ABSORPTION OF BACTERIALLY-SYNTHESIZED FOLATE ACROSS THE LARGE INTESTINE PRE-TRIAL I AND II: IN-VIVO BEHAVIOUR OF PLACEBO CAPLETS WITH PH-SENSITIVE COATINGS DESIGNED FOR COLON TARGETING

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Nutritional Sciences
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Absorption of bacterially-synthesized folate across the large intestine pre-trial I and II: In-vivo behaviour of placebo caplets with pH-sensitive coatings designed for colon targeting

Master of Science, 2008
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ABSTRACT

The purpose of this study was to design and test the effectiveness of two pH-dependent coatings in delivering intact barium sulfate caplets to the large intestine. In the future, this work will enable us to non-invasively assess the absorption of folate across the human colon. Barium sulphate caplet cores were coated with Eudragit L100 and S100, in either a 1:0 or 3:1 ratio. Each formulation was administered to ten volunteers, and monitored in-vivo via fluoroscopy. Test caplets with 3:1 coating formulations had 40% higher colon-targeting specificity compared to 1:0 caplets, and tended to begin dissolving at a later time after administration ($p=0.09$). The total time from administration to complete dissolution was also significantly longer for 3:1 coated caplets ($p=0.003$). These results suggest that barium sulphate caplets with a 3:1 (Eudragit L100:S100) coating formulation ratio would be a suitable delivery system for investigating the absorption of folate across the large intestine.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

RTC	Radiotelemetric capsule
PABA	Para-aminobenzoic acid
5MTHF	5-methyltetrahydrofolate
RFC	Reduced folate carrier
NTD	Neural tube defect
Hcy	Homocysteine
SCFA	Short chain fatty acid

LIST OF APPENDICES

1. OVERALL INTRODUCTION

The large intestine is the most distal segment of the human digestive tract, which begins at the ileo-cecal junction and ends at the anus. It is approximately 1.5m in length, and is divided into several regions, including the cecum, the ascending, transverse, descending, and sigmoid colon, as well as the rectum and anal canal (1). As an organ, some of the main functions of the large bowel include the formation, storage and elimination of fecal material (1), as well as the absorption of water and electrolytes (2,3). The luminal environment is generally viscous in nature, especially in more distal regions, and has a pH level that is close to neutral (4).

The colon also harbours a large population of viable microorganisms (5-7) that are known to be involved in the fermentation of polysaccharides and proteins (8,9), which, in turn, contributes to fecal bulking, and the formation of short chain fatty acids (SCFA) (10,11). Several species of microorganisms found in the colon also support the biosynthesis of certain B-vitamins, such as folate (12). Human studies have shown that the amount of folate synthesized by the intestinal microflora may exceed dietary intake by 3- to 5-fold (13). The mechanisms of bacterial folate synthesis have been well-defined; however, the role that this natural vitamin source plays in overall host folate status is still under investigation. At the level of the enterocyte, evidence from both animal and human studies has suggested that dietary folate may have a specific influence on colonic health, especially in terms of modulating the risk of colorectal carcinogenesis (14-19).

Folate uptake by human colonic cells has been demonstrated consistently in-vitro (20,21). Furthermore, it has been shown that these cells have the ability to transport folate by a similar mechanism as enterocytes originating from the small intestine (22,23).

Research in animals suggests that the impact of folate-producing microorganism on the vitamin status of the host may be influenced by diet. In a study by Krause et al., rats fed diets containing human milk solids had higher cecal counts of bifidobacteria and improved folate status compared to those fed milk solids from other sources (such as cow and goat) (24). The difference in colonic bacterial profiles among human milk fed rats may be attributed to their higher intakes of oligosaccharides (25,26), a type of indigestible carbohydrate that is known to be bifidogenic (27-30). Absorption studies in rats and piglets have enabled us to measure the bioavailability of folate or para-aminobenzoic acid (PABA), a folate precursor, after its direct injection into the cecum. In these studies, folic acid and bacterially synthesized folate were not only taken up from the colon, but also found to be incorporated into various organ tissues (31,32).

Current evidence in humans suggesting that bacterially synthesized folate can be absorbed across the large intestine, and influence folate status, is primarily observational. In 1997, Houghton and colleagues proposed that the folate status of adolescents could be influenced by promoting bacterial folate synthesis in the large intestine through diet, specifically the intake of non-starch polysaccharides (33). More recent research in infants has revealed the existence of a large pool of folate in human feces that consist primarily of short-chain 5-methyltetrahydrofolate (5MTHF), a form that is readily absorbed across the small intestine and incorporated into the circulatory system (13). Despite these promising results, there is a persistent lack of direct evidence to quantify the absorption and percent bioavailability of bacterially synthesized folate from the large intestine. This gap in the literature is mainly due to the absence of an appropriate and feasible means for studying nutrient absorption across the colon.

Current methods used for administering nutrient test doses to the large bowel are limited to the rectal route. Perfusions, enemas, and suppositories have been used to investigate the colonic uptake of certain B-vitamins (34) and, more recently, various fatty acids (35-38). Although these methods have provided valuable insight into the absorptive capacity of the large intestine, the findings from such studies should be interpreted with caution. Firstly, the site-specificity of rectal infusions is limited to the distal colon, and thus the relative contribution of more proximal regions to overall nutrient absorption may be underestimated. Secondly, the potential impact of laxative use, for bowel preparation purposes, on colonic absorption processes is not clear. Furthermore, the invasive nature of these methods may lead to low tolerance and acceptability among subjects.

Alternatively, the absorption of orally administered drugs across the human large intestine has been investigated by targeting their release in the large bowel area using a variety of specialized delivery systems. These delivery systems are often designed to rely on one or a combination of certain colon-specific features for their functionality, including intra-luminal pH (39-44), transit time (45-52), luminal pressure (53-55), and bacterial activity (56-61). Coated pH-dependent systems have been investigated extensively, and are available commercially for clinical use (62), particularly for the treatment of inflammatory bowel disease. The colon-specific targeting of these drug delivery systems has generally not played an integral role in treatment efficacy since most have been found to achieve remission rates of 40-80% in such disease states as mild to moderately active ulcerative colitis (39). Therefore, among the pH-dependent products that are available on the Canadian market, very few have been appropriately formulated to ensure that complete drug delivery occurs distal to the ileo-cecal junction. For large

bowel nutrient absorption studies, however, the site-specificity of a controlled-release delivery system has important implications in the quality of results, as many micronutrients are readily absorbed from the small intestine.

To date, there are virtually no colon-targeted dosage vehicles that have been designed for the purpose of delivering nutrients to the colon. Should this be accomplished, the absorption of folate across the intact and healthy human large intestine could be directly evaluated. Therefore, the main objective of this study was to design and test a suitable orally administered controlled-release system for the delivery of a vitamin dose to the human colon. The findings reported here were obtained from the results of in-vitro disintegration tests, and an in-vivo pre-trial series, where the transit time and dissolution profile of two different pH-dependent caplet coatings were evaluated and compared in healthy adults.

2. REVIEW OF THE LITERATURE

2.1 COLON-TARGETED DELIVERY SYSTEMS

2.1.1 Measurement and determination of gastrointestinal motility and pH

2.1.1.1Methods for measuring intestinal motility

The motor and transit characteristics of the gastrointestinal tract have been investigated extensively by scientists and clinicians using a variety of methodologies (63). Each technique has its own limitations in terms of accuracy and reliability, which tend to be diminished as technology advances. Imaging methods, such as fluoroscopy, can provide qualitative or semi-quantitative information on the motor activity of the gastrointestinal tract. The oral administration of a contrast material, such as a barium sulphate suspension, allows the detection of peristaltic actions and the subsequent movement of intestinal contents. Fluoroscopic images are valued for their high resolution (64); however the number of gastrointestinal events that can be captured is limited due to the risk of radiation exposure. Also, the use of barium as a contrast agent is not always recommended since, in some cases, it may have a physiological effect on the motility patterns of the stomach and small intestine. Alternatively, gamma-scintigraphy is an imaging method that does not require the use of contrast media, since it involves the detection of radionuclide tracers as they travel through the alimentary tract in the form of a labeled meal. Although the recorded images are not high in resolution, such as those associated with radiography, this computer-assisted technique allows the transit times of intestinal contents to be easily quantified.

Ultrasonography is another well-established imaging technique that uses high frequency sound impulses to detect organ boundaries and intra-luminal spaces. Real-time ultrasonic imaging has been employed for the study of gastro-duodenal transit (65,66). In

cases where imaging equipment or facilities are not available, the hydrogen breath test may be used as a simple indirect method for assessing oro-cecal transit times (67). It involves the ingestion of a substance, such as lactulose, that is not absorbed in the small intestine, but readily metabolized in the proximal colon by intestinal bacteria. The hydrogen gas, that is produced through bacterial fermentation and taken up into the blood stream, is measured as it is expelled via the lungs. Despite the simplicity of this technique, it should be used with caution since breath hydrogen levels can be influenced by such factors as intestinal bacterial overgrowth, antibiotic use, physical exercise, and hyperventilation.

In vivo intubation methods, such as manometry can be used to evaluate the motility patterns of all regions of the gastrointestinal tract by detecting changes in intra-luminal pressure caused by contractions of the muscular wall. Manometric pressure recordings can be obtained through miniature transducers or low-compliance water-perfused tube systems (68). This technique tends to be limited by its inability to detect more subtle changes in pressure, and the recordings may be affected by the presence of food particles or anatomical distortions caused by intubating the intestinal tract.

Methods for measuring gastrointestinal motility without intubation include radiotelemetry, and bioelectric impedance. Radiotelemetry is an in-vivo technique that involves the use of a capsule that contains a pressure-sensing device, and a radio-transmitter. The radio-pill is swallowed while tethered to a thread and the AM or FM signals are received by aerials that have been placed around the midsection of the subject. The main limitation of this device is signal loss, which can be minimized by improving the sensitivity of the receiving equipment. In addition to radiotelemetry, bioelectrical impedance is another non-invasive means for monitoring motility that involves the

measurement of fluid volumes in organs. This technique is often applied to the study of gastric emptying, and involves the use of electrodes, which are placed on the surface of the skin at anterior and posterior positions around the organ region of interest (69). Voltage fluctuations signify contractions of muscular organ walls, and subsequent decreases in organ volume. This method tends to be more sensitive and accurate than manometry (70), and is often preferred due to its simple and portable equipment, as well as its continuous output with immediate results that are easily interpreted.

The contractile activity of luminal walls can be evaluated by monitoring the frequency and intensity of membrane action potentials. Electromyography involves extracellular recordings of changes in electrical potentials generated by smooth muscle, and thus can be used to characterize the motor patterns of the gut wall or assess the affect of drugs and hormones on contractile activity. A similar technique, electrogastrography, measures the electrical activity of the stomach through electrodes that are surgically implanted in the mucosa or epigastric skin surfaces. This technique has been used to detect gastric dysrhythmias and assess the causes of abnormalities in gastric motility.

2.1.1.2 Gastrointestinal transit rates

During and shortly after the ingestion of a solid meal, the motor activity of the stomach is thought to include an initial lag phase followed by frequent low amplitude contractions that continue as long as food remains in the lumen (63). This pattern of activity is suitable for grinding digestible food into small particles within the stomach and expelling it through the pylorus (71). In the fed state, gastric emptying times of solid particles can range from 30 minutes to over 15 hours, and they tend to be highly dependent on the caloric value and composition of the ingested meal (71-74). There is

also some evidence to suggest that gastric motility may vary according to circadian rhythm. A study by Goo et al. (1987) demonstrated significantly longer half-emptying times of a solid meal in the evening compared to the morning (75). At the end of the digestive period, the stomach enters an interdigestive state known as the migrating myoelectic complex (76), which has four phases of contractile activity that cycle approximately every 2 hours (77). The third interdigestive phase has been termed the "housekeeper" wave; since it consists of the most intense proximal and distal contractions that can empty the stomach of cellular debris, bacteria, and any remaining indigestible particles that did not pass through the pylorus during the digestive phase (63,78,79).

Unlike the stomach, the motility of the small intestine is generally not influenced by the consumption of food (80). It also appears to be unaffected by age (81), physical activity (82), or pathological conditions. Small intestinal transit times have been reported to be very consistent with a mean value of 180±60 minutes (83,84). The variability in small bowel transit in humans tends to be less than gastric emptying, while the transit variability in both digestive organs tends to be lower within individuals than between (85). The rate of entry of ingested particles into the colon is thought to be controlled at the ileo-cecal junction (72,86), however, the mechanisms of this effect have not been elucidated. Some investigators suggest that the flow of chyme into the colon is controlled by the muscular contractions of a sphincter in conjunction with the motility patterns of adjacent large and small intestinal regions (63,87).

Compared to the stomach and small intestine, the transit characteristics of the human large intestine are less well-defined. While the overall flow of its contents tends to be slow (63), the specific motor activity of various large bowel regions may be influenced by food intake. The group of Jouet et al. (1998) measured the contractile

activity of the ascending and descending colon before and after ingestion of a 1000 kcal low residue liquid meal. They reported that, during the fasted state, the colon has lower mean motility in distal regions than in proximal regions (88). During the first 30 min after the ingestion of a meal, tonic contractions were detected in the ascending and descending colon, with a distal-to-proximal pressure gradient that lasted for at least 3h after the meal. The authors concluded that these regional differences in colonic motor activity may be responsible for postprandial mixing of the colonic contents. It is also believed that the transit of food particles through the colon is size-dependent and susceptible to diurnal variation, with large particles passing through more rapidly (89,90), and decreased transit during slumber (91). Furthermore, colon transit rates have been reported to be increased in certain disease states, such as ulcerative colitis (92); and decreased in the presence of other conditions, such as cholelithiasis (93).

