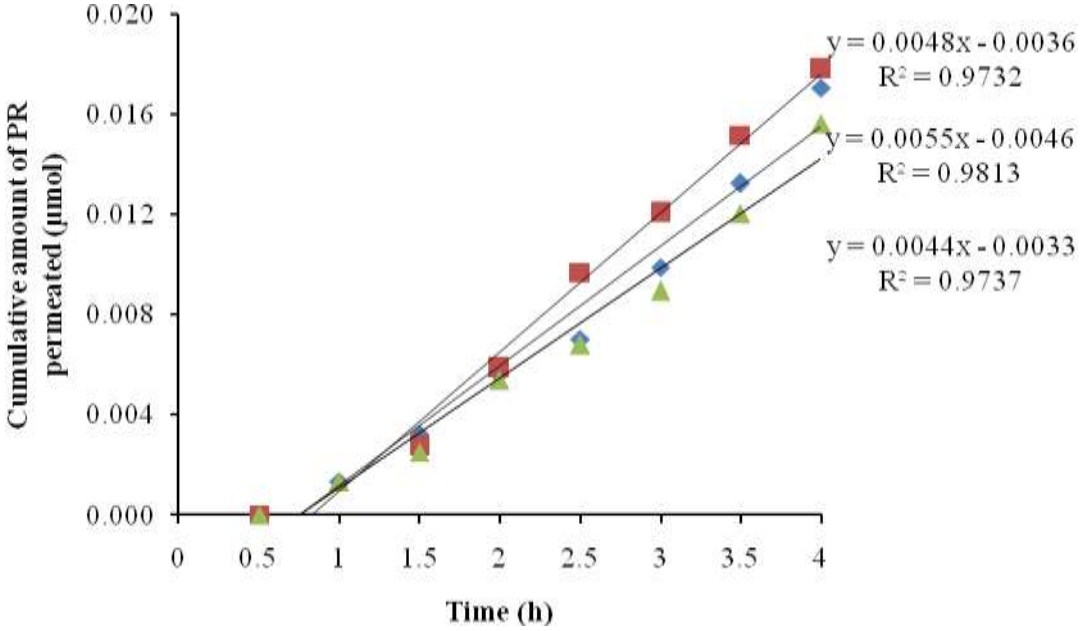

Figure 41 The linear regression line of amount permeated of PR with 2.0 %w/v of NaTDC through porcine colon at various time intervals

Table 19 P_{app} of PR in the permeation test through the isolated colon epithelium with and without various concentrations of CSG (n≥3)

P_{app} x 10^{-6} cm/s			
5-ASA+PR (n=5)	5-ASA+PR +0.01%CSG (n=5)	5-ASA+PR +0.5%CSG (n=4)	5-ASA+PR +2.0%CSG (n=4)
0.172	0.241	0.309	0.687
0.344	0.034	0.378	0.790
0.447	0.412	0.412	0.344
0.378	0.619	0.378	0.447
0.034	0.275		

Table 20 P_{app} of PR in the permeation test through the isolated colon epithelium untreated and treated with various concentrations of CSG at first 30 min (n≥3)

P_{app} x 10^{-6} cm/s			
5-ASA+PR (n=5)	5-ASA+PR +0.01%CSG 30 min (n=4)	5-ASA+PR +0.5%CSG 30 min (n=4)	5-ASA+PR +2.0%CSG 30 min (n=4)
0.275	0.687	0.653	0.653
0.172	0.550	0.344	0.241
0.344	0.412	0.584	0.515
0.309	0.378	0.722	0.515
0.241			

Table 21 P_{app} of PR in the permeation test through the isolated colon epithelium with and without various concentrations of NaTDC (n≥3)

P_{app} x 10^{-6} cm/s			
5-ASA+PR (n=5)	5-ASA+PR +0.01% NaTDC (n=4)	5-ASA+PR +0.5% NaTDC (n=3)	5-ASA+PR +2.0% NaTDC (n=3)
0.309	1.332	1.649	1.649
0.515	1.264	1.306	1.890
0.378	1.230	1.581	1.512
0.619	1.537		
0.206			

Table 22 Effect of CSG on TEER values of the isolated colon epithelium before and after the drug permeation test

Samples	TEER	
	before	after
5-ASA+PR (n=5)	111	94
	102	96
	109	92
	122	107
	108	99
5-ASA+PR +0.01%CSG (n=5)	107	78
	101	72
	116	73
	121	82
	134	93
5-ASA+PR	109	66

+0.5%CSG (n=4)	122	73
	122	59
	105	69
5-ASA+PR +2.0%CSG (n=4)	120	51
	92	32
	131	51
	110	59

Table 23 Effect of CSG treated at first 30 min on TEER values of the isolated colon epithelium before and after the drug permeation test

Samples	TEER	
	before	after
5-ASA+PR (n=5)	107	100
	103	98
	99	91
	107	97
	125	100
5-ASA+PR +0.01%CSG 30 min (n=4)	122	41
	120	60
	102	15
	139	77
5-ASA+PR +0.5%CSG 30 min (n=4)	133	51
	114	60
	135	66
	133	69
5-ASA+PR +2.0%CSG 30 min (n=4)	143	108
	144	93
	99	74
	115	88

