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The aspirin augmented standardized lactulose mannitol test as a measure of the ‘health’ of the gastrointestinal tract

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

at

MASSEY UNIVERSITY

Palmerston North, New Zealand

Ivana Roosevelt Sequeira

2015
DEDICATED TO

My father, Roosevelt Franklin Sequeira


For teaching me to never give up.

I miss you every day.
In this thesis, I studied the ‘classical’ lactulose mannitol test for intestinal permeability that has been used to measure the integrity of the intestinal mucosa and thus to provide an index of recovery from inflammatory bowel disease (IBD) and from autoimmune diseases such as coeliac disease. Perusal of the literature indicates that the protocol for the test has not been standardized and a variety of different test protocols have been used. Hence there are differences in the duration of urinary sampling, the doses of the two test probes, the volumes of fluid consumed during the test and the administration of the test during the fasted or fed state. There is therefore a need for a standardized test.

The bulk of the research conducted in this thesis was to develop an optimal protocol with a standardized osmolarity (720 osmol l⁻¹) for the test solution that contained 10 g of lactulose and 5 g of mannitol dissolved in 100 ml of water. Similarly the total fluid intake was standardized to 700 ml. The volumes of fluid consumed over the experimental period were also standardized in order to control for any osmolar effects of the test drink and to hydrate the subjects sufficiently to enable them to produce half-hourly urine samples of a reasonable volume.

The rates of excretion and the timings of the peaks in the excretion of mannitol and lactulose were found to vary over time in healthy subjects. Hence the rate of mannitol excretion peaked during the first two hrs whilst the rate of lactulose excretion peaked at four hrs. The correlation between urinary excretion with intestinal transit times were confirmed using a wireless motility capsule. The work with the wireless motility capsule indicated that the probe sugars were in the small intestine from 2½ - 4 hrs and in the proximal colon from 4½ - 6 hrs following dosage with the test solution. Hence a sample
collected during the 2½ - 4 hr period is best for assessing permeability of the small intestinal mucosa in healthy subjects. The wireless motility capsule also confirmed that the standardized dose of the lactulose mannitol did not influence gastric transit time or that through the small intestine and large intestine. These findings confirmed that the standardized test was determining absorption during transit of the test sugars through the small and the large intestine.

The effect of co-dosage with 600 mg of aspirin in the standardized test was then examined as a means of assessing the effect of a reproducible noxious stimulus on the absorption of the sugar probes. This agent augmented small intestinal permeability to lactulose and decreased its permeability to mannitol. Furthermore dosage with aspirin amplified the effect of a pre-existing adverse stimulus such as smoking. Hence the aspirin augmented test could conceivably be used to ‘unearth’ sub-clinical inflammation. Further work explored the effect of an antioxidant, ascorbic acid, on mucosal permeability. The results showed that, rather than mitigating the adverse effects of aspirin, ascorbic acid augmented intestinal permeability.

In summary the work in this thesis has enabled the development of a standardized test that optimizes the ability of the lactulose mannitol test to detect clinical disorders of absorption. Further, augmenting the test with a single dose of aspirin may be useful as an index of gut health or robustness.
ACKNOWLEDGEMENTS

‘The only limits are, as always, those of vision’ - James Broughton

I take this opportunity to present an unedited gratuitous expression to a few people that deserve special mention for their role in the accomplishment of my thesis.

My ‘academic parents’ here at Massey, Prof. Roger Lentle and Prof. Marlena Kruger, for caring and believing in me even when I doubted myself - your unfailing expectations, unwavering support and guidance helped fuel my determination.

Roger, my ‘gastro guru’, has always believed in my abilities. His passion and enthusiasm was often contagious. In reviewing my work and writing, he offered painstaking comments and always respected my voice. His comments and suggestions often evoked a mixture of gratitude (for the detailed attention to my arguments), exhilaration (as he always dramatically improved it!) and awe (“why didn’t I think of that?”). Thank you Roger for ‘damaging my brain’, for always reiterating ‘don’t you worry lass’ and for the constant kind words that motivated me to do better.

Marlena has been the calming influence throughout my work. Had it not been for the consideration that she gave to my application in 2009, a PhD in New Zealand would not have become a reality. As the voice of reason and a solid sounding board she often came up with issues that I would not have otherwise identified. I express my deep gratitude to her for thinking me worthy of this opportunity, for her encouragement and continuous support.

I gratefully acknowledge Dr. Roger Hurst and the New Zealand Institute for Plant and Food Research for the funding received during the course of this project. Dr. Roger
Hurst’s advice and guidance during the project has been invaluable. Routine meetings with both Rogers’ in attendance have never been dull!

Chris Booth and Michelle McGrath have been more than lab managers associated with the project, but friends whom I could count on and ‘go to’ when work got the better of me. Also Corrin, Shampa and others that gathered during morning tea, I appreciate the constant encouragement and healthy doses of caffeine you’ve shared with me. Dr. Gordon Reynolds, Prof. Patrick Morel and Dr. Fran Wolber, whose brain I picked to often review my writing, thank you for your patience with reading and offering me invaluable advice.

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My parents, Roosevelt and Lorena, brother Delano and god father Carl have been my pillars of strength and greatest support. Thank you for nurturing my goals and ambitions and supporting me during my strife. Much love to you.

Thank you all for being a part of my journey. The stress, excitement, hard work, sleepless nights and sheer joy at finally feeling like I have made a ‘discovery’ is unimaginable.

A special mention to ‘Damien’, the HPLC system, he was steadfast and rock solid when there were very many samples that required analysis!

To all the participants without whom none of the studies would have been possible, I am very grateful. Approval for all research described in this thesis has been obtained from the Massey University Human Ethics Committee Southern A.
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2. Doctoral student gains international recognition.
   Manawatu Standard. February 25th 2014

3. Contains sweeteners.
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   (http://definingnz.com/contains-sweeteners)

   Our Changing World. Radio NZ.
   (http://www.radionz.co.nz-national-programmes/ourchangingworld#audio-20161296)
## ABBREVIATIONS

<table>
<thead>
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<th>Definition</th>
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<tbody>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AJC</td>
<td>Adhesive junctional complex</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>ATL</td>
<td>Aspirin triggered lipoxin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri phosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>CLO</td>
<td>Campylobacter like organism</td>
</tr>
<tr>
<td>COX</td>
<td>Cycloxygenase</td>
</tr>
<tr>
<td>Cr-EDTA</td>
<td>Chromium labelled ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl-phosphotidyl choline</td>
</tr>
<tr>
<td>DHA</td>
<td>Dehydroascorbic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
</tr>
<tr>
<td>FOS</td>
<td>Fructo-oligosaccharide</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut associated lymphoid tissue</td>
</tr>
<tr>
<td>GHP</td>
<td>Glutathione peroxidise</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
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<tr>
<td>GOS</td>
<td>Galacto-oligosaccharide</td>
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<tr>
<td>GST</td>
<td>Glutathione s-transferase</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
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<tr>
<td>IBS-D</td>
<td>Irritable bowel syndrome with diarrhoea</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecules</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus amebocyte lysate</td>
</tr>
<tr>
<td>LI</td>
<td>Large intestine</td>
</tr>
<tr>
<td>LMR</td>
<td>Lactulose mannitol ratio</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTB</td>
<td>Leukotriene B</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated phospho/protein kinase</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylic acid transport</td>
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<tr>
<td>MD</td>
<td>Molecular dynamic</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NFKB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NHE3</td>
<td>Sodium-hydrogen antiporter 3</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain receptors</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>O/E</td>
<td>Observed to expected</td>
</tr>
<tr>
<td>OTC</td>
<td>Over the counter</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PDA</td>
<td>Photodiode array</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PGHS</td>
<td>Prostaglandin endoperoxidase synthase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RID</td>
<td>Refractive index detector</td>
</tr>
<tr>
<td>RMA</td>
<td>Reduced major axis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SGLT1</td>
<td>Sodium glucose transporter 1</td>
</tr>
<tr>
<td>SI</td>
<td>Small intestine</td>
</tr>
<tr>
<td>SIBO</td>
<td>Small intestinal bacterial overgrowth</td>
</tr>
<tr>
<td>SLR</td>
<td>Simple linear regression</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SVCT</td>
<td>Sodium dependent secondary active transport</td>
</tr>
<tr>
<td>Tc-DPTA</td>
<td>Technetium-99-labelled diethylenediaminepeptolytic acid</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UWL</td>
<td>Unstirred water layer</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZO</td>
<td>Zona occludins</td>
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Overview of the thesis
1.1. Introduction

Hippocrates stated nearly 2000 years ago that ‘all disease begins with the gut’ and we now recognize the pivotal role that gut health plays in our overall health and wellbeing\(^1\). Thirty years ago Dubos and Schaedler\(^2\) suggested the gut was an ecosystem and since then important research has been carried out to unravel the role that the gastrointestinal (GI) tract plays in the maintenance of overall health\(^1\).

As defined by the World Health Organization (WHO), ‘health’ is no longer thought to be a “state of complete physical, mental, and social well-being” nor is it “merely the absence of illness and infirmity”\(^3\). Diagnostic and screening tests, used by the medical profession, infer illness or an underlying condition based on values in a ‘reference range’ or ‘interval of tolerance’ assigned to a particular measurement. For example, ferritin levels are used to diagnose anemia. Generally the healthy range/interval is determined with respect to the reference group in the population with a level of variance/deviation from the mean that was a presumed range of values. Given that the presumed healthy range of values is normally distributed in the population, a result that turns out to be greater or lesser than 2.5 standard deviations (SD) from the mean is interpreted as having less than 2.5 % probability that it could be within the healthy range than strongly indicating the likelihood of illness. For example, a result showing ferritin levels normal within 2.5 SD of the mean would not guarantee ‘healthy’ iron stores, especially during conditions associated with inflammation, infection, liver disorders and malignancies that potentially mask iron depletion\(^4\). Similarly, acute exercise and physical activity can increase ferritin levels to disguise or contribute to identification in the ‘illness’ group\(^5\). In short, the statistical paradigm cannot ensure/explain health in the sense of wellbeing as persons within the range defined as normal may yet be predisposed to illness in a particular sense, e.g. women are likely to become anaemic during or after menstruation.
More recently a definition for a state of ‘health’ is ‘the ability of an individual or an organ system to ‘adapt’ to change, i.e. resilience\(^3\). This idea was first put forward by French physician, Georges Canguilhem, in his book called *The Normal and the Pathological* \(^6\), who viewed health as the ability to adapt to one’s environment. As health is a dynamic property and not a fixed entity and varies from one individual to another, the concept challenges the statistical classification of health into normal and abnormal states. The dynamic complexity of function is evident in the gut, as the intestine must balance two conflicting functions. It must allow selective absorption of required nutrients from the intestinal lumen whilst preventing the entry of harmful entities and toxins including microorganisms, antigens, and other pro-inflammatory factors. The latter has been termed the 'barrier' function. In the face of such dynamic complexity, it is hard to determine the state of health. Hence clinicians use to the old concept of ‘normality’, i.e. the absence of disease. Ergo, positive aspects such as ‘adequate’ function, i.e. effective digestion and absorption; the absence of illness diagnosed by a ‘normal’ histology of a biopsy or a negative campylobacter-like organism (CLO) test; the balance of conflicting factors such as absence of dysbiosis in intestinal microflora; a pro-tolerant rather than a pro-inflammatory immune state and a general state of well-being substitute as more ‘static’ parameters/measure.