2.1.1.3 Methods for measuring intestinal pH

A range of naso-intestinal intubation techniques have been employed to measure the pH levels of the stomach and upper small bowel. Aspiration of gastric fluids allows for direct and cross-sectional measurements of the pH of the stomach. Glass electrodes, on the other hand, have been used with portable intubation systems to take ambulatory measurements of pH fluctuations in the upper gut (39,94). While the aforementioned methods may be precise and well-correlated with each other, they are limited by various factors that can affect the accuracy of results. Hypersalivation and reflux through the pyloric sphincter, caused by intubation, may dilute aspirates and artificially raise pH levels. Furthermore, gastric fluid may be transported along the tube and lead to underestimations of intra-luminal pH in the small intestine. In the large intestine,

mucosal pH has been measured by inserting a pH probe through the biopsy channel of a colonoscope (95). While this method is useful for exploring relationships between colonic pH and pathologies, it is limited in the number of locations and periods of time that can be recorded. Furthermore, the effects of fasting and laxative use for the purpose of bowel preparation may alter the luminal surface and lead to pH readings that are not representative of the unprepared colon. In 1987, McNeil et al. performed in vitro pH measurements of colonic mucosa from rats and humans. The results showed that the mucosal surface acts as a sort of microclimate and its pH level does not necessarily reflect those of the intestinal lumen (96).

Various tubeless techniques have been tested with low success rates due to their indirect, nonspecific, and time consuming methodologies. The radiotelemetric capsule (RTC), however, has provided promising results since its invention in 1957 (97) and further development over the following four decades (98). The RTC consists of a reference and pH-sensitive electrode which transmits ambulatory pH readings from the intestinal lumen with a radiofrequency transmitter (99). It is battery powered and approximately 26mm in length x 7mm in diameter. The radio-pill is orally ingested, and its location determined via fluoroscopy, signal strength, and recorded changes in pH (4). Results obtained from an RTC have been shown to be strongly correlated to direct pH measurements of aspirates (100). Methodological problems associated with radiotelemetric pH-capsules include poor signal quality, as well as pH and frequency drifts (101,102). These limitations can be addressed, however, through improvements in equipment design and calibration methods (103).

2.1.1.4 Normal pH profile of the gastrointestinal tract

The gastrointestinal pH profile of healthy subjects (n=66) was investigated by Evans et al. using radiotelemetric capsules. As expected, gastric pH was highly acidic in the fasted state with a range of 1.0 to 2.5. The mean pH in the proximal small intestine and terminal ileum was 6.6±0.5 and 7.5±0.4, respectively. An abrupt fall in pH, to a mean of 6.4±0.4, was recorded as the capsule passed into the cecum, followed by a progressive rise from the ascending to the descending colon with a final value of 7.0±0.4 (4). The results from this study are in general agreement with other investigations of gastrointestinal pH using radiotelemetric devices (4,98,101-106).

A recent review by Nugent et al. (2001) summarized several factors that affect normal gastrointestinal pH levels (39). In the foregut, hydrogen and bicarbonate ion secretions by gastric and intestinal mucosa are major determinants of intra-luminal pH. Specifically, the gastric contents are buffered by alkaline secretions from the pancreas, which raises the pH level of the proximal small intestine. Subsequent bicarbonate excretions from the small intestinal mucosa lead to a further gradual rise in pH until the terminal ileum (4). A fall in luminal pH in the cecum is, in part, attributable to the carbohydrate fermenting action of colonic bacteria and the generation of SCFAs and hydrogen ions (9,107-109). SCFAs, especially butyrate, are absorbed and metabolized as a principal energy source by the colonic epithelium (110). As a result, a gradual fall in SCFA concentration may contribute to a rise in pH in the distal colon. In the rectum, a slight drop in pH may occur due to fecal stasis, since the colonic bacteria can continue to ferment any remaining carbohydrates. Ammonia is known to be formed in the colon due to the bacterial metabolism of protein, amino acids, and urea. Despite this metabolic

process, a high protein diet is less influential on intra-luminal pH levels than bicarbonate and organic acids (111,112).

The effect of transit time on pH levels in the colon is variable and inconsistent. Theoretically, a shortened transit time could increase pH by reducing the time available for fermentation. Faster motility could also result in decreased pH levels by causing carbohydrate-starved bacteria to produce more lactate (113). Luminal pH is further affected by intestinal pathologies, namely inflammatory bowel disease. Reduced mucosal bicarbonate secretions, increased bacterial lactate production, and impaired SCFA absorption and metabolism may contribute to lower colonic pH when the mucosa is inflamed (102,113).

2.1.2 Types of delivery systems

Orally administered pharmaceutical preparations are commonly designed with non-specific release mechanisms, and thus will dissolve in the acidic environment of the stomach to provide rapid drug absorption. Controlled-release formulations, however, will delay or extend the release of drug throughout the gastrointestinal tract (114,115), and are often designed to exploit one or a combination of characteristics that are unique to a target organ. In the case of targeting drugs to the colon, transit time, intra-luminal pressure, intestinal microflora, and intra-luminal pH are some of the features that have been used to design a range of specialized delivery systems. These unique formulations can be further subdivided into multiple-unit (e.g. pellets, granules or microspheres) (116-118) or single-unit (e.g. tablets and capsules) (3,119) systems. Multiple-unit systems generally permit more uniform drug dispersion throughout the lower gastrointestinal tract, and are often more reliable in terms of complete dose delivery (120). Single-unit

systems tend to have more variable 'all-or-nothing' release profiles, although their simplistic design, low manufacturing cost, and site specificity potential have made them more popular (3).

2.1.2.1 Time-dependent

Time-dependent delivery systems rely on the low variability of small intestinal transit rates to target drug release in the proximal colon. The formulations are often designed to promote dissolution after a pre-determined lag time of 5 or 6 hours post administration (45-47,49). Although several drug-release systems have been developed using time-dependent mechanisms, very few have demonstrated consistent in vivo results in terms of colon-targeting. This variability is mainly attributable to the unpredictable nature of gastric emptying, which can range from a few seconds to a number of hours, depending on the size of the delivery system, as well as the amount of food ingested by the individual. In some cases, a viscous intra-luminal environment may also affect the efficacy of time-dependent formulations, since they tend to rely on hydrodynamics to induce swelling or erosion of a soluble polymeric layer. In light of these limitations, the time-dependent concept has been more successfully applied to colon-targeted systems in combination with other time-independent mechanisms (45-52).

2.1.2.2 Pressure-dependent

Delivery systems have also been designed to utilize intra-luminal pressure that is generated throughout the alimentary tract via muscular contractions of the intestinal wall. The intensity and duration of these contractions tend to vary in different regions of the gut, with the colon having higher luminal pressure due to the combination of haustral

contractions and a more viscous environment (1). These pressure differences have been the basis of development for certain controlled-release formulations (53,54,121,122). In general, the disintegration properties of a gelatin capsule are altered via the thickness of an inner ethylcellulose coating, which provides protection from destructive peristaltic waves (55). The colon-targeting success of these delivery systems has been somewhat inconclusive, and very little in vivo performance data has been obtained from human studies (53,123). Furthermore, there is limited evidence-based knowledge on the variability of gastrointestinal pressure values among healthy individuals, and how they might be affected by other luminal factors such as pH or motility.

2.1.2.3 Enzyme-dependent

More recent research interests in the field of colon-targeted delivery systems have been focused on utilizing bacterial action to promote drug release in the large bowel (58,60,61). Bacterial fermentation takes place almost exclusively in the colon of healthy individuals, since large and diverse microfloral populations are unique to this intestinal region (7,124). Polysaccharides such as pectin (57,125-128), guar gum (129-136), and amylose (59,115,116,137-139), have been applied as natural polymers to solid dosage forms as matrix agents, or coatings. Upon entering the colon, intestinal bacteria will promote drug release by degrading the outer polysaccharide layer or inner excipient. Although this drug delivery concept has produced promising results, several formulations have not been developed beyond phase I clinical trials, due to the requirement of complex manufacturing methods, or the novelty of the technology (62).

2.1.2.4 pH-dependent

The pH-dependent delivery system operates on the assumption that intra-luminal pH levels are very acidic in the stomach, and gradually rise towards neutrality from the duodenum to the terminal ileum (4,39). The formulation often includes an enteric coating that is resistant to the acidic conditions of the stomach and becomes soluble at the more neutral pH levels (62). In 1953, Rohm & Haas (Darmstadt, Germany), created enteric coating products made of acrylic-based copolymers under the trade name, Eudragit. A series of Eudragit products have since been developed and tested for their in-vivo dissolution properties. Eudragit S100 is a copolymer of methacrylic acid and methyl methacrylate with a normal solubility threshold of pH 7.0, which has been used by several groups in colon-targeted formulations (40-43,121,140,141). Eudragit L100 is structurally similar to S100, although it is chemically designed to have a lower pH threshold (6.0), and thus is often used to promote drug release in the small bowel. Today, Eudragit products are often employed in commercialized pharmaceutical preparations for the treatment of inflammatory bowel disease (39,55,142-145).

Despite the broad use pH-dependent systems, the colon-targeting performance of these dosage forms tends to have high inter-individual variability due to inherent differences in ileo-colonic pH profiles among healthy people (4,103). These inconsistencies have prompted the investigation of mixed coating formulations, which involves combining the solubility characteristics of different Eudragit copolymer types. Some groups have mixed Eudragit S100 and Eudragit L100 in various proportions to improve the in-vitro dissolution profile and in-vivo site specificity of pH-dependent systems (44,146,147). The combination of these copolymers allows for the creation of

new coating formulations with customized solubility characteristics, including a pH threshold between 6.0 and 7.0.

2.1.3 Monitoring delivery systems and nutrient absorption in the human gastrointestinal tract

2.1.3.1 Endoscopy

Gastroscopy allows a real-time examination of the stomach cavity, and has been used in the evaluation of intra-gastric disintegration profiles of orally administered drugs (148,149). Endoscopic analyses can also be used for determining the nature of interaction between drug-containing tablets or capsules and the intra-luminal mucosal tissue. Graham et al. (1990) compared the distribution of potassium chloride from tethered capsules and free tablets in the stomach using a gastroscope. It was found that the potassium chloride released from both a capsule and a tablet was generally held in place by gastric mucus, although the tablet crystals tended to be less adhesive and dispersed in a net-like pattern (150). Despite the advantages of real-time analysis, gastroscopy may not be an appropriate method for investigating the dispersion and absorption of vitamins, since they are often taken up in the small intestine. The invasive nature of the procedure can also cause significant discomfort to the subject, and thus acceptability tends to be low. Furthermore the pharmacological effects of pre-medication drugs (such as sedatives), as well as the presence of the endoscope itself may cause abnormal gastrointestinal behaviour (69).

2.1.3.2 Gamma-scintigraphy

Gamma-scintigraphy was first used in 1976 to investigate the fate of pharmaceuticals in vivo (151), and is now commonly used to evaluate drug delivery to

the gastrointestinal and respiratory tract (152). The technique involves the use of a specialized camera to detect scintillation properties of a gamma-emitting material, such as technetium (^{99m}Tc), that is incorporated into a pharmaceutical formulation. Although, scintigraphic methods are ideal for obtaining quantitative measures of drug release in the gastrointestinal tract, certain errors that are related to other sources of radiological activity must be accounted for. This background noise can be caused by natural isotopes, cosmic rays, and the imaging equipment itself, and must be subtracted from the total reading to obtain a net count from the source of interest (69). Certain attenuation factors are also required to maintain accuracy, such as the absorption effect of intervening tissues and bone, as well as natural radioactive decay of the gamma source itself (69)

Other limitations associated with this technique may include the feasibility of using radionuclides, especially at the level of the manufacturer. Radiopharmaceuticals must be produced in a facility that has the capacity to handle radioactive material. They also must be labeled as close to the time of administration as possible to accommodate the short half life of commonly used isotopes, such as technetium (^{99m}Tc, 6 hours) and indium (^{111}In, 2.8 days) (153). Furthermore, the production of gamma-emitting delivery systems must be scaled down in order to limit radiation exposure. Small batch sizes may not be suitable for larger clinical trials, and are also prone to formulation inconsistencies, which can alter in-vivo dissolution behaviour (69). It should also be noted that the in-vivo dissolution characteristics of nutrient-containing delivery systems have not been evaluated using gamma-scintigraphy, and thus the effect of radio-labeling on the distribution and absorption of a vitamin is not known.

2.1.3.3 Radiology

Radiological techniques, such as fluoroscopy, have traditionally been used to assess the motor behaviour of the gastrointestinal tract (154,155) using orally ingested contrast media. This concept has also been applied to the in-vivo evaluation of controlled release oral delivery systems by incorporating a radio-opaque material (such as barium sulphate) into the formulation itself (156). This combined use of radiology and pharmacokinetics has been used by groups, such as Marvola, et al., to assess different types of delivery systems with respect to the relationship between drug release location, and subsequent absorption (157,158). The main advantages of fluoroscopic monitoring methods are related to the use of inert insoluble radio-contrast materials for visualizing purposes. A commonly employed material, barium sulphate, is generally compatible with most delivery system components and coatings, and has not been found to interfere with in-vivo drug absorption due to its extremely low bioavailability (153). In contrast to radioisotopes, barium sulphate is very stable and does not require specialized facilities or time-sensitive procedures to be handled safely. In powder form, it can be incorporated directly into capsule or tablet formulations, and is appropriate for scale-up production.

The major physiological risk associated with fluoroscopy is repeated radiation exposure, although recent advances in imaging technology have allowed the required radiation dose per image to be decreased (63,159). In terms of monitoring the in-vivo characteristics of orally administered delivery systems, the major disadvantage of this imaging method is the qualitative nature of the data collected. Despite these noted limitations, however, fluoroscopy has been successfully employed to track delivery systems, as well as other orally administered monitoring devices, through the gastrointestinal tract. Fallingborg et al., used fluoroscopy to localize radiotelemetric

capsules to determine the pH profile of different intestinal regions (103,106). Gastrointestinal transit rates have also been evaluated using this imaging technique by confirming the anatomical location of a tethered pressure-sensitive radio-pill (63).

