In vitro and in vivo evaluation of flax seed polymer and chitosan combination as a carrier for colon specific drug delivery

Keten tohumu polimeri ve sitozan kombinasyonun kolon spesifik ilaç dağıtım taşıyıcısı olarak in vivo ve in vitro değerlendirilmesi

Vijaya Raghavan,
Prof.
Sri Ramakrishna Institute Of Paramedical Sciences, Pharmaceutics, coimbatore, Hindistan
c_vijayan_2k@yahoo.com

Janaki G,
Sri Ramakrishna Institute Of Paramedical Sciences, Pharmaceutics, coimbatore, Hindistan

Ramakrishnan A,
Sri Ramakrishna Institute Of Paramedical Sciences, Pharmaceutics, coimbatore, Hindistan

Abstract
Purpose: The present investigation is aimed to use an inexpensive, nontoxic naturally available flax seed polymer and chitosan combination as colon-specific drug carriers and to study the influence of chitosan on the release characteristics of the formulation.
Materials and Methods: Core tablets of mesalazine were prepared by wet granulation with starch paste and were compression coated with coating formulations containing different weight ratios of flax seed polymer and chitosan 2:3, 3:2 and 4:1. The tablets were subjected to in vitro drug release studies in simulated colonic fluids (4% w/v of rat cecal contents). In vivo evaluation was performed in six healthy human volunteers.
Results: The compression coated flax seed polymer and chitosan tablets were found degraded by colonic bacteria of rat cecal contents in simulated colonic fluids at the end of 26 h indicating the susceptibility of the formulation to the rat cecal contents. In vitro studies in pH 6.8 phosphate buffer containing 4% w/v rat cecal contents showed that the cumulative percentage of mesalazine after 26 h were 52.16±0.06, 64.10±0.08 and 98.00±0.19 (mean ± s.d) respectively for tablets containing different weight ratios of flax seed polymer and chitosan 2:3, 3:2 and 4:1. In vivo studies conducted in six healthy male human volunteers for the various formulations revealed that the drug released was intiated only after 5h (i.e) transit time of small intestine and the bioavailability (AUC $_{0-t*}$)of the drug was found to be 196.97±3.02, 245.8±5.10 and 910.51±9.61 (mean s.d) respectively for tablets containing different weight ratios of flax seed polymer and chitosan 2:3, 3:2 and 4:1. Formulation C6 which contains 110mg of chitosan when compared to formulation C4 and C5 containg 330mg and 220mg of chitosan is the suitable ratio for the better release of the drug which in turn having higher bioavailability.
Conculusion: The results of the present study indicates that compression coated tablets containing 4:1 ratio of flax seed polymer and chitosan held a better dissolution profile, higher bioavailability and hence a potential carrier for drug targeting to colon.

Key Words: **Chitosan; Drug Delivery Systems; Flaxseed; Kolon; Mesalazine.**

Özet
Amaç: Sunulan çalışma, ucuz ve toksik olmayan keten tohumu ve sitozan kombinasyonunu kolon spesifik taşıyıcı olarak kullanarak, formülasyonun serbestleme özellikleri üzerine sitozanın etkisini araştırmayı amaçlamaktadır.
Gereç ve Yöntem: Meselazin tablet özleri nişasta hamurlu ıslak granül olarak hazırlandı ve ağırlıklarına göre 2:3, 3:2 ve 4:1 oranlarında keten tohumu polimeri ve sitozan içeren formülasyonları sıkıştırılarak kaplandı. In vitro serbestleme çalışmalarında bu tabletler kolonik ortamı taklit eden bir sıvıda (Rat çökal içeriğinin ağırlık/hacim olarak %4'ü) ve in vivo deneylerde 6 sağlıklı gönüllüde çalışıldı.
Bulgular: Sıkıştırılarak kaplanmış keten tohumu polimeri ve sitozan tabletlerinin 26 saatin sonunda kolon ortamına benzer sıvıda rat çökal içeriğin kolonik bakterileri tarafından parçalandığı bulunması, formülasyonun rat çökal içeriğine duyarlılığına işaret etmektedir. Ağırlık/hacim oranı %4 olan rat çökal içeriği bulunduran fosfat tamponu (pH:6,8) ile yapılan in vitro çalışmalar, 26 saat sonar mesalazinin kümülatif yüzdelerinin 2:3, 3:2, ve 4:1 oranlarında keten tohumu ve sitozan içeren tabletler için sırasıyla 52,16±0,06; 64,10±0,08 ve 98,00±0,19 (ortalama ± standart sapma) olduğunu gösterdi. Altı gönüllüde yapılan in vivo çalışmalar ilaç serbestlenmesinin 5 saatlik ince bağırsak geçiş zamanından hemen sonra başladığını ve ilacın biyoyararlanımının 2:3, 3:2 ve 4:1 oranlarında keten tohumu polimeri ve sitozan içeren tabletler için sırasıyla 196,97±3,02; 245,8±5,10 ve 910,51±9,61 (ortalama ± standart sapma) olduğunu ortaya çıkardı. Yüz on mg sitozan içeren C6 formülasyonu 330 ve 220 mg sitozan içeren C4 ve C5 formülasyonları ile karşılaştırıldığı zaman daha iyi ilaç salımı ve bu nedenle daha yüksek biyoyararlanıma sahip olan elverişli oran olarak bulundu.
Sonuç: Çalışmamızın sonuçları 4:1 oranında keten tohumu polimeri ve sitozan içeren sıkıştırılarak kaplanmış tabletlerin daha iyi çözünme profiline, daha yüksek biyoyararlanıma ve bu nedenle kolona hedeflenen ilaçlar için potansiyel bir taşıyıcı olma özelliğine sahip olduğunu göstermektedir.