1.2. The GI tract - a selective filter to the changing dynamic luminal environment

The intestinal epithelium adapts morphologically and functionally in response to the constantly changing internal and external environment\(^7\). Whilst the principle functions of the GI tract have been perceived to be limited to the digestion and absorption of nutrients, electrolytes and water homeostasis, the anatomic and functional arrangement of
the GI tract indicates an important function of this organ is its ability to act as a 'filter' and a ‘barrier’ to selectively absorb nutrients.

The barrier function of the intestine is comprised of three cooperative functions. An immune barrier, composed of locally acting factors such as, secretory IgA, intra-mucosal lymphocytes, Peyer's patches/nodules, mesenteric lymph nodes and of the systemic host defence represented mainly by the reticuloendothelial system; a biological barrier, which is made up of commensal intestinal flora of the gut lumen responsible for colonization resistance; and a mechanical barrier, consisting of the lining intestinal epithelial cells and the associated tight junctions. Failure of the barrier function of the intestinal epithelium is now recognized to be a cornerstone in the pathogenesis of inflammatory bowel diseases and, coupled with secondary innate and adaptive immune responses, is considered to play an important role in the genesis of intestinal inflammation.

The gut has a complex system that involves a number of defence mechanisms to reinforce the barrier property. To summarize, the intestinal mucosa controls the density of endogenous microflora at the apical membrane through the secretion of toxic molecules such as defensins and immunoglobulin A (IgA). Mucus allows particles 6 nm or smaller to diffuse to the mucosa whilst larger molecules like luminal antigens and microorganisms are trapped in the mucus and passed distally during peristalsis. The inward diffusion of potentially pro-inflammatory agents is further inhibited by tight and adhesion junctions located in the spaces between adjacent epithelial cells. Intra and extra cellular pattern recognition receptors, enable enterocytes to identify and distinguish commensal and pathogenic organisms and appropriately regulate the immune response to them. Within the sub epithelial layer adaptive immune responses maintain a balance between ‘pro-tolerant’ and ‘pro-inflammatory responses’ to any antigenic substance that penetrate beyond the epithelium.
Defects in this complex defence system can lead to a bias towards the release of pro-inflammatory mediators that initiate the inflammatory process; e.g. via the activation of nuclear factor kappa B (NFκB)\textsuperscript{15,16} through the pattern recognition receptors (PRRs) on epithelial and dendritic cells (DCs) in inflammatory bowel diseases (IBDs). Thus in a ‘healthy’ gut, the gut associated lymphoid tissue, the commensal bacteria and the intestinal epithelial barrier together contribute to the equilibrium between tolerance and immunity to microbial antigens.

1.3. How can we quantify gut ‘health’?

‘Gut health’ \textit{per se} is a term that lacks clear definition\textsuperscript{1}. An adequate measure of health would account for physiological changes and adaptations within the gut in response to a perturbation. The gut is constantly challenged and must maintain an appropriate equilibrium to sustain health. Hence were Georges Canguilhem’s ideology to define health as ‘the ability to adapt’ to be used, then a ‘dynamic’ measure of gut health would be one that measures a response to a noxious challenge. Such a challenge should be standardized, i.e. by its disturbance and magnitude, so that an assessment of recovery, from the challenge, could be reproducibly quantified. Health could then be inferred from the response to and recovery from the challenge.

There are many parameters on which such a test could focus, intestinal permeability based on the dual sugar absorption test, would be a suitable parameter for assessing the barrier property of the epithelium, i.e. the integrity of the mucosa\textsuperscript{1}. Indeed a number of enteropathies including IBD are associated with loss of the integrity of the epithelial barrier. The assessment of intestinal permeability is well established in the surveillance of recovery from a number of chronic inflammatory diseases of the intestine such as coeliac...
and Crohn’s disease; and has also been used to determine drug toxicity\(^\text{17}\). The classical test involves the ingestion of two sugar probes, each of different size, and permeability is assessed based on the recovery of these sugars in urine.

Epithelial permeability is, under normal circumstances tightly regulated, being affected by exogenous (e.g. bacterial attachment and toxins and drugs) and endogenous agents (e.g. cytokines and signaling molecules). Assessment of the permeability of the intestine effectively determines the diffusivity of two sugar probes, e.g. mannitol and lactulose, across the epithelium each by the transepithelial/transcellular routes or the paracellular pathway. The former is generally associated with transport of the smaller sugar probes across the epithelial cell and can occur via receptor mediated processes or non-specific endocytosis\(^\text{18}\). On the other hand, the latter is associated with the transit of the larger sugar probe through the tight junctions (TJ) that link adjacent epithelial cells\(^\text{19}\) as described in detail in Chapter 2. The dynamic interaction between these two pathways allows the rate of absorption of various nutrients to be up regulated after a meal, e.g. glucose\(^\text{20, 21}\).

1.4. **Intestinal Permeability - potential 'dynamic' measure of gut health**

The epithelial barrier can be made permeable with a standardized noxious stimulus such as a non-steroidal anti-inflammatory drug (NSAID). Intestinal permeability tests, like the dual sugar absorption test, can then be used to evaluate the response and/or recovery of the epithelium from the standard stimulus. The measures of resistance to and recovery from the stimulus, provides information regarding the resistance to the noxious stimulus and the speed of recovery to normal function. The standardized pro-inflammatory stimulus
is biologically relevant as it replicates the changes in mucosal integrity that are thought to occur during pro-inflammatory conditions such as Coeliac disease and Crohn's disease.

Hence in the advent of the delivery of the noxious stimulus, a simple non-invasive clinical test of intestinal permeability has the potential to provide a dynamic assessment of 'gut health'. The stimulus can be site specific, i.e. can cause a temporary increase in small intestinal permeability, and safe. Developing the stimulus augmented test also has the potential to test adverse effects of various foods or drugs and compare the mucosal response with that of the standardized stimulus. Similarly, it is possible to assess if a food or drug mitigates the mucosal changes induced by the standardized stimulus when co-administered with it or if it reverses the effect, i.e. whether the agent induces a preventative or restorative effect on permeability.

1.5. Perspective of the thesis

The aim of the research detailed in this thesis was to establish a dynamic method for measuring gut health based on the ‘classical’ dual sugar absorption test for intestinal permeability, with the view that it could eventually be used to test the effects of food ingredients or nutraceutical agents on gut health.

The procedure was to firstly choose/identify an appropriate method, to suitably refine the method, and to check that the refined method was able to accurately and reproducibly quantify the permeability probes in urine. A review of the literature indicated that a number of methodologies have been used to quantify urinary excretion of the sugar probes which included enzymatic assays, calorimetric assays and chromatography. Following careful consideration of the limitations of each method, high performance liquid chromatography (HPLC) was chosen as the preferred technique. A range of columns, i.e.
monosaccharide H⁺ - columns, cation exchange columns and anion exchange columns, and
detectors such as absorbance detectors (ultraviolet, photodiode array), refractive index,
florescence and mass spectroscopic detectors for HPLC have been previously used. A
simple, reliable, reproducible, cost effective method that did not involve derivitization of
analytes was required. Accordingly, the methodology was standardized and calibrated by
using an appropriate column and detector system that optimally detected urinary
 saccharidic probe sugars.

Moreover, a test procedure was further developed that standardized fluid intake
and optimized urine sampling time to refine the permeability test. A literature search
showed that differing volumes of fluid have been administered during the course of the
test\textsuperscript{28}; in some studies food was allowed two hrs after the test solution was administered\textsuperscript{29-31},
while other study protocols used different urine collection periods that ranged between
2 - 24 hrs\textsuperscript{32,33} and probes with similar molecular weights which were used interchangeably\textsuperscript{34}
at different doses\textsuperscript{22,35}. Such discrepancies in the study protocol precluded meaningful
comparisons between studies. This is due to the fact that the volume of fluid\textsuperscript{36}, its nutrient
content\textsuperscript{37,38} and its consumption along with solids\textsuperscript{39,40} influence GI residence time, the
absorption and consequently the quantities of the sugar probes recovered in urine. Hence a
classical/static test protocol was designed to restrict fluid intake to 700 ml over a six hr
urine collection period. This was done in order to limit the reported effect of diuresis on
the urinary recovery of the sugar probes. No food was allowed during the study period.
Urine samples were collected every half-hour over a six hr period so as to allow the
dynamics of absorption of each sugar to be tracked and the recovery period used in clinical
tests to be optimized.

It is noteworthy that in reports multiple sugar probes having similar molecular
weights were used and were at times administered simultaneously\textsuperscript{34}. Given that the
permeability depends on the diffusion of molecules through simple pores, it is likely that
the probes will compete for pore space. Thus the quantities of the various probes that are
recovered will depend on the number and relative proportion of pores available for
absorption. Ideally the sugar probes are water soluble, hydrophilic, lipophobic,
non-metabolized and readily quantified in urine. However some probes had been shown
to be absorbed by active transport, available through dietary sources, have brush
border activity and need to be radio labelled. Hence after reviewing the potential
limitations of the commonly used paracellular and transcellular marker probes, lactulose
and mannitol were chosen. As these sugars are degraded in the colon by enteral
microflora, they can be used to optimally assess small intestinal permeability. The
dynamics of absorption of lactulose and mannitol were determined and compared to two
other probes that are often used interchangeably with these sugars.

The sensitivity of the lactulose mannitol test was further explored using a
standardized noxious stimulus that has been reported to increase intestinal permeability to
larger probe sugars. Various exogenous agents that are reported to increase tight junction
permeability were reviewed. The findings indicated that the effects of ethanol/alcohol
consumption and cigarette smoking on permeability are not limited to the small intestine,
with contradictory reports to the effect of smoking on tight junction permeability. Therefore a non-steroidal anti-inflammatory drug seemed like the practical choice given
that the drug could be chosen based on its safety profile, general use and the effect on the
small intestine. Previous studies have used drug such as indomethacin, ibuprofen,
naproxen, sulindac; however, aspirin was selected as it met the criteria and is well
researched.