Fluoroscopy may also be successfully applied as a novel method for monitoring the transit and dissolution characteristics of nutrient-containing colon-targeted delivery systems. Although the quantitative output of scintigraphy has generally been preferred for studying the intestinal delivery of drugs, the use of radioactive isotopes makes this imaging technique a less feasible option when appropriate pharmaceutical manufacturing facilities are not available. Furthermore, the chemical stability of contrast agents, such as barium sulphate, may be more suitable for nutrient absorption studies in the large intestine, since the colonic transit time of a delivery system could exceed the half life of a radioisotope label.

2.1.3.4 Monitoring folate uptake from the large intestine

The presence of folate in the blood stream is generally used as an indicator of vitamin absorption from the small intestine; however the degree to which blood levels can reflect the absorption of bacterially synthesized folate from the colonic lumen has not been confirmed. The group of Kim et al. have used endoscopic methods to determine the colonic mucosal folate concentrations of human subjects, and reported direct correlations between mucosal and blood folate levels (160,161). These results demonstrate that blood folate may be a reliable indicator of colonic vitamin status; however, they do not provide quantitative evidence for the relative contribution of bacterially synthesized folate to the appearance of this vitamin in local tissues or the systemic circulation. Furthermore, it is possible that the level of folate found in the colonic mucosa was

underestimated since endoscopic procedures must be performed on a 'clean' bowel, and thus the naturally occurring populations of folate-producing intestinal bacteria were likely depleted. Therefore, our success in determining the bioavailability of bacterially synthesized folate in the large intestine depends on our ability to quantitatively deliver this vitamin and its precursor, PABA, to the intact large bowel. A folate-containing delivery system can be used to by-pass the small intestine, and monitoring methods, such as fluoroscopy, can be employed to verify the approximate anatomical location of dose release and subsequent absorption.

2.2 FOLATE

2.2.1. Chemical structure and properties

Folate is an essential water-soluble vitamin of the B group. The term *folate* generally refers to both synthetic and natural forms of the vitamin, which are structurally related (162,163). *Folic acid* is the synthetic and fully oxidized form of folate. It has a chemical structure and nutritional activity similar to that of natural folates, and is the most common form of the vitamin used for supplementation and food fortification (164,165). Natural folates exist primarily as reduced, one-carbon-substituted forms of pteroylglutamates, which consist of a pteroyl group [a pteridine ring (2-amino-4-hydroxy-pteridine) attached through a methylene bridge to para-aminobenzoic acid (PABA)] and attached glutamate residues (166) (Figure 2.2.1). Pteroylglutamates can differ in the number of glutamyl residues attached to the pteroyl group. Intracellular folates have been found to contain 5-8 glutamate residues (polyglutamates) (165), while those found in serum are monoglutamated.

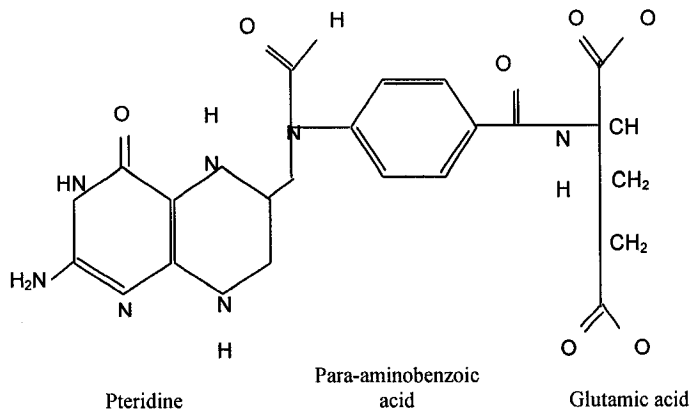

Figure 2.2.1: Structure of a one-carbon substituted folate

In the body, an important function of polyglutamated folate species is likely intracellular retention, and they are thought to have higher affinity for enzymes that utilize folate as a cofactor (166). The biologically active form, tetrahydrofolate, appears to act as a cofactor in multiple biochemical reactions by donating or accepting one-carbon units from such compounds as glycine and serine (165,167). 5-Methyltetrahydrofolate (5MTHF) has been the most common form found in both plant and animal tissues (163), and is likely the predominant form in the human circulation (168). The stability of folate is pH-dependent with the reduced forms most stable at pH>8 and pH<2 and least stable between pH 4-6. While the synthetic form, folic acid, appears to exhibit much greater stability than the reduced folates, all forms of folate can differ in their susceptibility to oxidative degradation, which is enhanced by oxygen, light, and heat.

2.2.2. Food folate

Since humans are incapable of de novo synthesis or long term storage of folate, a frequent and adequate supply of this B-vitamin must be obtained from the diet. Naturally rich sources of folate include yeast extracts such as Marmite, liver, kidney, leafy green vegetables, citrus fruit, and fermented dairy products (169). The adequacy of usual folate intake in a population can vary depending on the availability of folate-rich foods and dietary preferences. In Finland, for example, it has been determined that rye is the best single source of folate since it is consumed regularly and, thus, contributes about 11-12% to the average daily folate intake of the Finish population (170).

Usual folate intakes can also be influenced by food fortification strategies, which have been mandated on a nation-wide basis in North America over the past decade.

National and provincial food intake surveys, conducted between 1990 and 1998, revealed that the dietary folate intakes of most 18-65 year old Canadians tended to be lower than the recommended dietary allowance (400ug/day for adults) (171,172). Due to this finding, and the large body of evidence correlating the periconceptional use of folic acid-containing supplements and the reduced risk of certain birth defects (neural tube defects such as spina bifida and anencephaly) (173-176), the addition of folic acid to white flour and enriched pasta and cornmeal (150ug/100g flour and 200ug/100g pasta) became mandatory in Canada in November 1998 (177). A recent report of dietary intakes in the province of Newfoundland indicated that the consumption of folate had increased by 70μg/d among reproductive-aged women, after the implementation of a fortification program (178). It is also interesting to note that bread, rolls and crackers (made with wheat flour) are now considered to be the largest contributors of folate in the American diet (15.6%), as demonstrated by a comparison of national survey data pre- and post-fortification (NHANES III and NHANES 1999-2000) (179).

Due, in part, to the potential adverse effects of high folic acid intakes, namely the masking of a vitamin B12 deficiency (180), other countries in the world have questioned the safety and efficacy of fortifying the food supply. Instead, some have advocated for improving folate status through dietary means. Since 2001, for example, the Swedish Food Administration and National Board of Health and Welfare recommended that all fertile women increase their folate intake to 400ug/day by eating folate-rich foods such as green vegetables, fruits, fermented milk products, and coarse rye bread (181).

2.2.3. Intestinal Transport

It is generally accepted that folate is mainly absorbed in the jejunum through a multi-step process involving transport across the brush border membrane, passage through the enterocytes and transport across the basolateral membrane (23,164). Before absorption across the enterocyte can occur, however, polyglutamyl folates are hydrolyzed to folylmonoglutamates (166) then absorbed via a specialized pH-dependent (182) carrier-mediated mechanism (12). The uptake of monoglutamates has been described as either a passive or active process. Passive transport may occur primarily when very high levels of monoglutamyl folates are ingested, although it is likely insufficient to sustain adequate folate levels on its own. Active transport has been regarded as the most important absorption mechanism in the bowel, and is likely necessary to prevent back diffusion of monoglutamates. Upon arrival in the enterocyte, folate monoglutamates have been traditionally thought to be metabolized to 5MTHF, which is the principal form of the vitamin in serum.

Folate receptors, which are found on cell membranes, transport folates via an endocytotic process, and tend to have a high affinity for folic acid (183,184). An isoform of the folate receptor, called folate receptor-α, is primarily involved in the transport of folate across epithelial membranes (183,184). While folate receptors have not been found to be highly expressed at the level of the gut, the reduced folate carrier (RFC) is thought to be the major bi-directional, carrier-mediated transporter of folate in the intestinal tract (12,185). In the proximal small intestine, the RFC has been found to have similar affinity for reduced (e.g., 5MTHF) and oxidized (e.g., folic acid) forms of folate (186), a characteristic that may be unique to the gut. Although it has been well established that the small intestine is the preferred site of folate absorption, the RFC is

likely also present in the large intestine, but at different levels in each region (12). Recent studies on human colonic epithelial cells have revealed a possible folate transport system in the large intestine that has similar mechanisms to that found in the small intestine (20,23) The existence of an efficient carrier-mediated folate transporter in the human colon may imply that endogenous folates in the large intestine (such as those synthesized by intestinal microflora) could be absorbed from this region and contribute towards total folate homeostasis.

Absorption across the colon

As mentioned above, evidence from several in vitro and animal studies has shown that folate is likely absorbed across the large intestine, and that the absorption process may be similar to that of the small intestine (13). In vitro studies of human colonic epithelial cells have demonstrated that the folate uptake system is likely under the regulation of an intracellular protein tyrosine kinase- (22) and cAMP-mediated pathway (12). In 2001, Dudega et al. conducted an in vitro experiment to examine the mechanism of folate uptake by purified basolateral membrane vesicles, which were isolated from the colonic mucosa of organ donors. The results showed that a pH dependent, DIDS[1]-sensitive, carrier-mediated uptake system is likely involved, and that the transport of folate across the human colonic basolateral membrane could be an electroneutral process (22).

In 2000, Said et al. used a rat model to examine the effect of dietary folate deficiency on the ability of the large intestine to absorb folate. Upon inducing folate deficiency with a low-folate diet that contained the antibiotic succinyl sulfathiazole (in

[1]Anion exchange inhibitor, 4,4'-diisothiocyanate-2,2'-stilbene disulfonic acid

order to decrease the contribution of bacterially synthesized folate to total folate level), there was a significant up-regulation in the steady-state mRNA level of RFC in the rat colon. From this finding, the investigators suggested that a folate deficient animal may try to maximize the uptake of bacterially synthesized folate in the large intestine (187). Since the authors also observed a similar up-regulation in RFC mRNA level in the small intestine of folate-deficient rats, they went further to propose that a similar regulatory mechanism may in fact exist in these two distinct regions of the intestinal tract (187).

2.2.4. Folate excretion

The liver has been found to play an important role in the uptake of dietary folate, and enterohepatic recirculation may play a major role in maintaining plasma folate concentrations by clearing non-methylated folates from the circulation and re-excreting them into bile as methylated species (166). In a study by Lin et al. (2004), it was found that the amount of folate that is released into the gastrointestinal tract via bile may be as large as 4.25 times the dietary intake (188). It was further estimated that approximately 65% of intracellular folate monoglutamates are eventually re-circulated to the gastrointestinal tract via bile, while the remainder are used for pteroylpolyglutamate synthesis. Lin et al. also found that the excretion of monoglutamated folates (and their oxidation products) in feces could represent almost 50% of usual dietary intakes

2.2.6. Folate bioavailability

Natural Food Folates

The bioavailability of a nutrient can be defined as the proportion of the ingested amount that is absorbed and becomes available for use and storage in the body (189). Early investigations on the intestinal absorption of naturally occurring food folate have

led to the general consensus that they are approximately 35-50% less bioavailable than pure monoglutamated folic acid (190-194). Other investigators have recently found that the bioavailability of certain natural forms of folate, namely 5MTHF, is actually similar to or even higher than folic acid. In a study by Pentieva et al. (2004), equivalent oral doses of 5MTHF and folic acid produced similar changes in plasma folate concentrations among folate saturated adult male subjects (195). Houghton and colleagues very recently reported that supplementing lactating women with 5MTHF appears to be more effective than equimolar supplementation with folic acid in maintaining maternal erythrocyte folate concentrations (2178 versus 1967 nmol/L for 5MTHF and folic acid, respectively; $p<0.05$) (196). Similar findings have been reported by the group of Lamers et al. (2006), who also demonstrated a dose-dependent increase in red blood cell folate concentration of 190nmol/L for every 100μg of supplemented 5MTHF (197). In light of these findings, it is conceivable that supplementing with natural folate derivatives (such as 5MTHF) is a suitable alternative to synthetic folic acid, especially since the natural form may have the added advantage of not masking the anemia of a vitamin B12 deficiency (195-197).

2.2.7. Folate and human health

Various disorders are thought to be under the influence of folate status or allelic variations in genes coding for folate-dependent enzymes (169). Specifically, folate nutrition has been implicated in the etiology of neural tube defects (NTDs) and several cancers (including colon cancer). Folate nutrition has also been associated with the incidence of anemia, cardiovascular disease, early pregnancy losses and preeclampsia, Alzheimer's disease, and certain affective disorders (164,198). While the development of most disorders can be explained within the context of folate-dependent one-carbon

transfer reactions, the precise underlying cause may be linked to a combination of factors. Folate has been found to be an important co-factor in the remethylation of homocysteine (Hcy) to methionine, and an elevated total plasma Hcy concentration has been considered to be a marker for a defect in folate metabolism or for folate deficiency. High plasma Hcy concentration has also been considered a risk factor for cardiovascular disease, NTDs, Alzheimer's disease, and colorectal cancer. It has thus been suggested that the Hcy-lowering effect may be an important aspect of folate-related health (164). Furthermore, the likely role of folate in purine and pyrimidine biosynthesis may imply that impaired folate metabolism can have an effect on DNA elaboration and/or gene expression (169).