Anahtar Kelimeler: **İlaç dağıtım sistemleri; Kalın bağırsak; Keten tohumu; Meselazin; Sitozan.**

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Corresponding Author:
Vijaya Raghavan,
Sri Ramakrishna Institute Of Paramedical Sciences, Pharmaceutics, coimbatore, Hindistan

Telephone : +91 - 422 - 2536143 (Res)
+91 - 422 - 2210075 Extn: 164 (off)
E-mail : c_vijayan_2k@yahoo.com

Introduction

Polymers remain the most versatile class of biomaterials, being extensively applied in medicine, biotechnology, food and cosmetic industries. Polymers used as biomaterials can be naturally occurring, synthetic or a combination of both. The ability of the natural polymers to act as substrates for the bacterial inhabitants of the colon together with their properties such as biocompatibility and biodegradability invites their use as colon carrier (1). They can be easily modified chemically and biochemically and they are also highly stable, safe, nontoxic, hydrophilic and gel forming.

Among the various routes of drug delivery, oral route is perhaps the most preferred to the patient and clinicians alike. However, oral drug administration has disadvantages such as hepatic first pass metabolism and enzymatic degradation within the GI tract which prohibits oral administration of certain classes of drugs. Colonic drug delivery has gained increased importance not just for the delivery of drugs for the local diseases of colon but also for its potential for the delivery of proteins and peptides. The colon presents less hostile condition for drug delivery because of less diversity and intensity of enzymatic activities and a near neutral pH (2,3,4,5). In addition, the residence time of dosage form is longer in the colon and avoidance of first pass metabolism. Even though various approaches are available for colon specific drugdelivery, the best approach is the use of carriers that are degraded exclusively by colonic bacteria. On reaching colon, the polymers undergo assimilation by microorganisms (6) or degradation by enzymes (7) or break down of the polymer back bone leading to a subsequent reduction in their molecular weight and thereby loss of mechanical strength. They are then unable to hold the drug entity any longer.

Flax seed polymer is a natural polymer derived from seeds of Linum usitatissimum Linn (Family: Linaceae). It consists of a straight chain oligomeric structure with an average composition of five secoisolariciresionol diglucoside (SDG) residues interlinked with four 3-hydroxy-3-methyl glutaric acid residues (8). Chitosan is a linear polysaccharide derived by N-acetylation of the natural polymer chitin, which is the second most abundant naturally occurring polymer in nature. Chitosan conists of linear 1-4 linked 2- acetamido-2- deoxy-β-D glucopyranose and 2-amino-β-D-glucopyranose unit. In the present study the flax seed polymer and chitosan in the ratio of 2:3, 3:2 and 4:1 were applied over the core tablet in the form of compression - coat and evaluated as a carrier for colon release studies simulated gastro intestinal fluids in the presence and absence of rat cecal content. In vivo studies were carried out in healthy human volunteers to evaluate their in vivo behaviour.