Table 24 Effect of NaTDC on TEER values of the isolated colon epithelium before and after the drug permeation test

Samples	TEER	
	before	after
5-ASA+PR (n=5)	110	96
	104	98
	103	93
	109	100
	126	118
5-ASA+PR +0.01% NaTDC (n=4)	92	18
	114	15
	115	19
	108	10
5-ASA+PR +0.5% NaTDC (n=4)	128	18
	107	10
	97	24
5-ASA+PR +2.0% NaTDC (n=4)	103	-6
	102	-2
	106	3

Table 25 Effect of CSG of TR index values of the isolated colon epithelium after the drug permeation test.

TR index			
5-ASA+PR (n=5)	5-ASA+PR +0.01%CSG (n=5)	5-ASA+PR +0.5%CSG (n=4)	5-ASA+PR +2.0%CSG (n=4)
0.033	0.046	0.035	0.051
0.036	0.044	0.037	0.042
0.035	0.045	0.033	0.047
0.035	0.034	0.031	0.041
0.034	0.041		

Table 26 Effect of CSG treated at first 30 min on TR index values of the isolated colon epithelium after the drug permeation test.

TR index			
5-ASA+PR (n=5)	5-ASA+PR +0.01%CSG 30 min (n=4)	5-ASA+PR +0.5%CSG 30 min (n=4)	5-ASA+PR +2.0%CSG 30 min (n=4)
0.032	0.047	0.044	0.039
0.036	0.051	0.040	0.037
0.035	0.051	0.038	0.036
0.038	0.045	0.041	0.035
0.034			

Table 27 Effect of NaTDC on TR index values of the isolated colon epithelium after the drug permeation test.

TR index			
5-ASA+PR (n=5)	5-ASA+PR +0.01% NaTDC (n=4)	5-ASA+PR +0.5% NaTDC (n=3)	5-ASA+PR +2.0% NaTDC (n=3)
0.033	0.045	0.037	0.032
0.032	0.049	0.036	0.035
0.032	0.043	0.031	0.029
0.035	0.042		
0.036			

Table 28 List of abbreviations

Symbol	Definition
°C	degree Celsius
>	more than
<	less than
%	percent
MW	molecular weight
pK_a	minus logarithm base 10 of K_a, -log K_a
w/v	weight by volume
cm^2	square centimeter
g	gram
min	minute
h	hours
NaCl	sodium chloride
pH	The negative logarithm of the hydrogen ion concentration
CS	chitasan
CSG	chitosan glutamate
NaTDC	sodium taurodeoxycholate
5-ASA	5-aminosalicylic acid
GI	gastro-intestinal
Approx.	approximate
Å	angstrom
SE	surface epithelium
CC	colon crypts
GC	goblet cells
LP	lamina propria
MM	muscularis mucosa
ZO-1	zona occludens 1

Table 28 (continue)

Symbol	Definition
ZO-2	zona occludens 2
ZO-3	zona occludens 3
TJs	tight junctions
Da	dalton
kDa	kilodalton
Caco-2 cells	colonic adenocarcinoma cells
SEM	Scanning electron microscope
DSC	Differential Scanning Calorimetric
TGA	Thermogravimetric Analysis
PR	phenol red
FTIR	Fourier Transform Infrared Spectroscopy
dQ/dt	permeability rate
A	diffusion area
C_0	initial concentration
TEER	trans-epithelial electrical resistance
P_{app}	apparent permeability coefficient
rpm	revolution per minute
SD	standard deviation
AVG	average
UV	ultraviolet
mg	milligram
µg	microgram
mL	milliliter
µL	microlitre

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Presentations

1.Jurairat Nunthanid , Uraiwan Pongpiriyajit, Manee Luangtana-anan, Pornsak Sriamornsak and Sontaya Limmatvapirat. “Preparations and Physicochemical Characterization of chitosan salts” The 3rd Annual Research Conference in Silpakorn Research, 28-29 January 2009, Nakornpathom, Thailand, Poster presentation.

2.Uraiwan Pongpiriyajit, Tanasait Ngawhirunpat, Pornsak Sriamornsak, Sontaya Limmatvapirat, Manee Luangtana-anan, and Jurairat Nunthanid. "Factors affecting *in vitro* drugs permeation through porcine colon" The 2nd Annual Research Conference in Silpakorn Research, 28-29 January 2010, Nakornpathom, Thailand, Poster presentation.

3.Uraiwan Pongpiriyajit, Tanasait Ngawhirunpat, Pornsak Sriamornsak, Sontaya Limmatvapirat, Manee Luangtana-anan, and Jurairat Nunthanid "An effect of chitosan hydrochloride on *in vitro* permeation of theophylline through porcine colon" The Sixth Thailand Materials Science and Technology Conference, 26-27 August 2010, Bangkok, Thailand, Poster presentation.