Neural Tube Defects

The possible role of folic acid in the prevention of congenital malformations was first proposed in 1964 (199) and, since then, the health benefits of folate before and during early pregnancy have been well established, especially with regards to NTDs. During embryonic development, at around 24 days post-conception (200), the neural tube is likely formed when a "flat sheet" of cells rolls up in a process called neurulation (174). Failure of this process to be completed properly may result in developmental defects of the brain, spine, and spinal cord (174). Two of the most common NTDs include spina bifida and anencephaly (200). While anencephaly is known to be a lethal defect, spina bifida tends to have a higher survival rate but with increased risk for morbidity and mortality throughout life.

Evidence derived from early observational studies led researchers to suspect an association between maternal folate status and the risk of NTD-affected births (201). By

the early 1990's, periconceptional folic acid supplementation was generally accepted as a means to reduce both the incidence and recurrence of NTDs (202). In 1991, a large multi-centered prevention trial was conducted in the United Kingdom by the Medical Research Council Vitamin Study Research Group in order to determine whether supplementation with folic acid around the time of conception could in fact prevent NTDs (174). Women of childbearing age with histories of NTD-affected pregnancies were randomized to a control or vitamin group, and their pregnancy outcomes were monitored. The results of this double-blind trial showed that an estimated 72% of potential NTDs were likely prevented by folic acid supplementation (174).

In a subsequent trial, Czeizel and Dudas investigated the extent to which folic acid supplementation could reduce the incidence of a first occurrence of NTDs (203). The study subjects, consisting mostly of women who were planning a first pregnancy, were randomized to receive a vitamin (containing 0.8mg of folic acid) or trace-element tablet commencing at least one month before conception. Out of 4,753 confirmed pregnancies, congenital malformations (including six cases of NTDs) were significantly more prevalent in the trace-element group versus the folic acid-containing vitamin supplement group ($p=0.02$) (203). Collectively, the results from these and other studies, have supported periconceptional folic acid supplementation initiatives, as well as the implementation of mandatory folic acid food fortification policies in several countries (including the U.S., Canada, Chile, Costa Rica, and Brazil) (201).

In Canada, the mean blood folate concentrations among women of childbearing age have increased since the onset of mandatory folic acid food fortification in 1998. In a study by Ray, the mean difference in pre- and post-fortification erythrocyte folate concentration among >38,000 Ontarian women (18-42 year old) was reported to be 214

nmol/L. Likewise, the incidence of NTDs was shown to decrease by ~48% among 337,000 Ontarian women between 1994 and 2000 (200). In a 10-year retrospective study (from 1991 to 2000) of Nova Scotian women, Persad et al. reported that the annual incidence of all open NTDs decreased significantly (by ~54%) after the implementation of folic acid fortification (200). Further data on provincial NTD incidence was reported in a public health review by Eichholzer et al., which stated that post-fortification values had fallen by 32% and 78% for Quebec and Newfoundland, respectively (200).

Colon Cancer:

Initial investigations into the possible effect of folate intake on colorectal neoplasia have been fueled by the observation that low intakes of fruits and vegetables tend to be positively associated with the prevalence of colon cancer (204). Some reports have stated that certain fruits and vegetables may be protective against colorectal cancer (CRC) through the provision of dietary fiber (205); however, their folate content may also play a significant role in chemoprevention. The exact mechanism(s) by which folate can influence the development of certain cancers have yet to be defined, however they are most likely related to the role of folate in one-carbon metabolism. DNA synthesis, stability, integrity, and repair are likely central to folate-dependent pathways, and aberrations of these events have been implicated in cancer development (206). Specifically, DNA hypomethylation appears to be an early step in colorectal carcinogenesis (207). Furthermore, compared to healthy non-neoplastic cells, cancer cells may have increased folate requirements since they proliferate rapidly, have accelerated DNA synthesis, and tend to display increased rates of folate catabolism.

The evidence to date on the relationship between folate and CRC has been demonstrated through data from prospective epidemiological observations, human intervention trials, and animal studies. Collective evidence from epidemiological studies (such as the Nurses Health Study) suggests an inverse relationship between dietary folate intake, or blood folate levels, and the risk of CRC or colorectal adenomas (208,209). In a recent meta-analysis of 7 cohort and 9 case-control studies, an inverse relationship between folate consumption and CRC risk was also revealed, which was stronger for dietary folate (folate from foods alone) compared to total folate (folate from foods and supplements) intakes (210). Among the cohort studies, a 25% lower risk of CRC was associated with the highest dietary folate intakes, which ranged from less than 103 to more than 422μg/day. Conversely, a non-significant 5% lower risk of CRC was observed among those with higher total folate intakes (more than 2430μg/day) (210).

Randomized clinical trials of folate supplementation (5mg/day) have also provided some evidence towards a reduced risk of colon cancer. In a study by Cravo et al. (1998), a significant decrease in DNA hypomethylation ($p=0.05$) was found among folate-supplemented subjects with histories of single adenomatous polyps (211). Similar effects of folate supplementation on DNA methylation were found by Kim et al. (2001), who also reported increased mucosal folate concentrations that were reflective of folate levels in the circulation (17). Further evidence in humans has shown that individuals with diseases of the gastrointestinal tract, such as inflammatory bowel disease (Crohn's disease or ulcerative colitis), tend to have a 10-40 fold increased risk of developing CRC compared to the general population (212). Inflammatory bowel disease-related folate deficiency, as exhibited by low blood folate values or elevated Hcy concentrations, has been frequently reported. Suboptimal folate status in inflammatory bowel disease

patients may be linked to inadequate dietary intake due to malaise, anorexia, elevated folate requirements due to bowel inflammation, and possible inhibition of folate absorption as a result of drug therapy (213). There is also evidence to suggest that folic acid supplementation in patients with IBD can reverse folate deficiency and reduce the accompanying risk of CRC (214). Cravo et al. (1995) reported direct and significant correlations between folate concentrations in the blood and the colonic mucosa ($r=0.60$-0.62, $p<0.001$) after 6 months of supplementation (5mg/day) among patients with inactive inflammatory bowel disease (215).

Most animal studies in this area have used chemical carcinogen-induced and genetically engineered rodent models of CRC. Results from these investigations have suggested that folate deficiency may predispose normal epithelial tissues to neoplastic transformation, and folic acid supplementation can suppress the development of tumors in normal healthy tissue (16). Contrary to these findings, however, animal models have also demonstrated that correction of a folate deficiency or folic acid supplementation can promote the development of tumors in tissue with established neoplastic foci (16).

Despite these findings, an inverse relationship between folate status and risk of CRC has not always been consistent or reproducible. One theory behind this discrepancy may be the potential role of elevated usual intakes of folic acid due to the implementation of a fortification program in North America. In a recent epidemiological review, the group of Mason et al. (2007) revealed a rising trend in CRC incidence in Canada and the United States that seemed to correlate with the advent of mandatory folic acid food fortification (216). These findings have been supported by the results from a randomized clinical study, recently conducted by Cole et al. (2007), in subjects with previous histories of large intestinal polyps. The authors found that daily doses of 1mg folic acid

for 5 years was associated with an increased risk of ≥1 colorectal adenoma (RR, 1.13) and ≥1 advanced lesion (RR, 1.67), as well as non-colorectal cancers (217). Although the collective findings from these studies do not imply causality, they do remind us that the relationship between folate status and colon health is not linear, especially in the case of chemoprevention. Furthermore, it is possible that microbial synthesis of folate in the large intestine may be a significant uncontrolled confounding factor that has contributed to the variability in the data collected (218).

2.3 INTESTINAL MICROFLORA AND FOLATE NUTRITION

2.3.1. Bacteria of the large intestine

Current knowledge of the microbiotic species that reside in the human gut is far from complete (219), however the colon is known to harbour a complex group of microorganisms that consists primarily of bacteria, as well as fungi and protozoa (5,6,220). Total populations in the large bowel range from 10^{11} to 10^{12} CFU/ml of contents (compared to $10^{3.5}$-$10^{6.3}$ CFU/ml in the distal small intestine) (221), which is ~10 times greater than the total number of somatic and germ cells in the human body (222). There also appears to be a characteristic spatial distribution of these organisms within the large gut. At least 4 colonic microhabitats have been described: the intestinal lumen, the unstirred mucus layer, the deep mucus layer, and the surface of mucosal epithelial cells (6).

Although the total number of microbes in the gastrointestinal tract seems to be similar in different human populations (220), the composition and activity of the indigenous microflora may be affected by the physiology of the gut, as well as changes in substrate availability, and luminal pH (28). In the proximal colon, microorganisms flourish as intestinal motility is low, and there is generally a plentiful supply of dietary nutrients, and a mildly acidic intra-luminal pH. All of these factors favour proliferation, and thus the highest microbial activity has been observed in this region of the large intestine. In the distal colon, bacteria grow more slowly due to lower substrate availability, and a luminal pH that often approaches neutrality (28). It has been estimated that >500 species of microorganisms coexist in the human colon (28,220,223), most of which are anaerobic. The strict anaerobes include the genera Bacteroides spp., Clostridium, Bifidobacterium spp., Atopobium spp., and the peptococcci. Facultative

anaerobes occur in much lower numbers (~1000-fold) and include lactobacilli, enterococci, streptococci and Enterobacteriaceae. Although the highest microbial activity has been observed in the proximal colon, molecular procedures have shown that aerobes, including E.coli, enterococci, and lactobacilli, also have metabolic activity in the rectum (28,220,221,223).

Although there is potentially a large degree of variability in the proportion of microfloral species between human adults (220), molecular studies have shown that the composition of the colonic microbiota within individuals is usually fairly stable over long periods (28,222). Any observed variations may be due to differences in environmental factors (such as diet), or other host-related factors, including genetics (222) and intestinal physiology (e.g., gastric acid and bile secretion, peristalsis and transit time) (5,220). This implies that mechanisms may exist to suppress the growth of subpopulations or promote the abundance of desirable bacteria (222). With present sampling technologies, fecal analysis is the only non-invasive method for determining the composition of intestinal microflora (220). Due to this technological limitation, differences in the human microflora at various anatomical sites have not been well documented. It is assumed, however, that the proportions and activities of the microflora change with passage through the intestinal tract. Several investigators have also suggested that individuals harbour their own distinctive pattern of intestinal microflora, that usually remains constant throughout adult life (223). Recently, a group of seven European labs joined efforts to develop molecular methods for elucidating the composition and activity of the human intestinal microflora across the lifespan (219). Upon comparing microbial populations of different age groups, it was found that the flora of elderly people was more

diversified than that of younger adults. In contrast, the microbial diversity of babies was found to be extremely low (219).

In general, intestinal bacteria may be divided into species that exert either harmful or beneficial effects on the host (28). Some species of microorganisms can enhance a host's resistance to infection by producing a range of antimicrobial agents that are active against other harmful bacteria. Observations have also suggested that microbes have evolved synergistic mechanisms to influence the colonic environment for their own benefit, and potentially that of the host (221). Bifidobacterium is a major group of saccharolytic bacteria in the human colon, and can constitute up to 95% of the total intestinal population in newborns and approximately 25% in adults. One of the potential positive effects of bifidobacteria on human health may be the production of folate (28), since mammalians lack the appropriate enzymes to produce this vitamin de novo.

2.3.2 Intestinal biosynthesis of folate

As mentioned above, humans are not capable of synthesizing folate and, thus, rely on other exogenous sources (dietary and perhaps bacterially synthesized) to maintain body stores of this vitamin. Through several decades of research, certain species of intestinal bacteria have been shown to be significant producers of folate (13). The characterization of folate-synthesizing bacteria has been accomplished through investigations of starter cultures that are used in dairy fermentation (224). In 2003, Crittenden et al. examined the level of folate produced in fermented milk products, and reported that Bifidobacterium isolates could synthesize folate, while lactobacilli actually depleted the folate levels of the medium. Mixed cultures of two folate-producing organisms (*Bifidobacterium animalis* and *Streptococcus thermophilus*) were found to

increase the level of folate in skim milk by more than six fold. On the other hand, inoculation with both a folate producing strain (*Bifidobacterium animalis*) and a folate utilizing strain (*Lactobacillus acidophilus*) resulted in little net change in the total folate concentration of the milk medium (225).

As demonstrated in-vitro by Sybesma et al. (2003), folate synthesized by L. lactis is enhanced (two-fold) when greater amounts of PABA are available, up to an apparent saturation concentration of 100μM. Some amount of folate synthesis was also observed in the absence of PABA, indicating that L.lactis likely has the ability to synthesize PABA (226). This study group also showed that certain strains of lactic acid bacteria are high folate producers (S.thermophilus), while others are primarily folate consumers (Lactobacillus). The excretion of synthesized folate from bacterial cells tended to vary from strain to strain, and appeared to favour low pH levels in the surrounding culture media. Folate distribution was also found to vary according to polyglutamyl tail length, since extracellular folates tended to have fewer glutamate residues than those identified within bacterial cells (226).

Early human studies have shown that the amount of folate excreted daily in the feces of adults (300-500 ug/d) can outnumber dietary intake (<100ug/d) by 3- to 5-fold, providing indirect evidence of in vivo folate biosynthesis by the intestinal microflora (13,227). The mechanisms of bacterial folate synthesis have been well-defined; however the role that this B-vitamin source plays in colonic health, and the overall folate homeostasis of the host, is still under investigation. One reason for this knowledge gap may pertain to the methodological difficulties of distinguishing between folate that has been synthesized by colonic bacteria from that which has escaped small intestinal absorption. In 1996, the study group of Camilo et al. partly addressed this gap by

following the fate of radiolabeled folates that were synthesized by bacteria in the small intestine of human subjects with modest bacterial overgrowth (227). The study protocol involved the introduction of tritiated PABA into the duodenum of the subjects to allow the investigators to distinguish between serum folates that were synthesized by bacteria versus those of other endogenous origins. The intestinal eluates of all subjects contained detectable quantities of tritiated folate products of bacterial synthesis, which were assimilated by the host. The authors estimated that the observed rate of bacterial folate synthesis in the proximal bowel was sufficient to influence the folate status of those with small intestine bacterial overgrowth. It could not be concluded, however, whether folate synthesized by bacteria in the large intestine is also available to the host (227).