Materials and Methods

Flax seed was obtained from local market Coimabtore, India. Chitosan was obtained from Central Institute of Fisheries Technology, Cochin, India. Mesalazine (5 - amino salicyclic acid) was obtained from Sun Pharmaceuticals Ltd., India. Microcrystalline cellulose and Magnesium stearate were obtained from Loba Chemic Pvt. Ltd., India. Starch was obtained from E-Merck (India)Pvt. Ltd., India. Talc and sodium lauryl sulphate were obtained from S.D. Fine Chem Ltd., India.

Extraction of Flax Seed Polymer. Extraction of flax seed polymer was carried out with organic solvents (9,10,11,12). Flax seed is milled and defatted. The defatted flax seed flour is extracted with 1,4-dioxane/ 95% ethanol (1:1 V/V).

Acute toxicity study for Flax seed polymer. Wistar rats (150-200g) maintained at standard laboratory conditions were used. A total of five animals were used which received a single oral dose (2000 mg/kg) of flax seed polymer. Animals were kept overnight fasting prior to drug administration. After the administration of the polymer, food was withheld for further 3-4 hours. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours and up to 14 days after drug administration (OECD guidelines).

Preparation of Core Tablets. Each core tablet (average weight 80 mg) for in vitro and in vivo studies consists of mesalazine (40 mg) microcrystalline cellulose (MCC 29 mg), dried starch (5mg), sodium lauryl sulphate (4 mg), talc (1.5mg) and magnesium stearate (0.5mg). Starch and sodium lauryl sulphate were added to obtain fast disintegration tablets (disintegration<1 min) of mesalazine.

The materials were weighed, mixed and passed through a mesh (250 μm) to ensure complete mixing. Drug and the excipients were homogenously blended and subsequently compressed into flat faced tablets (80 mg)

using a single punch tablet machine (Cadmach, Ahmedabad, India). The thickness of the core tablet was 0.2 mm and their crushing strength was checked. It was about 3.5 kg/cm^2.

Preparation Of Compression-Coated Tablet. The mesalazine core tablet was compression-coated with different quantities of coating material such as Flax seed polymer, and Chitosan taken in the ratio of 2:3, 3:2 and 4:1. Half the quantity of the coating material was placed in the die cavity. The core was carefully positioned in the center of the die cavity and was filled with other half of the coating material. The coating material was compressed around the core at an applied force of 470 kg using 9 mm round, flat and plain punches. The crushing strength of the compression coat tablet was 4.75 kg / cm^2.

Tablet Evaluation. Five tablets from each batch were powdered individually and a quantity equivalent to 40 mg of mesalazine was accurately weighed and extracted with a suitable volume of methanol. Each extract was suitably diluted and analyzed spectrophotmetrically at 490 nm.

Ten tablets from each batch were evaluated for uniformity in tablet weight. Six tablets from each batch were examined for friability using a Roche-type friabilator. (Tropical Equipment Pvt. Ltd., Mumbai, India) and hardness using a Monsanto - type hardness tester (Camp bell, Mumbai, India).

Invitro Drug Release Studies Without Rat Cecal Content (13). The compression coated tablets of mesalazine were evaluated for their integrity in the physiological environment of stomach and small intestine under conditions mimicking mouth to colon transit. These studies were carried out using a USP XXIII dissolution rate test apparatus (apparatus I, 100 rpm, 37°C±0.5°C). The tablets were tested for drug release for 2 hrs in 0.1 N HCI (900 mL) as the average gastric emptying time is almost 2 hrs. Then the dissolution medium was replaced with pH 7.4 phosphate buffer (900 mL) and tested for drug release for 3 hrs as the average small intestinal transit time is almost 3 hrs. At the end of the periods, two samples each of 1 mL were taken, suitably diluted and analyzed spectrophotmetrically at 490 nm .

Then the dissolution medium was replaced with pH 6.8 phosphate buffer. The drug release studies were carried out for 21 hrs (usual colonic transit time is 20-30 hrs) and 1 mL samples were taken at different time and replaced with 1 mL of pH 6.8 phosphate buffer. The samples are diluted and analyzed spectrophotmetrically at 490 nm .

In vitro drug release studies using rat cecal content (13). The compression-coated mesalazine tablets were evaluated for their integrity in the physiological environment of stomach and the small intestine under condition mimicking mouth to colon transit. These studies were carried out using a USP XXII/XXIII dissolution rate test apparatus (Apparatus 1, 100 rpm, 37°C). Following the tablets were tested for drug release for 2 h in 0.1 N HCl (900 mL) as the average gastric emptying time is about 2 h, the dissolution medium was replaced with pH 7.5 phosphate buffer (900 mL) and tested for 3 h as the average small intestine transit time is about 3 h. At the end of time periods, the samples from both each of 1 mL were taken separately, suitably diluted and analyzed for mesalizine content using spectrophotmetrically at 490 nm.