A half-hourly urine sample collection regime was instituted, both with and without
the aspirin challenge, to enable the dynamics of absorption of each sugar to be determined.
Previous studies assessed permeability based on the ratio of the two sugars derived from cumulative samples. The half-hourly regime allowed the identification of the timing at which the peak in urinary excretion occurred. Moreover it allowed the rate of excretion of the sugars over the six hr period to be traced. Following this the suitability of a single dose of aspirin as an appropriate stimulus to augment the test was investigated. It was conducted on the premise that were aspirin to alter GI residence time or the physicochemical properties of the sugars, it could alter patterns of excretion of the sugars.

In a similar manner the administration of a nutrient during the test could also affect GI motility patterns. Therefore nutrients could decrease the proportion of sugar probes that are absorbed in the small bowel and increase the proportion in the large bowel\textsuperscript{48}. They could also reduce transit of a substance present in high concentration from the stomach to the large bowel to enhance absorption by increasing residence time in the small intestine. Alternatively, nutrients could increase residence time of gut contents in the lower bowel to engender extensive fermentative digestion.

The dynamic aspirin augmented test was therefore standardized based on the timing at which the sugars resided in the small intestine. Based on the temporal patterns of excretion of each sugar, three phases were identified, which likely corresponded to the emptying of the column of digesta from the stomach; to the small intestine; and its subsequent movement into the proximal colon over the six hour collection period. These time period’s over which the sugars resided in the stomach, small intestine and colon were confirmed using a wireless motility capsule, SmartPill\textsuperscript{®}. The non-invasive technique was chosen over several other expensive and protracted methods\textsuperscript{49, 50} such as the paracetamol absorption test\textsuperscript{51, 52} (gastric transit), $^{13}$CO$_2$ acetate breath test\textsuperscript{53, 54} (gastric and small bowel transit), scintigraphy\textsuperscript{49, 55} (whole gut transit) and radio opaque markers\textsuperscript{56} (whole gut and colonic transit) as detailed in Chapter 6. GI residence time was then determined based on
ambient pH, temperature and pressure recordings from the intestinal lumen\textsuperscript{49} obtained from the SmartPill.

Finally, the ability of the dynamic standardized lactulose mannitol test to determine the effect of food ingredients and nutraceuticals on gut health was assessed. This was important if the effects of substances that promote gut health by decreasing permeability to noxious materials using the test are to be meaningfully compared.

1.6. Structure of the thesis

The thesis is presented with a literature review that details the rationale behind the use of the lactulose mannitol test to assess intestinal permeability and the benefits of using \textit{in vivo} tests of intestinal permeability rather than other methods. I also describe the rationale underlying the use of an NSAID such as aspirin, as a noxious stimulus, that augments transient inflammatory changes within the GI mucosa. Subsequently I detail the methodological considerations underlying the development of a high performance liquid chromatography technique to quantify sugar probes in urine along with the calibration and testing of the sensitivity of the methodology. This is followed by five manuscripts based on published papers that describe the results from work using the standardized lactulose mannitol tests of permeability. The thesis concludes with a summarizing discussion that critically explains the results of the work and its significance and relevance to clinicians and the food industry. The conclusion finishes with recommendations for future work.
1.7. References

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CHAPTER 2

Review of literature
2.1. Factors influencing mucosal permeability

This section of the review discusses the factors that influence permeability and diffusion of molecules across the small intestinal mucosa. It emphasizes the significance of intestinal permeability as a measure of the integrity of the intestinal mucosa in health and disease.

2.1.1. Elements that constitute the barrier property of the intestine

The intestinal tract is a single contiguous layer of absorptive enterocytes which comprises of about 80% of the total population of small intestinal epithelial cells. These cells are interspersed with a mixture of secretory cells such as mucus producing goblet cells, endocrine and immune cells. The epithelial lining functions as a 'filter' and a 'barrier' to ensure non-passage of harmful environmental agents whilst being selectively permeable to nutrients. The process is dynamically regulated and determined by the interaction of several components which include the unstirred water layer, phospholipid layer of the apical and basolateral membrane, surface mucus coat, epithelial layer and endothelial factors.  

The small intestinal epithelial surface is made up of a series of finger-like projections called the villi that extend several microns into the lumen. Each villi are surrounded by 6 - 8 crypts which dip down into the underlying mucosa. The entire epithelial layer is replaced every 2 - 6 days from a population of stem cells that are continually dividing at the base of the crypts. The resultant undifferentiated cells migrate up the walls of the crypts onto the adjacent villi where they are transformed into epithelial cells. Normally, enterocytes reach full maturity about half way to two thirds up the villi reflecting a villus base-to-tip gradient.
The bulk of nutrient absorption occurs via the shaft of the villi. During differentiation as the enterocytes migrate toward the villus tip; they start to express transporter proteins as well as enzymes such as disaccharidases. Maximal active absorption has therefore been reported to occur in the apical region of the villi whilst absorption by passive diffusion is considered to be augmented in the lateral region of the villi. Little absorption is thought to occur from the intervillous crypts at the base of the intervillous space. The associated crypts are principally concerned with secretions of digestive agents and renewal of the ongoing loss of endothelial cells from the villus tip. Hence for example, for a nutrient like glucose bulk absorption is reported to occur within the terminal 5% of the villus while less than 2% is absorbed from the mucosa surrounding the intervillous space by solvent drag.

Villi were considered to increase intestinal surface area but whether this is necessary to augment digestion and absorption is debatable. An alternative view is that the villi reduce shear forces that are exerted on the mucosa by bending in the direction of the flow of the digesta. This occurs as the lateral surfaces of the villi are orientated perpendicularly to the direction of flow of digesta within the lumen and to the direction of propagation of contractile activity. The tonic and phasic contractions of the intestinal walls cause the distance between the intervillous spaces to vary. As a result the relative stasis between the adjacent villi is suggested to influence mixing in the intervillous space and contribute to the unstirred water layer (UWL).
2.1.2. The pre-epithelial layer – the first barrier that possibly alters diffusion gradients and rates of absorption

The constituents of the pre-epithelial layers vary along the length of the gastrointestinal tract; with a very thin or absent adherent mucus layer in the small intestine\textsuperscript{10} and collectively form the UWL. Broadly speaking the total thickness of the mucus layer is the thinnest in the jejunum (and areas overlying the M cells of Peyer’s patches) and greatest in the stomach and colon\textsuperscript{11}. The absorptive surfaces of the apical brush border of enterocytes are overlayed by 400 – 500 nm thick glycocalyx, a 5 – 10 μm thick mucus layer and an aqueous layer of chyme that measures between 0.1 - 1.5 mm\textsuperscript{12,13}. The glycocalyx, although thinner in comparison to the rest of the mucus layer, forms an effective size exclusion barrier at the apical membrane that impedes the access of harmful substances\textsuperscript{14}.

Micro-rheological measurements indicate that at lower pH the mucin gel is heterogeneous with a wide range of pore sizes, some of which are sufficient to allow 100 – 200 nm particles to move within the gel\textsuperscript{15}. The process of diffusion is governed by the physical size and arrangement of mucin fibers within the gel and varies inversely with the molecular size of solutes and is influenced by the net charge and hydrophobicity of the diffusing molecule\textsuperscript{16}. For example, the presence of a large number of polyvalent cationic groups may cause the molecules to bind to the negatively charged oligosaccharide side chains of the mucins. Conversely, highly anionic proteins may undergo repulsion from like charges on the oligosaccharide side chains of mucin preventing them from entering and diffusing through the gel. Most globular molecules such as proteins and antibodies are thought to readily diffuse\textsuperscript{17,18} through the mucin gel whilst large molecules of cylindrical or pentameric form are thought to be relatively reduced\textsuperscript{19}.
There is increasing evidence that the mucin layer functions together with the effect of solvent drag generated by the absorbing enterocytes, limits the passive diffusion of molecules by acting as a particulate and selective molecular sieve\textsuperscript{19}. Luminal mixing efficiently delivers the solubilized contents of the lumen to the brush border surface\textsuperscript{20} and favours the absorption of nutrients at the apical membrane\textsuperscript{21}. Fluid absorption\textsuperscript{22} and ‘solvent drag’ will tend to cause the concentration of the solute to rise in the UWL in the immediate vicinity of the apical membrane thereby increasing concentration gradients across the UWL. Such changes would increase the diffusion gradient of the molecule to favour the cellular absorption (provided that it is in low concentrations in the cytosol). Diffusion of molecules is limited at the inter villous aqueous space separating the lumen from the crypts (\textasciitilde 600 \textmu m) than at the at the villous tip (25 \textmu m)\textsuperscript{20}.

Determinations of the UWL have shown that ‘smaller’ molecules having a higher diffusion coefficient appear to encounter an apparent thicker UWL in comparison to ‘larger’ molecules which will alternatively have a lower diffusion coefficient and thus encounter a thinner UWL.\textsuperscript{23} Hence, as a consequence of their lower rate of lumenward diffusion the ‘larger’ molecules, would be influenced to a greater extent by solvent drag and would thus be drawn towards the apical membrane more effectively than the ‘smaller’ more mobile molecules\textsuperscript{24}. For molecules that rapidly transit the UWL, given that the rate of absorption is greater than the rate at which the molecule reaches the apical membrane, favourable concentration gradients (as a result of solvent drag) from lumen to brush border build up. For molecules that have lower membrane permeability, studies by Levitt and co-workers showed that they tend to accumulate along the villus-crypt axis to increase the surface area and correspondingly the transmembrane gradient across it to facilitate diffusion and absorption\textsuperscript{25}. 
2.1.3. The epithelial layer – the barrier that selectively absorbs nutrients via diffusion via the transcellular and paracellular route

A body of work suggests that in healthy epithelium, absorption via passive diffusion of nutrients occur transcellularly via aquaporins (AQPs). AQPs are thought to be responsible for osmotically driven water movement. The discovery of these water channel proteins in 1992 and their subsequent expression along the GI tract has to an extent helped our understanding of water transport\textsuperscript{26, 27}. Structural studies have revealed that the pore size plays an important role in permeability of not only water but larger solutes\textsuperscript{28} suggesting that AQPs are not limited to just water transport. The expression of AQPs in the digestive tract has revealed at least six isoforms in the small intestine\textsuperscript{29, 30} with variation in the reported tissue and cellular localization of AQP proteins\textsuperscript{31}. Due to the location of AQPs along the basolateral membrane, it has been suggested that water transport possibly occurs at the apical membrane via co-transport and leaves the cell by osmosis through the AQPs. However, the transcellular absorption of solutes via AQPs still remains to be established.