Recent research efforts have been more focused on the amount and fate of folates that are synthesized by microorganisms in the large bowel. The study group of Lin and Young (2000) examined 8 strains of lactic acid bacteria, and found that certain species of Bifidobacterium had the highest level of folate accumulation at 100ng/mL. Interestingly, in 6 out of the 8 strains examined, the form of folate that was found to accumulate the most was 5MTHF (228). Studies of the fecal folate content of human adults and infants have revealed similar results. In 2004, Kim et al. studied the folate content of feces from formula- and breast-fed human infants (n=22), and found that a large proportion of the total fecal folate (approximately 2/3) was predominantly in the form of 5MTHF. Additional analyses revealed that 52.5±30.1% of the total 5MTHF was monoglutamylated, a form that can be readily absorbed across the small intestine. Furthermore, it was estimated that the depot of folate existing in the large intestine of nursing human infants may represent, on average, ~63% of the adequate intake level for infants under 5 months of age (65ug/day) (13). It was, thus, hypothesized that the folate

produced by bacteria in the large intestine could be absorbed and play a significant role in maintaining overall folate status.

This hypothesis was tested in a subsequent study by Asrar et al. (2005), where a tracer dose of tritium (^{3}H)-labeled PABA was injected into the cecum of piglets (n=6). It was found that 12.6% of the injected ^{3}H -PABA was converted, by the intestinal microflora, into ^{3}H -folate, which was absorbed across the large intestine and incorporated into the liver (0.4%) or excreted into the urine (15%) of the piglets (31). From these results, it was predicted that approximately 18% of the dietary folate requirement for the piglet could be met through its absorption across the large intestine (31). The findings from this study were also comparable to those of a rat study with a similar protocol, where 6.9% of the administered ^{3}H-PABA was recovered as bacterially synthesized ^{3}H-folate (32).

In humans, indirect data do exist to suggest that bacterially synthesized folate in the colon may impact folate status (31). Results from an observational study in female adolescents led Houghton and colleagues (1997) to propose that increases in serum folate could be secondary to an increase in dietary fiber intake (and hence the quantity of fermentable substrate reaching the colon) through enhanced intestinal microbial growth (33). A study by Wolever et al., in human diabetics, also attributed an increase in serum folate content to an enhanced colonic bacterial population through the use of miglitol, a drug that inhibits carbohydrate digestion (229). This, and additional evidence from animal studies (24,230), has lead researchers to hypothesize that dietary manipulation of the profile of microorganisms in the large intestine can alter folate production and, in turn, influence overall host vitamin status (31). To date, there a lack of direct evidence to

support this hypothesis, since a feasible method for measuring the absorption of bacterially-synthesized folate across the human large intestine has not bee developed.

3. ABSORPTION OF BACTERIALLY-SYNTHESIZED FOLATE ACROSS THE LARGE INTESTINE PRE-TRIAL I AND II: IN-VIVO BEHAVIOUR OF PLACEBO CAPLETS WITH PH-SENSITIVE COATINGS DESIGNED FOR COLON TARGETING

3.1 DATA CHAPTER INTRODUCTION

3.1.1 Rationale

Research in the area of colon targeted delivery systems has been driven by the need to better treat local disorders of the large intestine (such as inflammatory bowel disease and irritable bowel syndrome) using pharmacotherapy (119,231). The colon has also been receiving attention as an alternative site for drug and micronutrient absorption; however, there has been little application of pharmaceutical technology to this area of research (11,12,232). Nutrient absorption across the human large intestine has previously been investigated using the rectal route, via suppositories and enemas. While these methods are reliable in terms of by-passing the small intestine, they are invasive, and their targeting specificity is limited to the distal colon (233,234).

Determining the absorptive capacity of the intact large intestine is important since certain essential vitamins, such as folate, are increasingly recognized as having significant roles in human health (especially in terms of fetal development and colon cancer). Large amounts of bacterially-synthesized folate have been found to exist in the colon; however, the percent bioavailability of this natural vitamin source is not known. Colon targeted pharmaceuticals may provide a feasible and non-invasive means for quantitatively delivering folate to the undisturbed human colon and measuring its uptake. One type of drug delivery technology that is likely suitable for this purpose is the pH-dependent system, which is currently approved for use in Canada. Several of these preparations are used to deliver anti-inflammatory drugs to the distal intestine in patients

with inflammatory bowel disease (39,55,142-145). Its mechanism is based on the assumption that intra-luminal pH levels gradually rise on passing from the acidic environment of the stomach to the relatively neutral terminal ileum (4,39).

The formulation of a pH-dependent delivery system often consists of a coating material that is chemically engineered to be acid-resistant but soluble at neutral pH ranges. One such coating material, called Eudragit (Degussa GmbH, Dusseldorf, Germany), is available for commercial use and has been applied to a variety of controlled-release pharmaceuticals. The Eudragit product line consists of polymer sub-types that are made up of different methacrylic acid and methyl methacrylate ratios to impart dissolution pH thresholds of 5.5 (Eudragit L30D-55), 6.0 (Eudragit L100), or 7.0 (Eudragit S100 and FS 30D). Although the site-specificity of these coating products can be quite variable, they are suitable for the treatment of inflammatory bowel disease since complete drug delivery to the large intestine is generally not essential. In contrast, the absorption of folate across the large intestine must be investigated using delivery systems that are designed to consistently release their contents past the terminal ileum, since this vitamin is known to be readily taken up by the small intestine.

Recent in-vitro studies have shown that combining the pH sensitivities of different Eudragit polymers, such as S100 and L100, may improve the site-specificity of colon-targeted delivery systems (44,235,236). This polymer mixing allows for the creation of coating formulations that will begin dissolving at pH levels that approximate the distal small intestine, while delaying the onset of drug release until the proximal colon by adjusting the coating thickness. Presently, there are no such delivery systems on the Canadian market that are appropriately formulated for investigating vitamin absorption across the large intestine. Therefore, the purpose of this study was to design a

suitable pH-dependent dose release system for future experiments involving the delivery of the B-vitamin, folate, specifically to the human colon. The objectives were to evaluate the disintegration profiles of various enteric coating formulations both in-vitro, and in healthy human subjects. The coating formulations that were investigated in-vitro, consisted of the copolymers Eudragit L100 and S100, which were combined in three different ratios (1:0, 1:1, and 3:1) and applied to placebo caplet cores at a range of thickness levels (9% - 14% w/w). Subsequent human studies were performed to compare the in-vivo dissolution characteristics of the (Eudragit L100:S100) 1:0 formulation (Trial 1), and 3:1 formulation (Trial 2) in the same subjects with a minimum 2-week washout period. The disintegration and transit time results from these in-vitro and in-vivo experiments are reported in the following sections.

3.1.2 Hypothesis

A pH-dependent delivery system coating can be formulated to quantitatively deliver a barium sulfate caplet to the large intestine of healthy adults.

3.1.3 Objectives

Caplet formulation

To test the in-vitro and in-vivo disintegration characteristics of two pH-dependent delivery system formulations.

Caplet transit

To assess the intra- and inter-individual variability of caplet transit times among healthy adults in the fed state.

3.2 METHODS AND MATERIALS

3.2.1 Placebo caplet formulations

Placebo barium sulphate caplets used in the present study were produced in the Good Manufacturing Practices compliant production facility of the Toronto Institute of Pharmaceutical Technology. The raw materials used, and the finished placebo caplets produced, were tested and released as per United States Pharmacopoeia requirements. The test caplet cores were made predominantly of a radio-opaque substance, barium sulfate, to allow them to be monitored in-vivo via fluoroscopy. The cores were coated with a pH-dependent polymer, designed to promote dissolution in the colon. Except where indicated, all formulation ingredients were purchased from Sigma (Oakville, ON).

Trial 1

Barium sulfate was blended with a binding agent (polyvinyl pyrillidone K90) and granulated with purified water using a high-shear mixer (Robot Coupe USA Inc., Jackson, MS). The resulting wet granules were dried at 35^0C in a tray dryer (to achieve a moisture level of 1.5-2%), then mixed with a diluting agent (microcrystalline cellulose), as well as a super-disintegrant (sodium starch glycolate; JRS Pharma, Patterson, NY) using a Maxi-Blend blender (GlobePharma Inc., New Brunswick, NJ). The final homogeneous mixture was compressed into tablet forms on a rotary press (Minipress II; GlobePharma Inc., New Brunswick, NJ) using convex caplet tooling (19.1mm x 9.7mm). Final caplet cores contained 73% (w/w) barium sulfate, 8% (w/w) polyvinyl pyrillidone K90, 14% (w/w) microcrystalline cellulose, and 5% (w/w) sodium starch glycolate for a total weight of 2876.7mg.

The caplet coating consisted of Eudragit L100 (Degussa GmbH & Co., Dusseldorf, Germany), a methacrylic acid copolymer with a solubility pH threshold of

6.0. After dispersing the coating copolymer in water, a plasticizer (triethylcitrate; Morflex Inc., Greensboro, NC) was added to increase the flexibility of the film. A glidant (talc) was also added as a suspension in order to prevent the coating from becoming tacky during the drying process. This aqueous mixture was then passed through a screen (297 microns) to eliminate sediment or agglomerates. The final coating solution was sprayed onto the barium sulphate caplet cores (LDCS-5 Hicoater, Vector Corporation, Marion, United States), at an atomizing pressure of 22-24psi, to achieve a target increase in caplet weight of 10 percent. The coated caplets were dried at 40^0C, and then sampled for in-vitro disintegration tests.

Trial 2

A second batch of placebo caplet cores was produced with approximately 10% less barium sulfate and a higher proportion of microcrystalline cellulose compared to the first batch of caplets. The purpose of this formulation change was to promote higher core disintegration rates and core friability by decreasing their density and increasing their solubility (237). In terms of in-vivo performance, this adjusted caplet formulation would, theoretically, ensure complete core disintegration within the large bowel in the event of prolonged coating dissolution times (due to a higher pH threshold). With these changes in place, the final core formulation had a total weight of 2638.9mg, and a component profile of 72% (w/w) barium sulfate, 8% (w/w) polyvinyl pyrillidone K90, 15% (w/w) microcrystalline cellulose, and 5% (w/w) sodium starch glycolate. All other aspects of the manufacturing process were the same as those used for Trial 1.

Two separate coating formulations were applied to the second batch of placebo caplet cores. These formulations were manufactured by combining the copolymers, Eudragit L100 and Eudragit S100, in 1:1 and 3:1 ratios. Since the solubility threshold of

Eudragit S100 (pH 7.0) is higher than that of Eudragit L100 (pH 6.0), the combination of these products produced coatings with intermediate pH thresholds, the exact value of which was governed by the relative proportion of each copolymer type used. The final coating formulations were applied to caplet cores at three different levels (9% and 13.6% of total caplet weight for 1:1 formulations, and 12.6% of total caplet weight for 3:1 formulations) in order to determine the effect of coating thickness on the disintegration rates of the delivery systems. All other aspects of the coating manufacturing process, and in-vitro disintegration tests, were the same as those used for Trial 1.

3.2.2 In-vitro disintegration tests

Trial 1

An initial random sample of 18 coated caplets was selected for vitro disintegration testing using a standardized procedure for delayed release (enteric coated) tablets (USP29/NF24) (238). The purpose of these tests was to confirm that the caplet coating formulation, and processing conditions, were appropriate for promoting caplet disintegration at a suitable rate and pH level for in-vivo colon-targeting. The tests were carried out using a standard six-tube basket assembly with a 10-mesh screen, a 1L disintegration beaker, and a 19x12x8 inch water bath (VK100 35-1200, Varian Inc., Cary, North Carolina). To simulate the impact of gastric and small intestinal conditions on the integrity of the caplet coating and core, 900mL each of 0.1*M* hydrochloric acid (pH 1.2) and 0.06*M* monobasic potassium phosphate (pH6.8) were prepared and equilibrated in a disintegration bath to attain a temperature of 37 ± 2^0C. The experimental conditions of all tests were maintained at a temperature between 35^0 and 39^0 C.

Using the basket-rack assembly, six individual caplets were immersed in 900mL of simulated gastric fluid (pH 1.2) for one hour, then removed and observed for any evidence of cracking, softness, or peeling. If they remained intact, the caplets were then transferred to 900mL of simulated intestinal fluid (pH 6.8) and continuously monitored for signs of disintegration. Caplets were considered to have 'passed' the test if complete disintegration (defined as the state in which a palpable caplet core could no longer be identified) occurred within 2 hours of submersion and agitation in the simulated intestinal fluid. If 1 or 2 caplets did not meet this criterion, an additional 12 caplets were tested for a target pass rate of 89% (16 out of 18 caplets).

Trial 2

The second batch of coated caplets was sampled and tested for disintegration properties using the same method as described above. Eudragit L100:S100 (1:1) coated caplets were tested in vitro according to coating thickness level (9% and 13.6%) at pH 1.2, 6.8, 7.0, and 7.5 (Table 3). Eudragit L100:S100 (3:1) coated caplets (12.6% coating level) were similarly sampled and challenged in-vitro at pH 1.2, 7.0, and 7.5. The results from these in-vitro tests were evaluated according to the same acceptance criterion applied in Trial 1, and further used to determine which coating formulation and thickness level would be most suitable for the in-vivo study.

3.2.3 Subjects and study protocol

Healthy adult males and females were recruited to achieve a target sample size of ten subjects who would each complete both in vivo trials. Exclusion criteria included the presence of intestinal disease (e.g. Crohn's disease or ulcerative colitis) or recent gastrointestinal surgery, as well as any other condition or use of medication that may affect usual digestive motility patterns or intestinal pH. Volunteers were also excluded if

they were habitual smokers, had consumed alcohol within 24 hours of the study day, or if they were pregnant. All subjects gave informed written consent for each trial (Appendix 1), and the study was approved by the Human Ethics Committee of The Hospital for Sick Children, Toronto.