The susceptibility of the various polymer coats to the enzymatic action of colonic bacteria was assessed by continuing the drug release studies in 100 mL of pH 6.8 phosphate buffered saline (PBS) containing 4% w/v rat cecal contents. The cecal contents were obtained from male albino rats after pretreatment for 7 days with various polymers (Each day 1 mL of 2% w/v of polymer dispersion - flax seed polymer / chitosan - was directly administered to the rats stomach through the Teflon tubing). Thirty minutes before the commencement of drug release studies, five rats were killed by spinal traction. Their abdomen were opened, the caecum were isolated, ligated at both ends, dissected and immediately transferred into pH 6.8 PBS which is previously bubbled with CO_2. The cecal bags were opened and their contents were individually weighed, pooled and then suspended in PBS to give a final dilution of 4% w/v. As the caecum is naturally anaerobic, all the operations were carried out under CO_2.

The studies simulating the drug release in colon were carried out in USP XXII/XXIII dissolution rate test apparatus (Apparatus 1, 100 rpm, 37°C) with slight modification. A beaker (capacity 150 mL, internal diameter 55 mm) containing 100 mL of dissolution medium immersed in water containing 1000 mL vessel, which is in turn placed in the water bath of dissolution apparatus. The coated tablets were placed in the basket containing

pH 6.8 PBS along with the rat cecal contents. The experiments were carried out with the continuous CO_2 supply into the beaker to simulate anaerobic environments of caecum. The drug release studies were carried out for 21 h (as usual colonic transit time is 20-30 h) and 1 mL of samples was taken at different time intervals. The volume was made up to 10 mL with PBS, centrifuged and the supernatant was filtered through a bacteria proof filter and this filtrate was analyzed for mesalazine content by spectrophotmetrically at 490 nm.

In Vivo Studies. Each study was carried out in six healthy male volunteers. A complete cross over design is employed. The study was performed in Latin square Design. Their liver and kidney functions were assessed to be normal by clinical and standard biochemical investigation. None of the subjects used alcohol or tobacco or had not taken any medication for a week prior to study. The purpose of the study was fully explained and each volunteer had given his written consent and was approved by the ethical committee of the institution.

Volunteers were fasted overnight and zero hour blood samples were collected early in the morning from each volunteer. Afterwards the various coated formulations of mesalazine were given to the volunteers alongwith 200 mL. of water. Food was withheld for a period of 2 h. The blood samples were collected at 0,3,5.... 32 h. The plasma was separated and the drug extracted and analyzed spectro-fluorimetrically at λ_{emi} 430 nm and λ_{exi} 315 nm (14,15).

Data Analysis. Data were generated by assuming the first order absorption and one compartment model with first order elimination. The maximum peak concentration (C_{max}) and time of its occurrence (T_{max}) were directly computed from the plasma concentration vs time plot. The elimination rate constant (K_{el}) was determined from the terminal phase of the log plasma concentration vs time profile by least square regression analysis. From this K_{el} is calculated as K_{el} = slope x 2.303. The elimination half life $t_{1/2}$ = 0.693/ K_{el}. The area under the plasma concentration time curve from $0 \rightarrow t^*$ ($AUC_{0 \rightarrow t^*}$) and from $0 \rightarrow \infty$ ($AUC_{0 \rightarrow \infty}$), area under first moment curve from $0 \rightarrow t^*$ ($AUM_{0 \rightarrow t^*}$) and from $0 \rightarrow \infty$ ($AUMC_{0 \rightarrow \infty}$), and mean residence time (MRT) were calculated using trapezoidal rule.

Statistical Analysis. The results obtained for invivo studies were subjected to statistical analysis using a computer program Instat (Graph Pad) for one way analysis of variance ($P<0.001$) followed by Dunnett's test.

Results

Preliminary acute toxicity studies which were carried out by the oral administration of a single dose of 2000 mg/kg b.w. of flax seed polymer caused neither mortality nor any signs of clinical abnormality in the tested animals during the observation of 14-day post administration of highest dose. The results have indicated that the polymer up to a dose of 2000 mg/kg was non lethal. The LD_{50} of the flax seed polymer falls under class 4 values as per the OECD guidelines. The biological evaluation was carried out at 2000 mg/kg dose levels.