Several works on permeability in healthy as well as the diseased epithelia have established that hydrolysed nutrient molecules diffuse across the apical membrane based on the intrinsic permeability properties of the epithelium\textsuperscript{32}. The villus epithelium here is considered to be made of two pore systems. According to the "transcellular model", smaller sized molecules are thought to transit via numerous small water pores situated in the enterocytes while large molecules transit only through larger paracellular pores of low incidence\textsuperscript{33} (Figure 2-1). It is generally accepted that the larger pores are present in the paracellular pathway and is associated with diffusion in the intercellular spaces between
epithelial cells regulated by tight junction (TJ) localized at the apical and apico-lateral membranes.

The "paracellular model" alternatively suggests that differences in permeation of smaller and larger molecules arise due to differences in the morphology of the TJ between adjacent enterocytes of the villus and those of the associated crypts. Hollander's paracellular hypothesis proposed that the enterocyte junctions near the crypts contain lower number of larger pores 5 - 6 nm (50 – 60 Å), in comparison to intermediate sized pores 1 - 1.5 nm

![Figure 2-1: Schematic representation of two hypotheses presented for the passive diffusion of molecules across the enterocytes. According to the transcellular model smaller sized molecules transit via numerous small "pores" present on the apical side membrane of the enterocyte whilst smaller and larger sized molecules transit via larger paracellular "pores" in the tight junction situated at the apical and apicolateral membranes of adjacent enterocytes. The paracellular model on the other hand proposes that the structure and density of the paracellular pores varies along the length of the intestinal villus with a larger number of coarser pores occurring at the bases than at the tips. The horizontal arrows provide a qualitative estimate of the number of each sized pore.](image)
(10 – 15 Å) at the villus base; and abundant small pores < 0.6 nm (< 6 Å) at the villus tip at the villus tip (Figure 2-1). Hence all pores would be permeable to smaller molecules while permeation of larger molecules would only be restricted to the pores at the crypts that are less accessible. This model reflected the successive and rapidly decreasing permeability across the crypt-villus axis as enterocytes are transferred from the proliferation zone in the crypts to the absorptive areas at the tips of the villi.

2.1.3.1. The TJ – a possible rate limiting barrier within the paracellular pathway

The membranes between adjacent enterocytes are connected to each other in the upper regions of their lateral membrane to form encircling junctional complexes. Each complex is continuous with those of the neighbouring cells forming a barrier which spans the mucosa. The TJ lie in the apical end of the lateral aspect of the enterocyte; the adherens junctions and the desmosomes lie deeper and constitute the complex and all three together are called the adhesive junctional complex (Figure 2-2).

Work in the last 25 years has focused on the importance of the TJ in regulating the passive diffusion of solutes and macromolecules. The TJ is made up of a multi protein complex that bridges the gap between the plasma membranes of adjacent cells and include 'claudins', 'occludins', junctional adhesion molecules (JAMs) and scaffolding proteins, such as zona occludins (ZO)-1, ZO-2, ZO-3. These proteins play a key role in the formation of the paracellular barrier. Although the precise roles of these proteins are unknown work has suggested that the claudins form the actual pores that determine charge selectivity of the paracellular pathway. Claudins from apposing cells fold to create the channel space with
charged amino acid side chains that line the channel to create the charge selectivity to molecules\textsuperscript{42}.

The TJ protein complex associates with the elements of the intracellular actin cytoskeletal system through ZO-1 which is a linker protein that ties the actin cytoskeleton to the transmembrane protein occludin\textsuperscript{43}. The cytoskeleton has been shown to regulate the integrity of the TJ\textsuperscript{43}. Details of the specific molecular interactions that link the tension/contraction of the perijunctional actomyosin ring to TJ regulation are not known. However, due to the lateral tension exerted by the cytoskeleton on the TJ, it has been hypothesized that any disturbance within the cytoskeleton could influence the TJ structure to

![Figure 2-2: Schematic depiction of the apical junctional complex. Tight junctions are connected to the apical actomyosin skeletal ring via the integral membrane proteins; occludin, claudin and junctional adhesion molecules (JAM) that interact with the cytoplasmic plaque proteins ZO-1, ZO-2, ZO-3. The tight junction proteins play a key role in the formation of the paracellular barrier by maintaining the structural integrity and regulation of the tight junction.](image)
alter permeability to ions and solutes\textsuperscript{44}. The effect is thought to be mediated through ZO-1 which connects the two structures\textsuperscript{45}.

2.1.3.1.1.\textit{Physiological regulation of the TJ disrupts barrier function to increase permeability.}

The integrity of the TJ is critical to the overall permeability of the intestinal epithelium. The various exogenous and endogenous stimuli in the environment surrounding the junction can potentiate rapid changes in structure and permeability of the TJ via membrane proteins and its associated cytoskeleton\textsuperscript{40}. This regulation of the TJ and the associated increase in permeability is recognized play a role in the leaky gut syndrome and inflammatory bowel diseases such as Crohn’s disease, ulcerative colitis and food allergies\textsuperscript{29}.

One of the first examples of the modulation of TJ by an extracellular event was first described in studies applying glucose to segments of guinea pig intestine mounted in an Ussing Chamber which caused a significant increase in paracellular permeability\textsuperscript{46, 47}. The existence of this alternative ‘diffusion’ driven pathway was studied in human and experimental animals\textsuperscript{48, 49}, isolated human and rodent small intestine\textsuperscript{50, 51} and intestinal epithelial cell lines\textsuperscript{52, 53}. Electron microscopy (EM) during Na\textsuperscript{+} glucose co-transport showed the dilation of the TJ structure and immunoelectron microscopic evaluation showed spatial dissociation between ZO-1 and the TJ\textsuperscript{54}. In addition to intra junctional dilatation, EM studies with active Na\textsuperscript{+} glucose co-transport also identified the condensation of the perijunctional actomyosin ring of the cytoskeletal framework\textsuperscript{55} through MLCK signalling\textsuperscript{50, 52, 56}.
It was also further suggested that glucose transport led to increased paracellular absorption via solvent drag or fluid drag. It was hypothesized that glucose transport caused sodium and glucose molecules to accumulate within the intercellular basolateral space to provide a driving osmotic force to promote bulk absorption of nutrients across the TJ along with fluid. Pappenheimer estimated that the process led to an amplification of transcellular nutrient absorption with 50 – 70 % of fluid absorption between the intercellular spaces6, 7.

Immuno regulatory cytokines have also been shown to modulate the TJs. Epithelial monolayers incubated with both gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) showed the reorganization of TJ proteins; ZO-1, occludin and claudins57. These cytokines serve as extracellular signals to alter permeability and is associated with an increase in MLCK, indicating that these cytokines too utilise the MLCK pathway to increase TJ permeability58. The reduction in TJ strands, strand breaks and alterations in TJ protein content and composition have been associated with the epithelial damage in Crohn’s disease59, 60 due to the increased levels of IFN-γ and TNF-α61, 62.

2.1.3.1.2. Other factors that increase permeability

Increased glucose absorption via the specific apical membrane active glucose transporter 2 (GLUT 2)63 has been suggested to bring about an increase in permeability by initiating cytoskeletal contraction. In the classical model GLUT2 was thought to be located solely in the basolateral membrane and to function as a site for outflow of glucose and fructose from the enterocyte. However it was shown that in the presence of luminal glucose the activity of the sodium glucose transporter 1 (SGLT1) promoted the rapid insertion of GLUT2 to the apical membrane64 via PKC βII65. The activation of PKC βII led to the contraction of the
cytoskeletal ring and the convex rounding of the apical membrane. This increased the surface area allowing greater area of contact of the luminal contents with the brush border membrane enzymes and contained transporters. As glucose luminal concentration of glucose fell, the mechanism reversed so that GLUT2 was inactivated and resorbed from the apical membrane. It is now known that SGLT1, along with the sodium-hydrogen antiporter 3 (NHE 3) via activation of p38 mitogen activated protein (MAP) kinase, activates MLCK leading to contraction of the perijunctional actomyosin ring. These two mechanisms are responsible for the glucose induced increase in permeability.

Endogenously produced nitric oxide (NO) contributes to increased intestinal permeability. NO in normal conditions has a role in gastrointestinal motility and vascular constriction; however sustained overproduction by inducible nitric oxide synthase (iNOS) during inflammatory conditions causes the depolymerisation of cytoskeletal filaments, cytoskeletal disassembly and disarray to increase permeability. Ethanol and pro-oxidants via the activation of nuclear factor κB (NF-κB) increase cytokine production that stimulate the induction of iNOS. The up-regulation of iNOS by inflammatory mediators results in the overproduction of NO and the production of peroxynitrite. This leads to the nitration and oxidation of actin and tubulin in the cytoskeleton.

Oxidative stress, including the formation of the superoxide anion, hydrogen peroxide and the hydroxyl radical have been shown to increase intestinal permeability and is seen in the pathophysiology associated with smoking, alcohol consumption and NSAID intake. Smoking has been reported to increase leukotriene levels (LTB4) 60 fold, to decrease prostaglandin E2 (PGE2) and increase nitric oxide synthase activity. Studies have demonstrated that the antioxidant defences of smokers are reduced, presumably from...
oxidative stress. There is an association between smoking and the genesis of chronic inflammatory bowel diseases such as Crohn’s disease, Coeliac disease and ulcerative colitis. While cigarette smoking has been shown to be a risk factor in the development of Crohn’s disease, smoking decreases the risk of ulcerative colitis. Permeability studies by Prytz et al. and Suenaert et al. showed that smoking tightens the TJs in healthy volunteers suggesting that this might explain the protective effect of smoking in ulcerative colitis. The latter hypothesis runs contrary to the reported effects of smoking on permeability.

Non-steroidal anti-inflammatory drugs (NSAIDs) are associated with a high incidence of gastrointestinal (GI) ulcers, perforation, haemorrhage and an exacerbation of IBDs. They are thought to deregulate the normal operation of the paracellular pathway by localized biochemical actions on the GI epithelium by effectively inhibiting cyclooxygenase (COX), generating free radicals and suppressing PGE2 synthesis. Given that PG’s have a protective action on mucosal integrity as they help promote blood flow and mucus, bicarbonate and other secretions, the decrease in the volumes of these secretions possibly increases the access of bacteria, toxins and antigens to the mucosa leading to inflammation. Further Oshima et al. demonstrated in vitro that NSAIDs such as aspirin could potentially alter permeability by decreasing the expression of TJ protein claudin.