After an overnight fast, each subject reported to the Clinical Investigation Unit in The Hospital for Sick Children at 7:45am on the morning of their clinic visit. Upon arrival, a screening interview and pregnancy test (Clearview hcG II, Wampole Laboratories, Princeton NJ) were performed, and current height and weight were determined using standard procedures (239) with a calibrated beam scale ($\pm$100g, Detecto, Webb City MO) and digital stadiometer ($\pm$0.01cm, Hightronic model #235, Quick Medical, Snoqualmie WA) (Appendix 2). The purpose of the screening interview was to ensure that each subject was eligible to participate. Questions pertaining to usual dietary practices and bowel habits were included, and females were asked to recall the date of their most recent menstrual period. A registered nurse, otherwise not involved in the study, was present during the consent and interview processes to ensure that inclusion criteria were met and anthropometric measures were taken properly.

The caplet was ingested between 0600 and 0800 hours with as much water as necessary, and immediately followed by a standardized breakfast (see section 3.2.4 'Dietary Intakes'). At approximately 2 hours post-dose, subjects reported to the Image Guided Therapy (IGT) department for a fluoroscopic image of the abdominal area. Subsequent fluoroscopic images were taken at approximately 60 minute intervals until complete caplet disintegration was observed or 1800 hours. Subjects were free to leave the Image Guided Therapy department between imaging sessions, and were asked to abstain from vigorous exercise.

3.2.4 Dietary intakes

A standardized breakfast, light lunch, and optional snacks, were provided on each study day. The standard breakfast was consumed immediately following caplet administration, and consisted of 500ml puffed rice cereal and 250ml non-dairy rice beverage (approximately 960kJ). The meal was designed to be low in energy and residue, with a nutrient distribution of approximately 85% carbohydrates, 5% protein, and 10% fat. Lunch consisted of vegetable soup, plain potato chips, a medium-sized apple, and a choice of beverage from cranberry or apple juice, to any type of decaffeinated soft drink, or water (approximately 2550kJ). Snack foods were optional, and ranged in energy content from 200 to 750kJ. Subjects were not permitted to consume any other foods after the standard breakfast while the caplet remained in the stomach. Once gastric emptying had occurred, all other meals and snacks were provided at the discretion of the volunteer, and water was available ad libitum throughout the study day.

3.2.5 In-vivo caplet transit and dissolution characteristics

The intestinal transit of each caplet was monitored using a fluoroscope (Infinix, Toshiba America Medical Systems Inc., Tustin CA) in the Image Guided Therapy department of The Hospital for Sick Children. Fluoroscopy was performed by a qualified medical radiation technologist, and each image required an average of 2 to 3 seconds of fluoroscopic exposure. The radiation entrance dose was approximately 20mRem per image, or a total of 120-160mRem for each study day. This level of exposure is roughly equivalent to a person's yearly dose of natural background radiation from both external (cosmic and terrestrial rays) and internal (air, food, and water) sources (240). The time

that each image was taken was recorded in order to determine the gastrointestinal transit time of the test caplets. The corresponding anatomical location of the caplets was also assessed for each image by a radiologist. Gastric emptying time was calculated as the mean time of images that were taken immediately before and after the caplet appeared to pass through the pyloric sphincter. Colon arrival time was calculated in a similar manner as the caplet appeared to move distally past the ileo-cecal junction. Small intestinal transit time was determined by subtracting gastric emptying time from colon arrival time.

The dissolution characteristics of each caplet were determined by assessing the time and anatomical location of initial and complete disintegration. Dissolution initiation time was defined as the mean of the two post-dose image time points that occurred before and after a clear disruption of the caplet coating could be observed. Complete disintegration time was recorded as the last image taken at the conclusion of the study day. The regions of the gastrointestinal tract where initial and complete disintegration took place were also recorded and compared across trials, as well as the total caplet disintegration time (complete disintegration time – dissolution initiation time). Finally, the total time from caplet administration to complete disintegration was calculated and denoted as total transit time.

3.2.6 Data analyses

Fluoroscopic images were reviewed by the study coordinator and radiologist to determine the anatomical location and dissolution state of the test caplets. Gastrointestinal transit and caplet dissolution data were compared across trials with paired t-tests using statistical software (SAS, version 9.1). Study day dietary intake records were analyzed for caloric value using the Canadian Nutrient File (241) or

manufacturer food packaging labels. The caloric and nutrient intakes of each subject were compared to his/her corresponding caplet transit profile to determine if the consumption of food was correlated to in-vivo caplet performance. The variability in caplet transit times between and within subjects was assessed using an intraclass correlation coefficient (242).

3.3 RESULTS

3.3.1 Subject characteristics

A total of 13 subjects (6 males and 7 females) were recruited for the study, ten of which completed both Trial 1 and Trial 2. One volunteer was excluded from the first trial prior to the consent process due to smoking. Two participants were excluded from the second trial. One of these subjects was diagnosed with cholelithiasis, and the other was prescribed medication that could affect gastrointestinal motility (e.g. codeine). The remaining 10 subjects did not differ significantly across trials in terms of weight, height, and body mass index (Table 1). The macronutrient and energy intakes of all participants throughout each study period also did not differ significantly across trials ($p>0.20$) (Table 2).

TABLE 1 Subject characteristics[1]

Characteristic	Trial 1	Trial 2	p-values
Gender (m/f)	4/6	4/6	N/A
Weight (kg)	63.3±11.1 (47.6-79.0)	64.2±10.7 (47.0-77.5)	NS
Height (m)	1.68±0.10 (1.52-1.85)	1.69±0.09 (1.59-1.84)	NS
Body Mass Index (kg/m^2)	22.6±4.8 (18.3-34.4)	22.5±3.7 (18.0-30.6)	NS
Age (y)	27±11 (20-49)	27±11 (20-49)	NS
Ethnicity (n)			N/A
Caucasian	5/11		
Asian	4/11		
Black	1/11		

[1]Values are expressed as means ± SD (min – max) unless otherwise noted. Significance of mean differences between trials determined with paired t-tests.

TABLE 2 Summary of study period energy intakes and food composition[1]

Food Component	Trial 1	Trial 2	p-values[2]
Total Energy (kJ)	4130±1472	4455±1661	NS
Breakfast Energy (kJ)	812±184	906±119	NS
Carbohydrates (g)	179.1±54.1	196.4±73.1	NS
Dietary Fiber (g)	12.2±3.3	12.5±3.5	NS
Protein (g)	13.5±2.8	16.5±10.1	NS
Fat (g)	24.7±15.9	25.2±13.2	NS

[1]Values are expressed as means ± SD unless otherwise noted.
[2]Significance of mean differences between trials determined with paired t-tests.

3.3.2 In-vitro caplet disintegration

All sampled caplets that were coated with Eudragit L100 only (formulation 1:0) did not show evidence of disintegration, softening or cracking when exposed to gastric simulation fluid (pH 1.2) for 1.5 hours (Table 3). The same caplets dissolved completely within two hours after they were transferred to a simulated intestinal fluid of pH 6.8. Similar results were obtained for the (Eudragit L100:S00) 3:1 coating preparation when test caplets were challenged at pH 1.2 for 1.5 hours, then exposed to an intestinal condition of pH 7.5. When tested at pH 7.0, however, the complete core dissolution times of 3:1 caplets were extended by an average of 60 minutes. The in-vitro disintegration test results of (Eudragit L100:S100) 1:1 coated caplets showed a trend towards slight coating disruption at pH 1.2 in approximately 33% of the sample (2 out of 6 caplets), which was consistent for both the low and high coating levels (9% and 13.6%, respectively). At pH 6.8 and 7.0, complete core disintegration was not complete by 240 minutes; especially among those caplets with greater coating thickness. Upon exposure

to an intestinal condition of pH 7.5, however, a sample of 1:1 test caplets with a 13.6% coating level dissolved completely within 120 minutes.

3.3.3 In-vivo caplet performance

The total average time from caplet administration to complete dissolution (total transit time) was significantly longer for caplets with mixed 3:1 coating formulation ratios (600±78min), compared to the caplets with 1:0 coating formulations (520±90min) ($p<0.01$) (Table 4). There was also a trend towards longer average times from administration to the first signs of coating disruption (dissolution initiation times) among 3:1 caplets ($p=0.09$), although the dissolution rates of their cores did not appear to differ significantly from those of the 1:0 caplets ($p=0.74$). In addition to these results, a smaller proportion of caplets in the second trial were observed to begin dissolving in the mid to distal ileum (1/10 versus 5/10 for 3:1 and 1:0 formulations, respectively) (Table 5). All other caplet transit outcomes, including stomach emptying, small intestinal transit, and colon arrival times, did not differ significantly between formulations.

TABLE 3 In-vitro dissolution profiles of caplets coated with different Eudragit L100:S100 formulation ratios[1]

	Eudragit L100:S100 (% coating level)			
pH Challenge[2]	1:0 (10%)	1:1 (9%)	1:1 (13.6%)	3:1 (12.6%)
pH 1.2	120 min: Coating remains intact	120 min: Coating remains intact in 4 of 6 (67%) caplets	120 min: Coating remains intact in 4 of 6 (67%) caplets	120 min: Coating remains intact
pH 6.8	45 min: Coating begins to dissolve 60 min: Disruption of caplet edges 90 min: 50% disintegration of core 120 min: 100% disintegration of core	135 min: Coating splits at caplet tips 240 min: 50-70% disintegration of core	135 min: Coating dissolves 240 min: 10% disintegration of core	NT[3]
pH 7.0	NT	135 min: Coating splits at caplet tips 240 min: 50-70% disintegration of core	135 min: Coating begins to dissolve and disruption of caplet edges 240 min: 50% disintegration of core	30 min: Coating begins to dissolve 75 min: Disruption of caplet edges 160 min: 50% disintegration of core 180 min: 100% disintegration of core

pH 7.5	NT	NT	15 min: Coating begins to dissolve	20 min: Coating begins to dissolve
			60 min: Disruption of caplet edges	30 min: Disruption of caplet edges
			75 min: 50% disintegration of core	75 min: 50% disintegration of core
			120 min: 100% disintegration of core	105 min: 100% disintegration of core

[1]Samples of 6 test caplets from each formulation batch were exposed to gastric simulation fluid (pH 1.2) for ≥60 min plus one other simulated intestinal condition (i.e. pH 6.8, 7.0, or 7.5) until complete disintegration was observed.
[2]Buffers were designed to simulate the luminal pH of the stomach (pH 1.2), the distal small bowel and the proximal colon (pH 6.8, 7.0, 7.5).
[3]NT indicates Not Tested

TABLE 4 Comparison of in-vivo transit times, dissolution times, and intra-subject variability of caplets coated with 1:0 or 3:1 (Eudragit L100:S100) formulation ratios[1]

	Eudragit L100:S100			
Timing (minutes)	1:0	3:1	p-values[2]	ICC[3]
Gastric Emptying	175±74	181±58	0.837	0.12
Small Intestinal Transit	183±73	241±103	0.333	-0.30
Colon Arrival	358±76	422±110	0.212	0.07
Total Caplet Transit[4]	520±90	600±78	0.003	0.71
Caplet Dissolution Initiation[5]	320±78	429±132	0.090	-0.08
Total Caplet Dissolution[6]	181±56	171±96	0.741	-0.54

[1]Values are expressed as means ± SD for 10 subjects unless otherwise noted.
[2]Paired t-test used to determine statistical differences between trials.
[3]The Intraclass correlation coefficient (ICC) approaches 1.0 when any given subject tends to have the same transit time values across trials (i.e. less variation within subjects). A negative ICC occurs when the within-subject variance exceeds the between-subject variance.
[4]Total time from caplet administration to complete caplet dissolution.
[5]Total time from caplet administration to the first signs of coating disruption.
[6]Total time from the first signs of caplet coating disruption to complete dissolution.

TABLE 5 Gastrointestinal location of dissolution initiation for 1:0 and 3:1 (Eudragit L100:S100) coated caplets[1]

	Eudragit L100:S100	
Intestinal Region	1:0	3:1
Ileum	5	1
Ileo-cecal Junction	1	2
Cecum	2	4
Ascending Colon	1	1
Transverse Colon	1	2
Total	10	10

[1]Intestinal region where the first signs of caplet dissolution were observed. Values are expressed as number of subjects (n).

In contrast to an apparent lack of difference in mean oro-cecal transit times between caplet formulations, the variability in these outcome measures tended to be high within subjects (Table 4). Within-individual variances were determined through the calculation of intraclass correlation coefficients using mean squares from two-way analyses of variance. The intraclass correlation coefficients associated with gastric emptying time (0.12), small intestinal transit time (-0.30), and colon arrival time (0.07) demonstrate low agreement in these outcomes within subjects across trials, relative to between subjects in each trial. Caplet dissolution times also tended to be highly variable within participants (-0.08 and -0.54 for initial and complete dissolution times, respectively), whereas the between-subject variance in total times from caplet administration to complete dissolution appeared to be large (0.71). A similar analysis of food consumption revealed high intra-subject agreement in total caloric intake values relative to the variability between participants (0.75) (data not shown).

3.4 DISCUSSION

3.4.1 In-vitro caplet disintegration

In the present study, the solubility profiles of three separate pH-dependent caplet formulations were evaluated through a series of in-vitro disintegration tests. The disintegration characteristics of each caplet type differed according to the relative proportions of Eudragit L100 and S100 that were incorporated into the coating formulation (1:0, 1:1, or 3:1 ratios), as well as the level of film thickness applied to the caplet core (9%, 12,6%, or 13.6% w/w).