Table I shows the composition of the compression coated mesalazine core tablet. The microcrstalline cellulose is added in the formulation as a direct compression adjuvant, since the flax seed polymer and chitosan do not produce sufficient hardness.

Physical Evaluation. Tablet weight varied between 678.2 and 682.5 mg, hardness between 4.5 and 5.5 kg/cm^2. The assay content of mesalazine varied between 98.6 and 99.8% and the friability ranged between 0.4 and 0.6%. Thus, all the physical parameters of the compressed tablets were practically within control.

***In Vitro* Drug Release Studies.** The percentage of drug released at different time periods from the mesalazine tablet compression-coated with coat formulation C4, C5 and C6 in 0.1 N HCl (2 h), pH 7.4 phosphate buffer (3 h) and pH 6.8 PBS (21 h) are shown in Figure 1. The results of the drug release studies carried out in the presence of 4% w/v rat cecal contents in pH 6.8 PBS are shown in Figure 2.

Given the above mentioned figures, it is clear that the drug was not released till 5 h, which indicates that the drug was not released in the presence of the 0.1N HC1 and pH 7.4 phosphate buffer.

***In Vivo* Studies.** The blood samples collected from the healthy human volunteers shows different pharmacokinetic parameters for the different formulations. The pharmacokinetic parameters are shown in Table II and the mean plasma concentrations of the different formulation are given in Figure 3.

The pharmacokinetic parameters and the mean plasma concentration hase shown that the drug from all the formulation was released only after 5 h and the formulation C3 shows better release.

Discussion

The successful delivery of drugs to the colon requires protection of drug from being released in stomach and small intestine. In the present study flax seed polymer and chitosan in the ratio of 2:3, 3:2 and 4:1 was applied over mesalazine core tablet and drug release studies were carried out under condition mimicking mouth to colon transit. The rationale for the admixture of chitosan along with flax seed polymer is due to its several favorable properties. It has been reported that chitosan enhance the absorption of various compounds across the mucosal barrier (12). Illum et al demonstrated the ability of glutamate chitosan to enhance the transport of insulin across the nasal mucosa of sheep and rat (17). Chitosan hydrochloride has been used to improve the bioavailability of buserelin in rats. Chitosan increases cell permeability by affecting paracellular and intracellular pathways. Chitosan causes relatively mild and reversible effects on epithelial morphology which makes it a promising absorption enhancing compound (16).

The *in vitro* release of the drug from tablets coated with coat formulation containing 2:3, 3:2 and 4:1 ratio of flax seed polymer and chitosan (C4, C5, and C6) was not found till 5 h of testing in simulated gastric fluids and intestinal fluids Figure 1 but on exposure to the dissolution fluid, the polymer got hydrated and formed a viscous gel layer that slowed down further seeping- in of dissolution fluid towards the core tablet. The hydration of coat seemed not to be affected by pH of the dissolution medium. Thus, flax seed polymer in the form of a coat was capable of protecting the drug from being released completely in the physiological environment of stomach and small intestine. To assess the integrity of the coats, the drug release were further continued for 21 h by replacing the dissolution medium with pH 6.8 PBS. At the end of the experiment (26 h), the cumulative mean percentage of drug released from coat formulations C4, C5, C6 were found to be 8.14±0.18, 14.20±0.80 and 24.28±0.14, respectively. This indicated that the polymer, coat would not permit the release of the bulk of the drug until the coat was broken.

The aim of the drug delivery system targeted to the colon was not only to protect the drug from being released in the physiological environment of stomach and small intestine, but also to release the drug in the colon after enzymatic degradation of colonic bacteria. Hence, the *in vitro* drug release studies were carried out in pH 6.8 PBS containing 4% PBS of rat cecal contents. At the end of 26 h of testing which included testing in simulated gastric and intestinal fluid, the percent of mesalazine released from the coated tablets with formulation C3 was found to be 98.00±0.19 and a lesser release 52.16+0.06, 64.10±0.08 respectively for formulations C4 and C5.

The release rate showed that the coat formulation C3 (440 mg flax seed polymer and 110 mg chitosan) produced better release of mesalazine. About 94.64% of the drug was released in the colon after protecting the drug form the stomach and small intestine. It was also evident from the results of drug release, in the presence of rat cecal contents that the maximum amount of the drug release occurred by the degradation of the coat material by the enzyme present in the cecal content.