The fact that oxidative stress and the genesis of reactive oxygen species (ROS) is implicated in the disruption of the barrier properties of the intestinal mucosa and increased permeability, suggests that antioxidants could have the potential to regulate the oxidant induced tissue damage. Tissues with relatively lower concentration of antioxidants are shown to be more susceptible to oxidant injury, with oxidative stress in inflamed intestinal mucosal cells resulting in reduced ascorbate levels by 35 – 73 %, to decrease the capacity of the
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mucosa to prevent oxidative damage and subsequent recovery\textsuperscript{88}. Active episodes of IBD; Crohn’s disease and ulcerative colitis are reported to be characterized by neutrophil induced oxidative stress\textsuperscript{89}. Evidence supporting the attenuation of the actin cytoskeleton of the mucosa in an animal model of colitis with antioxidant therapy\textsuperscript{87}, suggests that antioxidants could be beneficial in regulating the deleterious effects exerted by pro-oxidants on the mucosa\textsuperscript{88}. Moreover given that antioxidants have also been shown to improve the balance between beneficial and deleterious microflora\textsuperscript{90} within the colon it could be reasoned that they could modulate immune responses so as to maintain the ‘barrier’ function of the mucosa.

2.2. Measuring intestinal gut permeability

Intestinal permeability, to measure the barrier function of the mucosa, has been determined using different methods. These methods will be discussed in this section of the review and include \textit{in vivo} human tests such as the dual sugar absorption tests, the C labelled breath tests and the paracetamol absorption test. \textit{In vitro} assessments include cellular methods such as the everted gut sac technique, the Ussing chamber, cell based models and the \textit{in vitro} gut models.

2.2.1. Dual sugar absorption test

In the early 1970s, Menzies introduced the use of test probes for the functional assessment of intestinal barrier dysfunction\textsuperscript{91}. It was hypothesized that in the healthy mucosa the diffusion of larger sized molecular probes was limited. However the loss of membrane integrity augmented the absorption of these molecules across the membrane. The loss of
membrane integrity or increased permeability has been quantified by the simultaneous oral
administration of two water soluble probe molecules of different sizes that are neither digested
nor metabolized\textsuperscript{92, 93}. Monosaccharides or polyalcohols, are thought to transit via the
transcellular route reflecting the degree of absorption of small molecules whilst disaccharides
are thought to transit via the TJs within the paracellular pathway to reflect the permeation of
larger molecules\textsuperscript{92, 94}. Hence the rate of sugars absorbed across the mucosa provides an
indication of the permeability of the mucosa. The excreted probe sugars are measured in urine
as urinary concentrations are reported to be higher than that in the plasma\textsuperscript{94}.

2.2.1.1. Probe molecules used in intestinal permeability testing

The probes used in permeability tests are water soluble, non-metabolized, non-toxic,
hydrophilic, lipophobic, absorbed via passive diffusion, not available through dietary sources,
not produced endogenously, excreted rapidly and completely in urine\textsuperscript{92}. Permeability to smaller
probes has been assessed using polyethylene glycol 400 (PEG 400), D-mannitol, L-rhamnose,
D-xylose and sucrose. Permeability to larger molecules has been determined with lactulose,
sucralose and cellobiose. Additionally, other markers with radio label tags such as chromium
labelled ethylenediaminetetraacetic acid ($^{51}$Cr-EDTA) and the technetium-99-labelled
diethylenediaminepeptolytic acid ($^{99}$Tc-DPTA) have been used to measure permeability to
larger probes. Characteristics of the commonly used probes have been cited in Table 2-1 from
available data.
Table 2-1: Characteristics of commonly used probes to assess intestinal permeability

<table>
<thead>
<tr>
<th>Tracer Molecule</th>
<th>MW (Da)</th>
<th>Cross Sectional Diameter (nm)</th>
<th>Radius (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 400</td>
<td>232-594</td>
<td>0.53</td>
<td>NI</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>150</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>182</td>
<td>0.67</td>
<td>0.38</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>164</td>
<td>0.83</td>
<td>0.49</td>
</tr>
<tr>
<td>Lactulose</td>
<td>354</td>
<td>0.95</td>
<td>0.62</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>354</td>
<td>1.05</td>
<td>0.50</td>
</tr>
<tr>
<td>Cr-EDTA</td>
<td>358</td>
<td>1.10</td>
<td>0.68</td>
</tr>
<tr>
<td>Tc-DTPA</td>
<td>549</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Sucralose</td>
<td>397.6</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>

References: Cobden I, 197893; Jenkins and Bell, 198794; Hollander D, 199234, Anderson ADG, 200495, Travis S, 199296. NI = no information available

2.2.1.2. Determination of intestinal permeability measuring urinary recovery of the probes

Two theoretical relationships have been used to predict and calculate the ratio of the probes. If the molecular weight (MW) of the sugar probe is less than around 15 times the MW of the solvent in which they are dissolved then the rate of diffusion of the probe through pores in the apical membrane of the enterocyte is inversely related to the square root of the MW. If their MW is greater than 15 times the size of that of the solvent then their rate of diffusion is inversely related to the cube root of their molecular size99. Accordingly the relationships predict that the ratio of the rates of diffusion of probes such as lactulose and mannitol typically lie between 0.73 and 0.8127. A similar value for the ratio of the two sugars is achieved (0.71 - 0.80) based on the Stokes-Einstein relationship27 which is the relationship between the
molecular radius and diffusion of the molecule. However in healthy human subjects the ratio of the rate of transit of the sugars across the small intestinal mucosa sugars is much lower, i.e. around 0.025\(^{100}\).

The urinary excretion of each sugar is quantified from urine sample collected from subjects over the experimental period using the following calculations:

**Excretion at time** \( t \) = \[ \text{concentration of the sugar (mg ml}^{-1} \text{) at time } t \] \times \text{total urine volume (ml) at time } t

\%
\text{Cumulative excretion} = 100 \times \left( \sum \text{excretion}_t \right) / \text{total dose of sugar ingested}

The **ratio** is calculated as the excretion of the larger sugar (mg h\(^{-1}\)) / excretion of the smaller sugar (mg hr\(^{-1}\)).

In practice intestinal permeability is diagnosed with a normal ratio of 0.03 used as a cut off value\(^{101}\). The ratio of the probes is used as incomplete dosages, reduced recovery, altered intestinal dilution, altered transit time, and altered body distribution similarly affect the recovery of both probes\(^{92, 98}\). Given that the rates of pre-and post-mucosal factors similarly affect both probes; the differences in the ratios from those of the theoretical values based on molecular diffusion seem to support the hypothesis that the permeability of the probes is based on their molecular size and on the differences in pores sizes in the small intestinal mucosa. Therefore, larger numbers of smaller pores in the mucosa admit mannitol but not lactulose whilst smaller numbers of large pores in the mucosa admit both lactulose and mannitol.
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2.2.1.3. The use of particular probes to measure permeability at particular sites

A number of factors influence the choice of probes used clinical assessment of intestinal permeability. Probes have been chosen based on the extent to which they are degraded in the GI tract by the enteral microflora. Probes such as lactulose, mannitol, cellobiose and rhamnose are readily degraded in the colon and are useful in evaluating permeability within the small intestine. Sucrose is considered an ideal probe to assess gastroduodenal permeability as it is completely broken down by the enzyme sucrase. Probes such as sucralose and Cr-EDTA are stable throughout the gut and are considered suitable for measuring large intestinal permeability.

2.2.1.4. Drawbacks of probes limit their use in tests

Although most of the above mentioned probe sugars have been used in various studies for permeability testing, a number of them have problems with consistency. The use of PEG in clinical test has reported inconsistent results. The shape of the molecule is cylindrical rather than globular having a smaller cross sectional diameter compared to other smaller probes (Table 2-1). It has a linear dimension and thus PEG behaves like a large probe instead of a small probe. The permeability of the probes may also be affected if the probes are soluble in lipids, allowing the molecule to permeate through the lipid membranes by a non-aqueous route. In vitro work demonstrated that PEG-600 could move across aqueous compartments when separated by lipid membranes. Similarly PEG 400 appears to have a degree of lipid solubility and has been shown to cross liposomes which are devoid of water pores. Therefore there could be incomplete urinary excretion of the probe to confound test results.
L-rhamnose is of a similar molecular weight to mannitol (Table 2-1), and the two are used interchangeably. However studies indicate that rhamnose may also have some solubility in lipids and is able to traverse membranes of erythrocyte and therefore could be incompletely excreted in urine. D-Xylose, another transcellular probe is reported to also be absorbed by a carrier mediated process in humans largely from the jejunum and hence cannot be relied upon to accurately assess permeability. D-Mannitol is produced endogenously in humans and naturally occurs in some vegetables albeit in negligible amounts that are insufficient to influence the test. Mannitol in cell based assays has been used as a paracellular marker. However the administration of the sugar with other larger probe sugars in vivo, has shown that mannitol is differentially absorbed via smaller pores, providing evidence that mannitol is a marker for transcellular permeation.

Amongst the larger sugars, cellobiose is hydrolyzed by intestinal lactase, and broken down in the jejunum at the brush border membrane. Lactulose, the most commonly used larger sugar, is a laxative and is reported to cause intestinal hurry which would decrease its absorption and that of the smaller sugar administered with it. Cr-EDTA is a useful marker however it cannot be used when radiolabelled probes are contraindicated.

While several combinations of larger to smaller sugars have been used to assess small intestinal permeability; the lactulose:mannitol and the cellobiose:mannitol combinations are commonly used. Alternatively, the lactulose:rhamnose or the cellobiose:rhamnose tests have been relied upon. However the potential source of error resulting from the metabolism of cellobiose and the absorption of rhamnose, have limited their use in permeability tests. Hence lactulose and mannitol are used preferentially to assess small intestinal permeability over a six hr period.
However, researchers have also demonstrated that a fraction of lactulose and mannitol remain in the colon following \textit{in vitro} degradation by colonic bacteria\textsuperscript{128}. Previous work on small intestinal permeability has reported the continuing urinary excretion of lactulose and mannitol for over six hrs after dosage from bulked urine samples collected over 8 – 24 hr\textsuperscript{118}. The source of this ongoing excretion, and times at which the bulk of probe containing digesta is likely to have traversed the small intestine\textsuperscript{129, 130} is controversial. One hypothesis is that it results from the passage of the sugars into, and continuing absorption from, the large intestine\textsuperscript{118, 120}. Evidence confirms that mannitol can be absorbed by the \textit{ex vivo} colonic mucosa as a result of solvent drag\textsuperscript{131, 132}. Hence, the continual absorption in the colon would require that the sugars must first traverse the extensive microbial biofilm that adheres to the colonic mucosa\textsuperscript{133} without being degraded.