The in-vitro dissolution characteristics of the caplets coated with Eudragit L100 only (1:0 formulation) at pH 6.8 were comparable to those reported by Khan et al. (2000), who used a similar in-vitro protocol (USP basket method) to test Eudragit L100-coated tablets at pH 6.5 (44). In both cases, the coating began to show signs of disruption after 30 minutes in simulated intestinal fluid, and at least 80% of the solid cores had dissolved within 120 minutes. The group of Kahn et al. also tested a (Eudragit L100:S100) 4:1 formulation at pH 7.0 and reported a dissolution initiation time of 30 minutes, which was also found in the present study when a 3:1 formulation was challenged in similar conditions.

The in vitro disintegration rate of caplets tested at pH 6.8 was delayed by at least 2-fold when Eudragit S100 made up 50% of the coating formulation (1:1 L100 to S100), and slower still when the thickness of the 1:1 coating was increased from 9% to 13.6% (w/w). At pH 7.0, however, the dissolution characteristics of (Eudragit L100:S100) 1:1 coated caplets were similar, regardless of coating thickness. This variability in disintegration profiles was also observed by Cheng et al. (2004), who compared the in-

vitro release characteristics of pellets coated with Eudragit L100 and S100 in 1:0, 1:1, and 4:1 ratios (236). The authors concluded that coating formulations with higher proportions of Eudragit S100, compared to L100, will lead to slower drug release in vivo, especially when intra-luminal pH levels are below 7.0. It was also suggested that changes to the coating formulation ratio may have a greater impact on dissolution rates than altering the coating thickness.

Based on the results from the present in-vitro study and the findings of other investigators, the (Eudragit L100:S100) 1:0 and 3:1 coating formulations were selected to be tested in-vivo, since their disintegration profiles were thought to be appropriate for promoting complete dissolution in the distal bowel of healthy adults. The rationale for this decision involved an examination of the relative caplet disintegration times of each formulation at specific pH levels, as well as the consideration of natural variability in gastrointestinal motility and acidity among individuals. These issues will be discussed further in the following sections.

3.4.2 In-vivo caplet performance

3.4.2.1 Dissolution characteristics

The total mean time from caplet administration to complete dissolution (total transit time) was approximately 80±64 minutes longer among (Eudragit L100:S100) 3:1 coated caplets, compared to those with 1:0 formulations (Eudragit L100 only) ($p<0.01$). Since both test caplet formulations appeared to dissolve in vivo at similar rates ($p=0.74$), the extended total transit times among 3:1 coated caplets may have been explained by their tendency to begin dissolving at a later time after administration (248±124min versus

145±74min for 3:1 and 1:1 formulations, respectively). These results indicate that the solubility threshold of the 3:1 coating formulation (pH 7.0-7.5) may have approximated the intra-luminal pH of more distal intestinal regions compared to the 1:0 formulation (pH 6.0-6.8). Several authors have investigated the pH profile of a healthy gastrointestinal tract using radiotelemetric devices (4,98,101-106). Collectively, the results from these studies have demonstrated a range of pH levels in the small bowel from an average of 6.25 in proximal areas, up to 7.5 in the most distal region. A consistent fall in pH, to values as low as 5.5, from the terminal ileum to the cecum has also been reported in most publications. This abrupt change in intra-luminal pH can be attributed to the presence of SCFAs, a by product of carbohydrate fermentation by colonic bacteria (9). Lastly, the pH level of the descending colon and rectum has been recorded as a range of 6.1-7.5 (39).

As mentioned previously, the dissolution profiles of different pH-dependent coatings have been applied to a variety of delayed release pharmaceutical preparations that are available commercially as treatment regimens for inflammatory bowel disease. One such product type is 5-aminosalicylic acid-containing tablets that are coated with either Eudragit L100 (Salofalk® and Claversal®) or Eudragit S100 (Asacol®) only (146). Since Eudragit L100 has a solubility threshold of pH 6.0, Salofalk® and Claversal® are generally used to treat inflammation in the distal small bowel, and have been reported to dissolve completely in this area at least 70% of the time (243). The higher pH threshold of Eudragit S100 (pH 7.0), on the other hand, has been applied to aminosalicylic acid formulations such as Asacol®, which are used to treat inflammatory disease in proximal regions of the colon (144). While Asacol® has demonstrated up to 80% dose release in

the large bowel of patients with mild to moderately active ulcerative colitis (244), these coated tablets have also been reported to remain intact and appear, un-dissolved, in a patient's stools (245). Thus it is clear, from the above observations, that promoting complete and consistent intra-colonic drug release with a pH-dependent mechanism may not be accomplished through the use of single Eudragit coating products.

In the present study, an improvement in colon targeting was observed when test caplets were coated with a mixture of Eudragit L100 and S100 in a 3:1 ratio. Specifically, 9 out of 10 caplets with this coating combination dissolved distal to the ileum, compared to 5 out of 10 caplets that were coated with Eudragit L100 only. A similar enhancement in site-specificity was found by Watts et al., who reported a $\geq$90% success rate in colon targeting when Eudragit S100 was added to a coating formulation of injection-molded starch capsules that were previously coated with Eudragit L100 only (146,147). These authors suggested that the use of a mixed coating formulation may have led to more reliable intra-colonic release by, not only adjusting the solubility pH threshold, but also through the addition of a time-dependent dissolution mechanism. In other words, if the pH level in the distal ileum of a particular subject was high enough to initiate disintegration, an additional lag time may have been sufficient to delay core release until the capsule had passed the ileo-cecal junction.

It should be noted that the longer mean total caplet transit time that was associated with the 3:1 coating formulation in Trial 2 of the present study was likely underestimated. Complete dissolution was not observed by the end of the study day in five subjects. A follow-up 24-hour fecal collection was successfully obtained from three of these participants, and analyzed with a fluoroscope. The stools of these three subjects

generally appeared opaque, indicating dispersion of barium sulphate, with no remnants of an intact caplet core remaining.

3.4.2.2 Gastrointestinal transit times

The mean gastric emptying times of both caplet formulations in the present study (approximately 180±60 minutes in both cases) were roughly equivalent or up to 2 hours faster compared to the range of emptying times in other investigations of single unit delivery systems with different levels of food intake (156-298min) (48,56,79,246). Since the emptying rates of single-unit systems tend to decrease as diameter and density increase (78,79,86,117), the comparative findings from the present study are somewhat surprising, since the caplets used here were generally larger and heavier than those commonly tested by others. An alternative explanation may relate to the known effect of reduced gastric motility with the consumption of meals with higher energy values (72-74,83). Since the breakfast meal size in the present study (960kJ) was comparatively smaller than those used in other similar investigations (1200-2500kJ), it is possible that this discrepancy played a role in promoting the observed differences in stomach emptying times. Despite these theories, however, the relative contribution of other uncontrolled factors known to affect gastric transit [such as stress (247), body posture (248), or random emptying (73)] should not be discounted.

The mean small intestinal transit times of both caplet formulations (183±73min and 241±103min for the 1:0 and 3:1 formulations, respectively) were generally in agreement with the accepted value of 180±60min (83). Small bowel motility is reported to be very consistent between individuals, and is apparently unaffected by the type of

delivery system administered or dietary condition (83,84). The consistency of small intestinal motility among most individuals suggests that any differences in the total time for each caplet to arrive in the colon were likely driven, for the most part, by the variability in gastric emptying times. Colon arrival times may also be affected by diet, since food consumption has been shown to stimulate colonic activity (the 'gastrocolonic response') (249), and increase the rate of ileal flow into the cecum (250,251). In the present study, the contribution of this effect to overall caplet transit times could not be directly measured, since the amount and timing of food consumption was not strictly controlled.

The variability in caplet transit outcomes of each subject was assessed in relation to the variability between all subjects for both trials using intraclass correlation coefficients. The low apparent agreement in gastric emptying, small intestinal transit, and colon arrival times within subjects was surprising since it contrasts the findings of other authors (79,85,252,253). These discrepant findings may have arisen due to differences in study design with regards to replication, dietary intakes, and imaging methods. While participants in the present study ingested two test caplets on separate occasions, the volunteers in a study by Coup et al. were administered four non-disintegrating tablets simultaneously, thus allowing for a comparatively higher number of replicates and greater test re-test reliability (85). Contrary to the present study, these authors also maintained strict control of dietary intakes both before and during the study period. This likely reduced inter- and intra-subject variability in the potential effect of food consumption on gastrointestinal motility. Furthermore, the in vivo transit characteristics of the aforementioned non-disintegrating tablets were examined using

gamma-scintigraphy with an imaging frequency of 15-20 minutes. Shorter intervals between scintigraphic images could have lead to more precise transit time calculations than those that were determined currently using hourly fluoroscopic sessions. Collectively, these methodological differences may have produced unforeseen sources of variability in the outcomes of the present investigation, and thus contributed to discrepant findings between studies.

3.4.2.3 Associations between caplet performance and subject characteristics

The small intestinal transit time of caplets with (Eudragit L100:S100) 1:0 coatings was strongly associated with total intakes of carbohydrates ($r=0.71$, $p=0.03$), and calories ($r=0.77$, $p=0.01$) throughout the study day (Table 6, Appendix 3). A near-significant relationship between small bowel transit times and total protein intakes was also evident for all test caplets, irrespective of coating type ($r=0.68$ and 0.62 for 1:0 and 3:1 formulations, respectively). Similarly, total protein intakes were positively correlated with the total transit times (from administration to complete caplet dissolution) of caplets with 1:0 coating formulations, although this association was not apparent for 3:1 coated caplets.

The significant correlation between small intestinal transit times of 1:0 coated caplets and total intakes of carbohydrates, protein, and energy is somewhat surprising since it is well established that small bowel motility is generally not influenced by meal size or composition (72,74,86). Because small intestinal transit time in the present study was defined by the arrival of the caplet in the colon, and not the terminal ileum, it is possible that food-dependent variations in the rate of caplet movement across the ileo-

cecal junction were responsible for this relationship. In a study by Khosla et al. (1989), the gastrointestinal transit of non-disintegrating tablets was monitored in healthy volunteers after they had ingested either a light or heavy breakfast meal (74). Under conditions of greater food consumption, the tablets appeared to spread in the small intestine, then re-group at the ileo-cecal junction before entering the colon. In a similar study by Wilson et al., a variable delay in the transit of single unit tablets was observed as they passed from the terminal ileum to the cecum (72).

A "stagnation" effect at the ileo-cecal junction has also been attributed to the consumption of dietary fiber. Spiller et al. (1987) investigated the postprandial ileo-colonic transit of low- and high-residue meals and found that cecal filling rates decreased when 4g of dietary fiber (in the form of guar) was added to the initial test meal (250). Since the amount and type of food consumed in the present study was not strictly controlled, it is conceivable that the colon arrival times of ingested test caplets were affected by differences in macronutrient intakes among subjects. This concept may be supported by high inter-individual variability in total caloric intakes, which was implied by a large and positive intraclass correlation coefficient (0.75) for this outcome measure.

3.4.3 Strengths and Limitations

Strengths

The present study is the first exploratory experiment to test the colon-targeting potential of a barium sulphate caplet coated with a pH-dependent formulation mixture of 25% Eudragit L100 and 75% Eudragit S100 in healthy adults. This delivery system will be used in future novel investigations of vitamin absorption to quantitatively deliver

folate to the colon. The same ten subjects participated in both Trial 1 and Trial 2, which allowed a more reliable comparative analysis to be made between caplet formulations. The ethnic diversity of the subjects that were recruited may have prevented biases in gastrointestinal characteristics due to food preferences (254). In order to maintain a high level of accuracy and low inter-tester variability, anthropometric measurements were always obtained by one of two nurse practitioners in the clinical investigation unit at The Hospital for Sick Children. All fluoroscopic images were also analyzed by the same radiologist in the Image Guided Therapy department of the hospital.

Limitations

Due to the exploratory nature of this study, the sample size was small, which may have reduced our ability to detect statistically significant differences in caplet performance across trials, as well as altered the strength or direction of some correlation statistics. In the case of dissolution initiation times, for example, a post-hoc analysis revealed that a sample size of 21 subjects may have been required in each trial to observe significant differences ($p<0.05$) between the caplet formulations. The fluoroscopic analysis of in-vivo caplet performance may have been subject to errors of precision and accuracy, since only bony landmarks and intra-luminal air accumulations can be used to determine a caplet's location in the gastrointestinal tract. While the fluoroscopic imaging schedule was adjusted to ensure minimal radiation exposure, the interval length between images (60 minutes) may have been limiting in terms of determining the actual post-dose time of certain caplet transit events (such as gastric emptying or colon arrival time). Furthermore, the direct comparison of imaging results from this study to those reported

by other groups is difficult, since scintigraphic methods have been employed in most of these cases. The use of gamma-scintigraphy may not be appropriate for investigating nutrient absorption across the large intestine since the short half life of some radio-isotope labels are not sufficient to allow the total transit of colon-targeted delivery systems to be observed. Furthermore, the production of radiopharmaceuticals is not highly feasible since they must be manufactured with small batch sizes in a facility that has the capacity to handle radioactive material. Lastly, the complete dissolution of two caplets in Trial 2 was not observed due to significantly extended total transit times. This, in turn, resulted in less complete caplet performance data, and the underestimation of some transit and dissolution times.

3.4.4 Conclusions

The current study has provided evidence to show that orally administered barium sulphate caplets (2600mg) that have been coated (at a 12.6% w/w level) with a mixed pH-dependent formulation of the copolymers Eudragit L100 and S100 in a 3:1 ratio will have a 90% colon-specific targeting success rate in healthy human adults. In other words, 9 out of 10 ingested caplets will not begin to dissolve until they have by-passed the stomach and small intestine.