The reason for colonic targeting property of flax seed polymer is that it is one of the richest source of plant lignans , secoisolariciresinol and matairesinol (18). The plant lignans are precursors of mammalian lignans. The main plant lignan precursors of flax seed secoisolariciresinol and matairesinol are converted by facultative bacteria in the colon to enterodial and enterolactone (Figure 4). So the ingested plant lignans are converted to mammalian lignans by gut bacteria (19,20,21).

Even though in vitro studies had revealed that a better release was obtained from the coat formulation C3 *in vivo* study using human volunteers was ultimate requirements to establish their credibility. The pharmacokinetic and mean plasma concentration (Table II and Figure 3) showed that the drug was released only after 5h indicating that the coat formulations (C4, C5, C6) have a capacity of preventing the drug release in the stomach and intestine. It was also indicated that the AUC ($_{(0-t^*)}$) of the formulation C3 was grater (547.81±3.4) when compared to other formulation.

In vitro drug release studies and *in vivo* studies using the formulations C4, C5, and C6 clearly indicated that the flax seed polymer and chitosan as a coat material over the core tablet was capable of protecting the drug being released in the physiological environment to stomach, small intestine and was susceptible to colonic bacterial enzymatic actions with resultant drug release in the colon. Thus, the study clearly hase indicated that the flax seed polymer and chitosan was a potential colon specific drug delivery carrier in which (4:1) 440 mg flax seed polymer and 110 mg chitosan proved itself a good carrier.

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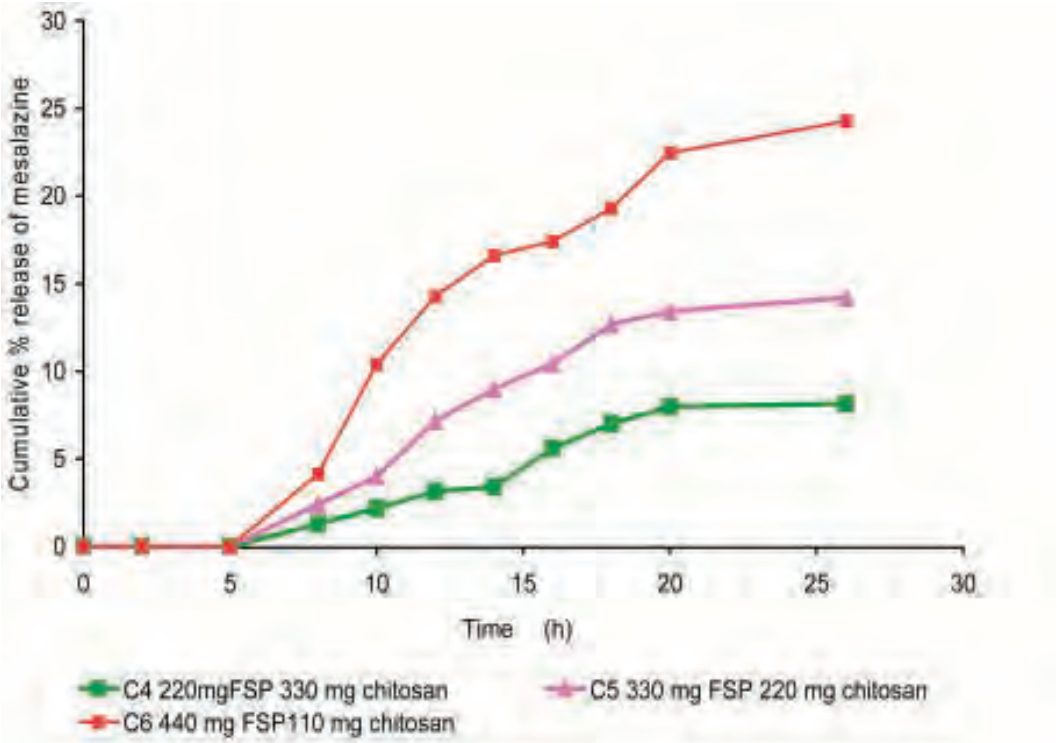