\textbf{2.2.1.5. Problems with dual sugar absorption tests}

Several physical factors have been thought to influence the permeation of the sugar probes to potentially influence the outcome of the test.

\textbf{2.2.1.5.1. Effect of osmotic fluid retention}

Poorly absorbed probes such as lactulose have been suggested to have an osmotic stimulus and accelerate intestinal transit to limit the absorption of concomitantly administered solutes\textsuperscript{110}. The administration of 5 g lactulose in an iso-osmolar solution is reported to decrease the cumulative 5 and 24 hr urinary excretion of smaller sugars in patients with ileostomies\textsuperscript{110}. However studies have used lactulose at doses between 1 - 10 g in sugar solutions. For example
lactulose at doses of 1 g\textsuperscript{120}, \textsuperscript{134}, 5 g\textsuperscript{97}, \textsuperscript{112}, \textsuperscript{113}, \textsuperscript{115}, \textsuperscript{118}, \textsuperscript{124}, \textsuperscript{127}, 6 g\textsuperscript{116}, 6.7 g\textsuperscript{126}, 7.5 g\textsuperscript{114}, \textsuperscript{135} and 10 g\textsuperscript{107}, \textsuperscript{111}, \textsuperscript{119}, \textsuperscript{121}, \textsuperscript{125} have been most commonly used. The effect of these various doses have on GI transit and effect on the absorption of co-administered smaller probe sugars have not been reported. Hence the dosage of probe sugars administered in tests requires to be standardized.

2.2.1.5.2. \textit{Effect of hyperosmolar stress}

Solutions above 1500 mosmol kg\textsuperscript{-1} have been reported to increase permeability of the intestinal mucosa in healthy subjects\textsuperscript{136}. The ingestion of hyperosmolar solutions have been shown to increase permeability of larger sugars such as lactulose, raffinose and dextrans (3000 Da)\textsuperscript{137}. Uil and co-workers\textsuperscript{121} showed that lactulose absorption, but not mannitol, increased following the administration of a hyperosmolar solution relative to that of an iso-osmolar solution. The magnitude of the effect is increased in the presence of osmotic fillers such as glycerol\textsuperscript{109}, \textsuperscript{127} and sodium chloride\textsuperscript{100}, \textsuperscript{113} which are used in preparation of some test solutions. As a consequence there is need for controlling the osmolarity of test solutions. It is also suggested that tests be conducted on fasted participants to standardize for any individual variation\textsuperscript{119} that may occur due to this effect.

2.2.1.5.3. \textit{Effect of fluid volume}

In a recent \textit{in vivo} study, in human subjects diuresis was shown to decrease the recovery of mannitol\textsuperscript{138}. Fluid loading and the related increase in diuresis has also been previously shown to affect the lactulose excretion\textsuperscript{139}. However there is debate as to whether fluid loading
affects the excretion of the sugars. Nonetheless it has been suggested that the intake of fluids be carefully monitored during the test and that the urine volume be accurately measured\textsuperscript{140}.

\subsection{Effect of timing of urine collection}

\textit{In vivo} permeability testing has been conducted over periods of time that vary from 2 - 24 hrs. Urine collections over 0 - 3, 3 - 5 and 5 - 24 hr broadly correspond with permeability in the proximal small intestine, distal small intestine and colon respectively\textsuperscript{118}. Akram and co-workers\textsuperscript{111} shortened the usual 5 - 6 hr collection period to 2 hr on the premise that lactulose and mannitol were each absorbed at the same rate along the length of the intestine. However it was recently shown that greater quantities of mannitol were absorbed over the 0 - 2 hr period whilst greater amounts of lactulose were absorbed in the 4 - 8 hr period\textsuperscript{118}. Hence varying proportions of each sugar were absorbed in the different segments of the gut\textsuperscript{120}. Around 14 \% of the administered 5 g dose (5 – 25 \%) of mannitol is absorbed over a 6 hr period. Conversely, less than 1 \% of the administered 5 g dose being absorbed of lactulose is absorbed\textsuperscript{141}. In humans, mean mannitol recoveries are reported to range from 16 \% - 20 \% whereas lactulose recoveries range from 0.2 \% - 0.8 \% of the ingested dose\textsuperscript{100} over a six hr period. These calculated ratios of the recovery of the probes would be changed/ altered were the timing of urine collection period to be shortened or lengthened.

\subsection{Gender differences}

Gastric emptying and hence the rate of on flow of digesta distally, through the GI tract, varies between subjects and with gender differences. The latter as women have a slower
rate of gastric emptying than men due to the effect of progesterone and estradiol, on the GI tract\textsuperscript{142}. However other studies\textsuperscript{118} reported no such gender differences whilst some studies have been inconclusive\textsuperscript{81, 82, 143}. It is possible that gender differences might alter the outcome of the tests\textsuperscript{82, 142}.

### 2.2.2. Carbon labelled breath tests

The method involves feeding subjects with a food or liquid with a substrate that contains a stable isotope, i.e. $^{13}\text{C}$, in place of the normally present $^{12}\text{C}$ atom. Following ingestion the labelled marker is emptied from the stomach into the duodenum where it is promptly metabolized. The $\text{CO}_2$, a by-product of the substrate metabolism, mixes with the body pool of $\text{CO}_2$ and $\text{HCO}_3^-$ and is subsequently exhaled as labelled $\text{CO}_2$. The rate at which the labelled carbon is excreted in breath reflects the rate at which the labelled food empties from the stomach. The ratio of the labelled $^{13}\text{CO}_2$ to normal $^{12}\text{CO}_2$ in expired air is determined by isotope mass spectrometry. At rest the amount of $\text{CO}_2$ produced is roughly constant per unit time and hence the total amount of $\text{CO}_2$ excreted per unit time is determined from a nommogramme based on body surface area\textsuperscript{144}.

This principle is used for $^{13}\text{C}$-octanoic acid to measure gastric emptying\textsuperscript{145}. Octanoic acid is a medium chain fatty acid that is rapidly absorbed in the duodenum and metabolized in the liver. For the test, one carbon atom in the molecule is labelled with $^{13}\text{C}$ isotope. Again in the urea breath test, urea is labelled with $^{13}\text{C}$ or $^{14}\text{C}$ and is orally administered and in the presence of \textit{Helicobacter pylori} in the stomach is hydrolysed by urease to produce labelled $\text{CO}_2$\textsuperscript{146} that diffuses into blood, is excreted by the lungs, and can be detected in breath. These tests
provide an indirect measure of gastric emptying and multiple testing is sometimes required with breath samples collected every 15 - 20 min over a 2 hr period into balloon like bags. There could also be potential interference from dietary factors when using $^{13}$C labelled compounds.

### 2.2.3. Paracetamol absorption test

The paracetamol absorption test, widely used as a marker for gastric emptying\textsuperscript{147-149}, has also been used in the assessment of small intestinal permeability\textsuperscript{150}. Orally administered paracetamol is poorly absorbed in the stomach and rapidly absorbed in the small intestine. Gastric emptying is a rate limiting step in the delivery of the drug to the small intestine and hence the rate of appearance of paracetamol in blood is reflective of the rate of gastric emptying\textsuperscript{151}. The test involves subjects ingesting a liquid drink containing the drug following which repeated blood samples are taken at regular intervals. Serum or plasma paracetamol concentrations from the samples are used to derive the rate of gastric emptying\textsuperscript{152}.

The diffusion of paracetamol across the small intestinal mucosal membrane occurs via the transcellular pathway\textsuperscript{153}. However, some amount of paracellular transport of the drug has also been reported\textsuperscript{154}. The paracetamol absorption test is easy to perform and is well tolerated by patients thereby avoiding stress-induced delay of gastric emptying\textsuperscript{155}. Although convenient to perform, the pharmacokinetics of paracetamol varies between and within individuals\textsuperscript{156-158} and repeated blood samples are required which limits the use of the test\textsuperscript{152}. 
2.2.4. **Cellular methods**

Several *in vitro* techniques have been used for assessing intestinal permeability and are favoured as they are less labour and cost effective in comparison to the *in vivo* methods. Each *in vitro* method has distinct advantages however are not representative of the *in vivo* physiological environment. Hence extrapolation of data to the *in vivo* situation might require additional data.

2.2.4.1. **Everted Gut Sac technique:**

The everted gut sac technique of the rat small intestine has been used as early as the 1950s to study the transport of sugars and amino acids from the mucosal to the serosal side\(^{159,160}\). More recently has been used to quantify the paracellular transport of hydrophilic molecules\(^{161}\). Mannitol flux (as a paracellular marker) showed an apparent permeability (P\(_{app}\)) of 1.5 x 10\(^{-5}\) to 1.7 x 10\(^{-5}\) cm s\(^{-1}\), a value similar to that reported with low molecular weight hydrophilic drugs in human perfusion studies\(^{162}\). Also, molecules that traverse the epithelial barrier via the transcellular route have been accurately quantified using the everted gut sac technique\(^{162}\). The oxygenated tissue culture media and specific preparation ensures that the tissue is viable for up to 2 hr and is suited to measure absorption at different sites across the intestine and into epithelial cells\(^{163}\).

2.2.4.2. **Thiry Vella loop**

The Thiry-Vella loop model has been used to study the physiology of epithelial transport, digestion and absorption and to determine regional differences in the absorption of
a molecule from the intestine or stomach. In the model, a loop of bowel with intact nerve and blood supply and lymphatic drainage is removed from continuity with the remainder of the intestine and the ends of the loop are brought to the skin surface of the abdomen by the creation of two stomas\textsuperscript{164, 165}. For jejunal loops the small bowel is divided at 10 and 30 cm distal to the ligament of Treitz, leaving the mesentry intact. The two ends of the 2 cm segment are then brought out the left side of the abdominal wall and sutured to the abdominal skin\textsuperscript{166}. For ileal studies a segment of distal ileum 10 – 30 cm proximal to the cecum is used\textsuperscript{166}. The perfusate is then introduced into the exteriorized loop and at regular time intervals the loop is emptied and the amount of substrate remaining in the solution determined\textsuperscript{167}.

### 2.2.4.3. Ussing Chamber:

The use of the Ussing Chamber to assess intestinal permeability is well recognized. The unique feature of this approach is that electrical resistance of the membrane can be measured during the course of the experiment, with the short circuit current and resistance across the membrane routinely used as indicators of intestinal tissue viability\textsuperscript{47, 168}. The technique allows the electrical resistance of the membrane to be measured from changes in current and corresponds to the integrity of the tissue. The short circuit current is used as an indicator of active ion transport taking place across the intestinal epithelium\textsuperscript{47}.