3.4.5 Future directions and clinical implications

The aim of the present study was to design a pH-dependent dose release system that would be suitable for the delivery of vitamins specifically to the human large intestine. A barium sulphate caplet with a 3:1 copolymer combination coating, such as

the one tested here, may be an appropriate vehicle to achieve this aim. More specifically, this delivery system formulation could be employed to determine if the B-vitamin, folate, is absorbed across the human colon and incorporated into the systemic circulation.

The protocol used in the present investigation may be applied to a future absorption study, although certain adjustments will be required. Firstly, in order to measure folate uptake, blood sampling procedures must be included and scheduled in conjunction with caplet dissolution times. According to the observed in-vivo disintegration profiles of placebo caplets, it is likely that the arrival and dissolution of a delivery system in the colon will not occur, on average, until 10 hours post-dose. This implies that earlier caplet administration, and/or an extended imaging schedule, should be incorporated to allow for sufficient time to collect blood samples. Secondly, the colon-targeting precision of the delivery system must be considered, since folate release in the small intestine (a known site of B-vitamin uptake) will lead to inaccurate absorption measures. In the present study, one out of ten (Eudragit L100:S100) 3:1 coated caplets did not successfully reach the ileo-cecal junction before dissolution had begun. One way to account for this variability in site-specificity may be to over-recruit by 10% (the approximate failure rate of placebo caplets) to ensure that complete data is obtained from an appropriate number of subjects. Furthermore, fluoroscopic images can be more specifically utilized to determine when delivery systems have entered the colon, and where (anatomically) dissolution occurs, thus requiring fewer images and lower radiation exposure.

The total energy and macronutrient intakes of subjects in the present study did not appear to have significant effects on the in-vivo dissolution performance of the (Eudragit

L100:S100) 3:1 coated caplets. This finding indicates that, once a delivery system has emptied from the stomach, the amount and type of food consumed throughout the remainder of the study day may not need to be strictly controlled. Conversely, the ingestion of a breakfast meal has been associated with longer gastric emptying times of large single-unit delivery systems (such as the one tested here) when compared to fasted administration. Therefore, omitting the breakfast meal in future work may be considered if this change will translate into a more feasible study day schedule.

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5. APPENDICES

APPENDIX 1

SickKids

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

TITLE OF RESEARCH PROJECT
Folate Absorption Across the Large Intestine: Study #2a **Part II** - Dissolution Characteristics of a pH-dependent Colon-targeted Delivery System

Investigators

	Contact Number
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Ashley Aimone MSc student supervised by Dr O'Connor	pager:

You have been invited to participate in a research study. Before agreeing to participate, it is important that you read and understand this research consent form. This form provides all the information we think you will need to know in order to decide whether you wish to participate in the study. If you have any questions after you read through this form, ask your questions to a doctor or study personnel. You should not sign this form until you are sure you understand everything on this form. You may also wish to discuss your participation in this study with your family doctor with respect to your health history and any medications you may be taking, in order to prevent any unnecessary harms to you should you decide to participate in this study.

PURPOSE OF THE RESEARCH

The purpose of our study is to test the performance of a drug-less pill in the human digestive tract. This pill is in the form of a caplet that has a special coating, which is designed to dissolve in the large intestine. We will give you one dose to swallow and find out how long it takes for the pill to reach your colon and dissolve by watching it with a fluoroscope (similar to an X-ray machine). A series of X-ray-like pictures will be taken of your midsection until the pill has reached the end of your colon. If this test pill performs well, its design may be used in another study to deliver vitamins to the human large bowel. The purpose of this second experiment would be to find out whether folate (a B-vitamin, sometimes called folic acid), made by bacteria in your large intestine, can be taken up and used by your body.

DESCRIPTION OF THE RESEARCH

Study Enrollment

You are being invited to participate in this study because you are a healthy adult with normal digestive tract motility.

Study Procedure

The study takes place at The Hospital for Sick Children (SickKids). If you agree to be in our study, we will need your participation for one test period that will take approximately 1 day to complete.

Pre-Study Day: Prior to your scheduled clinic visit, you will be asked to come in to the Clinical Investigation Unit (CIU) at the Hospital for Sick Children for a brief screening interview. You will also be given one test caplet, a standardized breakfast meal, and the necessary instruction for home caplet administration and study day preparations. Female subjects will be asked to perform a pregnancy test within 24 hours of the scheduled clinic visit, the results of which will be confidential.

Study Day: You will be scheduled to arrive at the Hospital for Sick Children CIU at 8 a.m. The entire study day will take up to 10 hours to complete and we will provide you with meals.

We will monitor the movement and dissolution of the caplet using a fluoroscope in the IGT (Image Guided Therapy) unit at SickKids Hospital. This procedure is like having X-rays (with approximately 10 times less radiation exposure) and will involve taking 2-3 second long videos of your midsection. This series of snap shots will begin approximately 2 hours post-dose and continue at approximately 60 minute intervals until the caplet has completely dispersed or up to 12 hours post-dose. At most, this will add up to a total of 14 images or 28-42 seconds of fluoroscopy, which is equivalent to less than half of your yearly exposure to natural background radiation from the sun and the Earth. In the event that the caplet has not completely dissolved in your colon by the time the last image is taken, we may ask you to collect your stools over the next 24 hours, which will be further analyzed with the fluoroscope. A feces collection kit will be provided by us, as well as instructions for sample

delivery. Study-specific meals and snacks will be provided throughout the day, starting from the time that the caplet has left your stomach.

New information from this study or other studies may affect whether you want to continue to take part in the study. If this happens, we will tell you about this new information.

POTENTIAL HARMS

Although the dose of radiation that is given with fluoroscopy is approximately 10 times less than a regular X-ray, there are certain risks associated with exposure to radiation. Routine precautionary measures will be taken to ensure that your radiation exposure is as minimal as possible. There are no other known harms associated with participation in this study.

POTENTIAL DISCOMFORTS OR INCONVENIENCE

You may be inconvenienced by having to travel to The Hospital for Sick Children. Study obligations also require you to take time away from your regular schedule.

POTENTIAL BENEFITS

To Individual Subjects
There are no direct benefits to volunteers from participation in this study.
To Society
Your participation is appreciated as the results will be used to better understand how the human digestive tract works, and whether a specialized pill can be used to deliver vitamins to the large bowel. The results of this study may be applied to another experiment that will allow us to find out whether the B-vitamin, folate, can be absorbed across the human large intestine.

CONFIDENTIALITY AND PRIVACY

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless the law makes us do this. For example, the law could make us give information about you in the following circumstances:

- If a child has been abused
- If you have an illness that could spread to others
- If you or someone else talks about suicide (killing themselves), or
- If the court orders us to give them the study papers

SickKids Clinical Research Office Monitor, employees of the company funding the study (NSERC), or the regulator of the study may see your health records to check on the study. For example, people from Health Canada Products and Food Branch, if necessary, may look at your records.

By signing this consent form you agree to let these people look at your records. We will put a copy of this research consent form in your patient health records.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members. Following completion of the research study, the data will be kept as long as required and then destroyed as required by Sick Kids policy. Published study results will not reveal your identity."

The results of the tests described will be used for research purposes only in the context of this study. We would need your permission and signed consent to send the test results to another professional involved in your care.

REIMBURSEMENT

We will pay for all your expenses for being in this study eg., meals, babysitters, parking and getting you to and from SickKids. If you stop taking part in the study, we will pay you for your expenses for taking part in the study so far. Participants will receive $100 on completion of the test period. This will cover travel expenses and a very modest reimbursement for lost wages.

PUBLICATION OF RESULTS

After completion of the study, the investigators plan to present study results at conferences, seminars and other public forums. Eventually the investigators plan to publish study findings in a research journal. You will remain completely anonymous.

ALTERNATIVES TO PARTICIPATION

Participation in the study is voluntary. If you choose not to participate, your family will continue to have access to quality care at Sick Kids. You do not have to participate in this study. If you choose to participate in this study you can withdraw from the study at any time.

PARTICIPATION

It is your choice to take part in this study. You can stop at any time. The care you get at SickKids will not be affected in any way by whether you take part in this study.

New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want to be in this study.

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you took part in this study.

We will give you a copy of this consent form for your records.

In some situations, the study doctor or the company paying for the study may decide to stop the study. This could happen even if the treatment given in the study is helping you. If this happens, the study doctor will talk to you about what will happen next.

If you become ill or are harmed because you took part in this study, we will treat you for free. Your signing this consent form does not interfere with your legal rights in any way. The staff of the study, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

SPONSORSHIP

The sponsor of this research is the National Science and Engineering research Council (NSERC). In certain situations, the study may be cancelled at the discretion of the investigator or the study sponsor even if you are benefiting personally. If this occurs, the investigators will discuss next steps with you.

CONFLICT OF INTEREST

I, and the other research team members, have no conflict of interest to declare.

CONSENT:

By signing this form, I agree that:

1) The study has been explained to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know about what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect my health care at SickKids.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my medical records will be kept private. You will give no information about me, unless the law requires you to.
6) I understand that no information about who I am will be given to anyone or be published without first asking my permission
7) I have read, and understood pages 1 to 6 of this consent form. I agree, or consent, to take part in this study.

Printed Name of Subject and Age	*Signature & Date*
Printed Name of person who explained consent	*Signature & Date*
Printed Witness' name (if the subject does not read English)	*Witness' Signature & Date*
Printed name and dated signature of qualified staff member who has witnessed the consent procedure	

If you have questions about this study, please call ***Ashley Aimone*** *at (telephone), or ((pager).*

If you have any questions about your rights as a subject in a study or injuries during a study, please call the Research Ethics Board Manager at (416) 813-5718.

APPENDIX 2

CASE REPORT FORM: Study 2a

PRELIMINARY STUDY REQUIREMENTS:

Subject meets study eligibility requirements (see Study Protocol) OYes ONo
Subject signed Informed Consent Form OYes ONo

PATIENT DATA:

Date of Birth (d/m/y): ____ / ____ / ____
Sex: OMale OFemale
Height (m): _____
Weight (kg): _____
Ethnicity: _____

DIETARY/MEDICAL HISTORY:

GI Disease: OYes ONo _____
Laxative use: OYes ONo _____
Other Motility Agents: OYes ONo _____
Pregnant: OYes ONo _____
Alcohol use: OYes ONo _____
Smoker: OYes ONo _____
Supplements: OYes ONo _____
Antacids: OYes ONo _____
Allergies: OYes ONo _____
Food Preferences: OYes ONo _____

CAPLET ADMINISTRATION:

Caplet ingested with meal: OYes ONo
Number of caplets administered:
Date (d/m/y): ____ / ____ / ____ Time (24h): _____

IMAGING EVENTS: Date (d/m/y): ____ / ____ / ____

Image Number	Time (24h)	Duration (sec)	Intensity (mGy)	Comments/Notes
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				

FOOD AND DIETARY INTAKES:

Study day & Date (d/m/y)	Time (24h)	Food Description and meal type	Portion size (g)	Folate content (ug)
		Dose meal:		
		Snack:		
		Lunch:		
		Snack:		
		Dinner:		
		Snack:		

STOOL COLLECTION:

Collection required: OYes ONo
Date of delivery /pick-up (d/m/y): ____ / ____ / ____
Other details: _____
_____

Fluoroscopy Results: _____
Date of scan (d/m/y): ____ / ____ / ____
Time of scan (24h): _____

COMPENSATION:
Reimbursement received? OYes ONo
Amount: $_____

Signature:_____

APPENDIX 3

TABLE 6 Summary of correlations between caplet transit times, dissolution time, dietary intakes, and the menstrual cycle

	1:0[1]		3:1	
	r	*p*-values	r	*p*-values
Gastric Emptying Time				
Breakfast Calories	0.33	0.315	-0.04	0.912
Total Calories	-0.45	0.167	-0.15	0.688
Carbohydrates	-0.33	0.345	-0.11	0.767
Dietary Fiber	-0.39	0.270	0.05	0.888
Protein	-0.30	0.408	-0.17	0.649
Total fat	-0.44	0.203	0.05	0.901
Day of Menstrual Cycle	0.36	0.554	0.26	0.673
Small Intestinal Transit Time				
Breakfast Calories	0.04	0.916	<-0.01	0.994
Total Calories	0.77	0.009	0.14	0.710
Carbohydrates	0.71	0.033	0.38	0.278
Dietary Fiber	0.55	0.127	-0.30	0.394
Protein	0.68	0.045	0.62	0.058
Total fat	0.56	0.115	-0.07	0.838
Day of Menstrual Cycle	-0.14	0.828	-0.83	0.082

Colon Arrival Time				
Breakfast Calories	0.39	0.269	-0.02	0.948
Total Calories	0.09	0.803	0.05	0.891
Carbohydrates	0.01	0.988	0.30	0.401
Dietary Fiber	-0.12	0.758	-0.26	0.473
Protein	0.14	0.715	0.49	0.151
Total fat	-0.001	0.997	-0.05	0.900
Day of Menstrual Cycle	0.37	0.536	-0.81	0.099
Dissolution initiation time^2				
Breakfast Calories	0.41	0.242	-0.55	0.101
Total Calories	-0.11	0.766	-0.08	0.823
Carbohydrates	-0.11	0.77	0.07	0.853
Dietary Fiber	-0.25	0.521	-0.32	0.369
Protein	-0.12	0.751	0.45	0.195
Total fat	-0.003	0.994	0.05	0.882
Day of Menstrual Cycle	0.57	0.321	-0.19	0.766
Total Transit Time				
Breakfast Calories	0.30	0.371	-0.28	0.435

Total Calories	0.45	0.165	0.25	0.484
Carbohydrates	0.55	0.102	0.24	0.511
Dietary Fiber	0.54	0.111	0.30	0.394
Protein	0.67	0.034	0.33	0.347
Total fat	0.38	0.273	0.48	0.162
Day of Menstrual Cycle	0.03	0.965	0.70	0.190

[1]Coating formulation ratio of Eudragit L100 to S100.
[2]Total time from caplet administration to the first signs of coating disruption.