Figure 1. Cumulative mean (± s.d.) percent drug released from mesalazine tablets (n=6) compression coated with different ratios of coating material containing 2:3, 3:2, 4:1 of Flax Seed polymer and chitosan in 0.1N HCI (2h), pH 7.4 buffer (3h) and pH 6.8 PBS (21h)

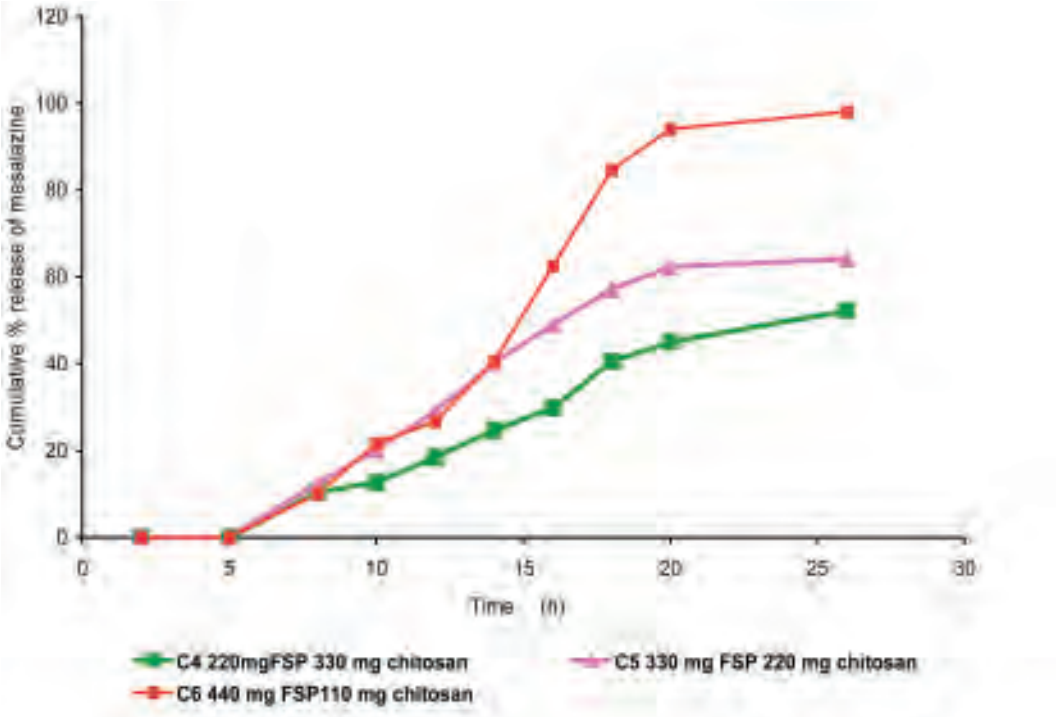

Figure 2. Cumulative mean (± s.d.) percent drug released from mesalazine tablets
(n = 6) compression coated with different ratios of coating material containing 2:3, 3:2, 4:1 of Flax Seed polymer and chitosan in 0.1N HC1 (2h), pH 7.4 buffer (3h) and pH 6.8 PBS containing 4% w/v rat cecal contents (21h)