Basically, the chamber consists of two halves that are mounted together containing the tissue specimen with the apical side isolated from the basolateral side. The active ion transport produces a potential difference across the epithelium and the generated voltage difference is measured using two voltage electrodes that are placed as near as possible to the tissue. The spontaneous voltage is cancelled out by injecting a counter current using two other current
electrodes that are placed away from the epithelium. This current externally injected is called
the short-circuit current and is the exact measure of net ion transport taking place across the
epithelium\textsuperscript{169}.

The Ussing Chamber technique is adopted to study regional differences in the
absorption of molecules as the investigator is able to mount the tissue from specific regions of
the gut\textsuperscript{170}. A comparison of interspecies data is also therefore possible. However as with other
\textit{in vitro} techniques physiological conditions cannot be maintained, i.e. blood and nerve supply,
rapid loss of tissue viability, changes in morphology and functionality of transporter proteins
during the process of surgery and mounting\textsuperscript{168}. It is therefore critical that fresh and viable
resections from surgery are obtained and more importantly tissue viability is maintained during
the experiment in the chamber. Other recommendations to ensure better results include that
the excised segment be instantly rinsed to remove debris, solutions be well oxygenated,
nutrients be added to the buffer, and low temperatures be maintained during preparation\textsuperscript{171, 172}.
Additionally careful handling of the excised segment is required so that the tissue is not
stretched during preparation to maintain the tip to crypt axis structure\textsuperscript{172}.

\textbf{2.2.4.4. Cell based models:}

A variety of cell monolayer models that mimic the \textit{in vivo} intestinal epithelium in
humans have been developed. However unlike enterocytes, tumor cells grow rapidly into
confluent monolayers and differentiate to provide an ideal system for transport studies\textsuperscript{168}. Cell
monolayers are grown on filter support in a multiple well format and permeability is measured
by the movement of molecules from one side of the cell monolayer to the other. The
adenocarcinoma cell lines HT-29 and Caco2 are most commonly used as they display a number of properties characteristic of undifferentiated intestinal cells.

Caco2 cells are considered by some to be a gold standard technique\textsuperscript{173}. The cells are derived from colorectal adenocarcinomas differentiate spontaneously into polarized intestinal cells possessing an apical brush border and TJ between adjacent cells and express hydrolases and transporters\textsuperscript{174}. Caco2 despite their colonic origin, express the majority of morphological and functional characteristics of small intestinal absorptive epithelial cells, including phase I and phase II enzymes, detected either by measurement of their activities toward specific substrates, or by immunological techniques\textsuperscript{175}.

Whilst these cultures are used to study the rate of absorption through the absorptive epithelial cells the major criticism is that the model does not assess transport across the mucus layer, the lamina propria and/or the muscularis mucosa. Additionally the size of pores in the monolayer, estimated to be $\sim 3.7 \pm 0.1 \text{ Å}$, is smaller than that in the mucosa of the human small intestine and hence express much 'tighter' TJs\textsuperscript{176}. Accordingly the epithelial resistance across the cell is much greater at about 234 ohms cm$^2$ whilst that in the typical small intestine is estimated to be in the range of 25 - 40 ohms cm$^2$ \textsuperscript{176}. Differences in culture conditions and composition of cell sub populations of Caco2 cells, derived from different laboratories, result in varying epithelial resistance values\textsuperscript{177}. These differences have been thought to affect the permeation of molecules through the 'larger' pores in the monolayer, i.e. the paracellular route\textsuperscript{174, 176}. This has been demonstrated using mannitol as a paracellular marker whose permeability can vary as much as a 100 fold depending on the source of Caco2 cells\textsuperscript{178}. 

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2.2.5. *In vitro* gut models

In comparison to the *in vitro* techniques, the *in vitro* gut models have been shown to be advantageous as they appear to be more physiologically relevant due to an intact blood and nerve supply in experimental animals and humans. The basic principle of perfusion experiments is that absorption is calculated from rate of disappearance of the probe from the perfused segment. Calculation of the effective intestinal permeability ($P_{eff}$) is dependent upon the hydrodynamics within the perfused segment, which in turn is determined by the perfusion technique, rate of perfusion and the motility of the intestine. The intestinal $P_{eff}$ is a direct measurement of the local absorption rate in humans and reflects the transport velocity across the epithelial barrier, expressed in centimetres per second.

In the triple lumen tube method, the perfusate and the GI fluids are mixed in a mixing segment, and at a distal end of the mixing segment from where the sample is taken is referred to as the inlet concentration at the test segment. A second outlet sample is taken at the end of the test segment, which is usually 20 – 30 cm distal to the mixing segment to calculate the rate of absorption. A major disadvantage of this method is that the composition of the perfusate changes as it can flow in either direction along both the mixing and the test segment. This makes it difficult to define the conditions of absorption and therefore to determine reference permeability at well-defined luminal conditions.

A multilumen tube overcomes the problem of proximal contamination due to the use of an occluding balloon proximal to the test segment. There is also a separate tube that terminates aboral to the balloon, for continuous drainage to prevent proximal leakage into the test segment. This method decreases proximal leakage and therefore the luminal composition is kept at equilibrium and permeability can be determined under defined conditions. However,
both these methods have the disadvantage that they have low recoveries of non-absorbable volume markers, usually PEG 4000, and they usually use high perfusion flow rates between 5 and 20 ml min\(^{-1}\). These flow rates are significantly higher than physiological flow rates of about 1 - 3 ml min\(^{-1}\).

A major concern regarding all perfusion techniques is that they perturb physiology, especially motility patterns. The choice of anaesthesia administered to the animals might interfere with absorption of the marker\(^{188}\). It is noteworthy that the disappearance of the markers from the perfusate may not always equate with absorption and hence it is suggested that samples from the portal and hepatic vein be additional taken to acquire information on first pass metabolism in the liver. An additional drawback is that the methodology is expensive and requires repeated measures to obtain statistically significant absorption data.

### 2.2.6. Conclusion

Of the various methods outlined in this section of the review none are suitable for general screening in ambulant human subjects. The following section of the review outlines the development of the dual sugar absorption test that can be used as a dynamic measure of health in the gut.
2.3. **Aspirin, a pro-inflammatory mediator that influences mucosal integrity; implications for the sugar absorption test**

Acetylsalicylic acid (aspirin) is a synthetic derivative of methyl salicylate produced from willow bark. Aspirin was initially marketed by Bayer Pharmaceuticals (1897) as an analgesic, antipyretic and anti-inflammatory agent. Since then it has also been found useful as an anti-thrombotic and vasodilator. The action of the drug is associated with decreased prostaglandin levels by inhibition of the enzyme endoperoxidase synthase (PGHS) commonly known as cyclooxygenase (COX).

2.3.1. **Pharmacology, metabolism and absorption of aspirin in the GI tract**

Aspirin has the molecular formula C₉H₈O₄ and molecular weight of 180.2 Da. The pharmacology of aspirin is unique and is characterized by the action of the reactive acetyl moiety and the salicylic acid (salicylate) residue (Figure 2-3).

Both these components are biologically active and act at different sites with equimolar ratios after release by the action of esterases that occurs primarily in the liver or by spontaneous hydrolytic cleavage of aspirin that occurs at a lower pH such as in the acidic environment of the stomach.
The circulating half-life of aspirin ranges from 5 to 16 min\textsuperscript{189}, the fall in concentration being associated with an equal rise in the concentration of salicylic acid. Salicylic acid has a longer half-life, 2 - 3 hr\textsuperscript{190}. It is metabolized primarily in the liver\textsuperscript{191} and is excreted by the kidneys either unchanged, or as salicyluric acid, salicyl phenolic glucuronide, salicyl acyl glucuronide and gentisic acid\textsuperscript{192}. The amount of free salicylate excreted is extremely variable ranging from 10 % to 85 % in human urine, depending largely on urinary pH\textsuperscript{193}. Acidic urine is reported to facilitate the reabsorption of salicylate by renal tubules, while alkaline urine promotes the excretion of the drug\textsuperscript{193}.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{aspirin_equilibrium.pdf}
\caption{pH-dependent equilibrium of ionized and non-ionized forms of aspirin}
\end{figure}

\textbf{2.3.1.1. Specific variation in the absorption of aspirin in different segments of the GI tract}

Both aspirin and salicylic acid are absorbed in the stomach and the small intestine, the rate of absorption being dependent upon the luminal pH. The un-ionized form is preferentially absorbed in the stomach whilst the ionized form is predominantly absorbed in the small intestine\textsuperscript{194, 195}.
2.3.1.1.1. **Stomach**

The main factors that determine gastric absorption are the rate of dissolution of the drug, the intra gastric pH and the rate at which it empties from the stomach. The hydrolysis of the drug commences in the gastric lumen. It has been reported that the low rate of hydrolysis in the gut wall allows approximately 68% of a 650 mg dose to reach the systemic circulation as aspirin.

The acidic pH (1.5 - 2.5) in the stomach has been shown to inhibit ionization of aspirin as it protonates the carboxyl group of the molecule rendering it neutral in charge. This makes the molecule more lipophilic and facilitates partitioning across the gastric mucosa, the rate of absorption being proportional to the concentration of the un-ionized drug. Cooke and Hunt demonstrated that in normal human subjects significant gastric absorption of aspirin occurred at pH 2.1, but when the drug was given as a solution buffered to pH 6 - 7, gastric absorption was effectively prevented. The buffering of aspirin or co-dosage with antacids accelerated transit through the stomach whilst the use of proton pump inhibitors or atropine delayed gastric emptying. Decrease in residence time reduced contact of the drug with the stomach and conferred a protective effect on the mucosa to result in fewer gastric side-effects.

2.3.1.1.2. **Small intestine**

Aspirin is absorbed mainly via passive diffusion in the upper small intestine. The progressive distal movement of the drug from the stomach into the relatively higher pH environment of the proximal small intestine, results in the de-protonation of aspirin. However unionized aspirin continues to be passively absorbed into the portal vein to be metabolized by the liver. In the jejunum where the pH is around 5.5, 10% is in the neutral
form \(^{202}\). Hence a significant quantity of aspirin is absorbed. Once in the enterocyte the ionized form cannot readily diffuse into the lumen \(^{201}\) owing to the lower pH of the cytosol.