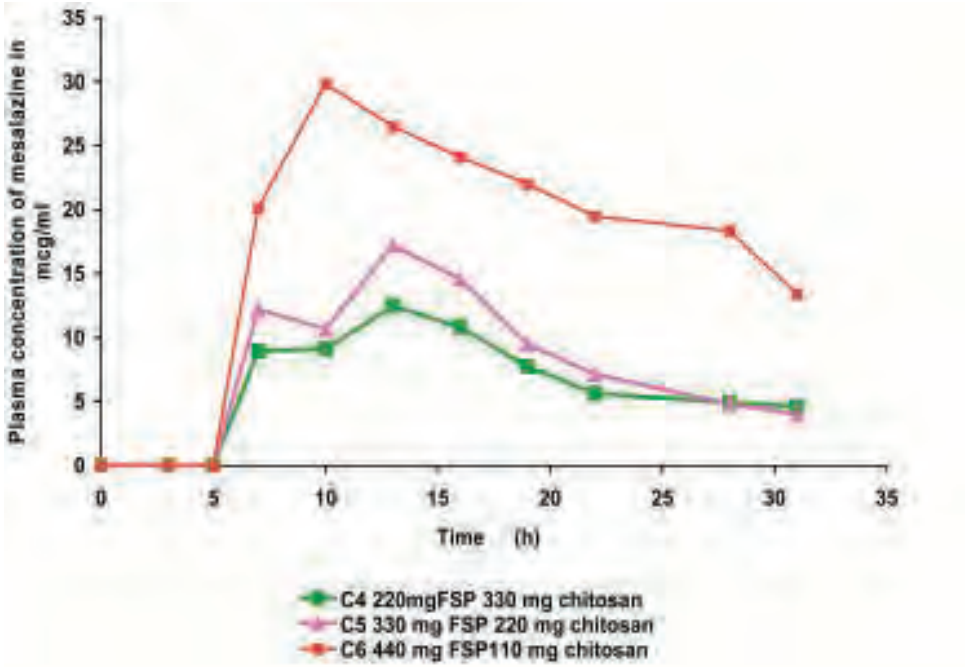

Figure 3. Mean plasma (±s.d.) concentration from measalazine tablets (n=6) compression coated with different ratio's of coating materials 2:3, 3:2 and 4:1 of flax seed polymer and chitosan in healthy human volunteers

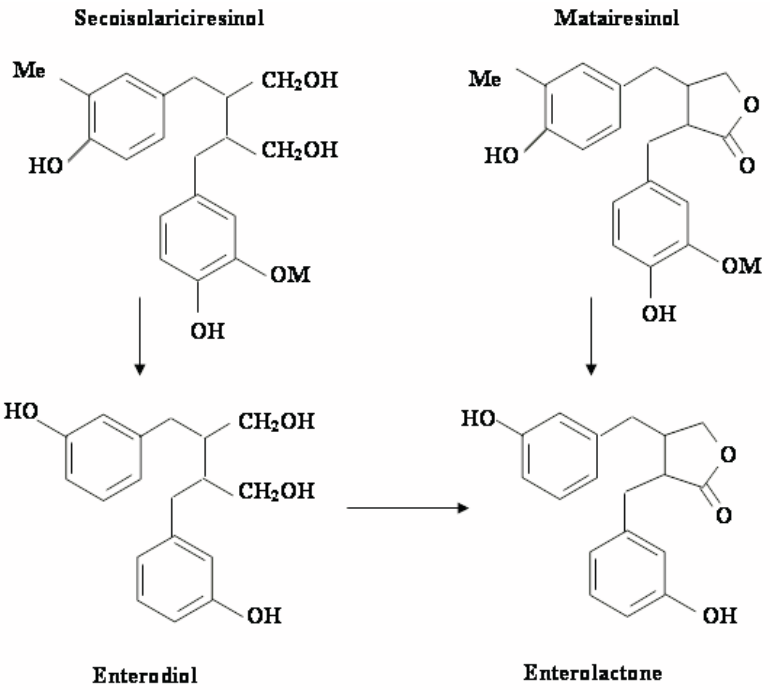

Figure 4. The main plant lignan precursors of flax seed secoisolariciresinol and matairesinol are converted by facultative bacteria in the colon to enterodial and enterolactone.

Table I. The composition of the compression coated mesalazine core tablet.

Coat formulation	Composition (mg)				
	FSP	**Chitosan**	**MCC**	**MgS**	**Talc**
C4	220	330	45	2	3
C5	330	220	45	2	3
C6	440	110	45	2	3

FSP: Flax seed polymer; **MCC:** Micro crystalline Cellulose; **Mgs:** Magnesium stearate; **Coat weight:** 600mg.

Table II. Pharmacokinetic parameters of mesalazine compression coated tablets obtained from the in vivo studies carried out in healthy human volunteers

Parameters	**C4 200mgFSP 330 mg chitosan**	**C5 330 mg FSP 220 mg chitosan**	**C6 440 mg FSP110 mg chitosan**
Cmax (µg/ mL)	12.46±0.46	17.2±0.82	29.84+0.32
Tmax (h)	13±0.0	13±0.0	10±0.0
Kel (h)	0.0227±0.03	0.064±0.05	0.036+0.08
$t_{1/2}$(h)	30.44±0.46	10.72±3.21	18.75+1.42
AUC_{0-t^*} (µg.h / mL)	196.97±3.02	245.8±5.10	547.81±3.4
$AUC_{0-\infty}$ (gh.h/ mL)	399.06±17.14	307.81±5.61	910.51+9.61

Values are mean SEM (n = 6 in each group)
Statistical analysis was performed using Instat (Graph Pad) one -way analysis of variance (ANOVA)
*P< 0.001 between the groups

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