It is important to note that carrier mediated transport of aspirin has also been described \(^{203}\). This was originally described by Fisher \(^{204}\) and subsequent work indicated the involvement of the monocarboxylic acid transport (MCT) system \(^{203}, 205, 206\). The MCT transporters are Na\(^+\) independent and H\(^+\) dependent and active transport is likely via MCT-1 \(^{207}, 208\). However, the relative contribution of active transport to the overall absorption of aspirin is unknown. The MCT-1 transporter has been identified in the stomach mucosa of rats and in the colonic mucosa of humans and pigs \(^{209}\). Expression of MCT-1 related proteins are also expressed in the rat and rabbit intestinal epithelium and in the Caco2 cells \(^{210}\). However there is a lack of human \textit{in vivo} studies confirming the presence of these transporters in the small intestine and their quantitative contribution to the overall absorption of aspirin.

### 2.3.2. Chemistry of aspirin and salicylic acid with biomolecules

Aspirin and salicylic acid have pro-inflammatory effects on the GI mucosa and alter the integrity of the GI mucosa and its function. The drug, like most NSAIDs, increases the permeability of the small intestinal epithelium \(^{211}, 214\), an effect that may contribute to the pathogenesis of a number of inflammatory bowel disorders \(^{213}\). Work by Smeucol \(^{215}\) and Maiden \(^{31}\) hypothesized that aspirin initiates GI injury firstly via its topical action on the mucosa however the precise sequence of events are less defined.
2.3.2.1. Inhibition of cyclooxygenase

Aspirin via the inhibition of COX has been shown to deplete prostaglandin synthesis, to increase gastric acid secretion, decrease mucosal blood flow, decrease secretion of mucus and duodenal bicarbonate production. A series of experiments conducted by Vane demonstrated that prostaglandin (PG) F\textsubscript{2\alpha} was inhibited by aspirin, indomethacin or sodium salicylate in a dose dependent manner\textsuperscript{216}. Again aspirin decreased PG levels and reduced platelet aggregation induced by thrombin\textsuperscript{217}. Further aspirin and indomethacin blocked the release of prostaglandins from perfused, isolated dog spleen\textsuperscript{218}. These experiments along with evidence that PGE\textsubscript{1} was a pyretic agent\textsuperscript{219} and that PGE\textsubscript{2} mimicked the inflammatory response\textsuperscript{220} led to speculation that PG might be involved in the genesis of fever and inflammation and that aspirin and other NSAIDs exert their therapeutic activity by preventing its biosynthesis.

COX-1 or prostaglandin endoperoxidase synthase was isolated in 1976. This enzyme converts arachidonic acid to prostaglandin H\textsubscript{2}, the precursor to a series of prostanoids\textsuperscript{221} (Figure 2-4). In 1991, a second COX encoded by a different gene was discovered. COX-2 is an inducible enzyme that is expressed in response to inflammatory stimuli released from bacteria such as lipopolysaccharides (LPS), cytokines released from macrophages like interleukin-1, mitogens and growth factors. COX-1 and COX-2 are of similar molecular weight, having 65 % amino acid sequence homology and near-identical catalytic sites\textsuperscript{221}. COX-1 contains two active sites: a heme with peroxidase activity, responsible for the reduction of PGG\textsubscript{2} to PGH\textsubscript{2}; and a cyclooxygenase site, where arachidonic acid is converted to hydroperoxyendoperoxide PGG\textsubscript{2}\textsuperscript{222}. The active site contains a long hydrophobic channel and most of the aspirin like drugs inhibit COX by excluding arachidonic acid from the upper portion of the channel\textsuperscript{223}. Residues of
Tyrosine (Tyr) and serine (Ser) are situated at the apex of the long active site. The acetyl moiety of aspirin inactivates COX through the acetylation of the serine residue. The resultant covalent bond causes the irreversible inhibition of COX.

Figure 2-4: Decreased PG synthesis due to aspirin induced COX inhibition. The prostaglandin precursor arachidonic acid that is normally cleaved from the cell membrane by phospholipase A2, is not able to be converted to unstable endoperoxidases ProstaglandinG2 and ProstaglandinH2. (Adapted from Awtry et al., 2000)
The most significant difference between the COX-1 and COX-2 isoenzymes which allows for selective inhibition is the substitution of isoleucine at position 523 in COX-1 with valine in COX-2. This difference makes the active site of COX-2 slightly larger than that of COX-1. Drug molecules such as the 'coxibs' have been shown to bind to this alternative site and are considered to be selective inhibitors of COX-2 (Figure 2-5). Aspirin acetylates serine-516 in COX-2 but due to the larger catalytic channel, arachidonic acid forms 15-R-hydroxyeicosatetraenoic acid (15-(R)-HETE) which is then converted by 5-lipoxygenases to the anti-inflammatory lipoxins. Hence whilst the acetylation by aspirin completely blocks the enzyme catalyzed oxygen uptake by COX-1, it does not do so for COX-2 and only alters the chemistry of the product so that 15-(R) –HETE is generated. The anti-inflammatory lipoxin also called as aspirin triggered lipoxin (ATL) has a protective effect on the gastric mucosa (Figure 2-5).

Studies have proposed that aspirin induced upper GI mucosal damage is a result of the systemic inhibition of COX-1 rather than inhibition of its isoform COX-2. However this hypothesis has been disapproved in COX-1 knockout mice which despite the lack of the COX-1 enzyme still showed GI injury, suggesting that COX-1 inhibition was not the only cause of the GI damage. The reason for this became clear when rats were treated with a combination of a selective COX-1 and a selective COX-2 inhibitors. Ketorolac, a preferential COX-1 inhibitor, was shown to cause gastric damage only when given in doses at which significant COX-2 inhibition occurred. Likewise celecoxib a COX-2 inhibitor produced significant injury only when both drugs were given in combination rather than when each of them was administered alone. Hence it seems likely that the inhibition of both COX isoforms is required for the development of GI damage.
Figure 2-5: Role of prostaglandins in the stomach and the effect of aspirin and selective cyclooxygenase 2 (COX-2) inhibitors. (a) In the absence of aspirin COX-1 and COX-2 confer gastro protection with each of the COX isoforms regulating different aspects of mucosal defence. Selective inhibition of either COX isoform does not, in itself, result in significant gastric damage. (b) Administration of aspirin inhibits both COX-1 and COX-2 to suppress prostaglandin synthesis to impair mucosal defence. In the process of COX-2 inhibition aspirin triggers the release of lipoxin (ATL) which partially counteracts the detrimental effects of prostaglandin suppression. (c) Selective inhibition of COX-2 activity by COX-2 inhibitors does not allow the formation of ATL to increase the gastric damage. (Adapted from Wallace et al., 2003)\textsuperscript{222}
The hypothesis that aspirin induced GI mucosal injury is principally from mucosal COX inhibition has been recently re-examined. Scientific evidence from both laboratory and clinical studies suggest the link is weak\textsuperscript{237-239}. Hence Whittle\textsuperscript{240} showed that small intestinal mucosal lesions developed only 48 hrs after the administration of indomethacin, by which time COX activity, which was fully inhibited less than 3 hr post dosage, had returned to normal. Again aspirin orally administered to healthy human subjects at doses of 10, 81 and 325 mg for 10 days caused GI mucosal damage that was not related to the levels of mucosal PG\textsuperscript{241}. This led the authors to conclude that decreased mucosal PG levels were pre-requisite but not exclusive for GI mucosal injury. However studies of aspirin induced GI erosions, ulcerations, and bleeding\textsuperscript{242-245} that were associated with decreased mucosal PG concentrations and/or COX activity, were either partially or completely reversed following treatment with exogenous prostaglandin analogues like misoprostol\textsuperscript{246, 247}.

Lichtenberger and colleagues\textsuperscript{248} proposed that aspirin caused GI damage by a non-COX mediated effect. The fact that aspirin, when administered systemically at comparable doses to that after oral administration, impaired the gastric barrier function to a lesser extent\textsuperscript{249, 250} than that after direct contact with the mucosa\textsuperscript{128, 249, 251} further supports their findings. Again, after oral administration of aspirin for a month the human gastric mucosa showed resistance to the injurious effects of the drug, this adaptive response was not linked to the recovery of COX activity\textsuperscript{252}.

Finally, the COX molecule is an integral membrane protein having a three dimensional structure\textsuperscript{223}. The conformation of the membrane-binding motive suggests that the enzyme integrates into only a single leaflet of the lipid bilayer\textsuperscript{231, 253}. Hence it seems likely that aspirin
could alter the biophysical properties of the lipid membrane to change the conformation of embedded proteins, like COX, to initiate the process of GI injury\textsuperscript{254}.

\subsection*{2.3.2.2. Interaction with mucus and membrane phospholipids}

Sigthorsson and co-workers showed in a rodent model that a topical effect in conjunction with the COX-I and COX-2 effects increased small bowel damage\textsuperscript{255}. The initial topical effect involved the interaction of aspirin and salicylic acid with phospholipids in the mucus layer, in the stomach and cellular membrane to alter their biophysical properties.

\subsubsection*{2.3.2.2.1. Mucus phospholipids}

The phospholipids are secreted by mucus cells form extracellular layers, or monolayers, that coat the luminal interface of the mucus gel layer or the plasma membrane itself\textsuperscript{256, 257}. It has been hypothesized that the phospholipids contribute to the barrier property of the GI tract by providing a resistance the movement of protons\textsuperscript{258, 259}.

Studies have revealed that after an initial hydrophobic intermolecular interaction, the free carboxyl group of the NSAID forms a strong electrostatic bond with the positively charged head group of zwitterionic phospholipids, such as dipalmitoyl-phosphatidylcholine (DPPC), to increase the solubility of the phospholipid and neutralize its surface activity\textsuperscript{260, 261}. This has been shown with aspirin and non-aspirin NSAIDs, such as indomethacin and naproxen\textsuperscript{261, 262}. Further work in a series of rodent experiments demonstrated that phosphatidylycholine (PC), the most abundant and surface-active of the gastric phospholipids, could protect rats from a number of ulcerogenic agents and/or
conditions including NSAIDs\textsuperscript{261, 263}. Work by Lichtenberger and co-workers extended this observation and demonstrated that associating NSAIDs with PC prior to administration prevented the drug from chemically associating with phospholipids to reduce interaction with the gel layer so as to maintain the hydrophobic barrier properties in the stomach\textsuperscript{260}. Subsequent studies have confirmed that the gastric toxicity of aspirin-PC and other PC-associated NSAIDs was markedly lower than the unmodified drug in rodent ulcer models\textsuperscript{261, 264}. Furthermore, unlike the COX-2 selective inhibitors, ulcer healing appeared to be accelerated significantly (compared with the effects of the unmodified NSAID) when rats with acetic acid-induced gastric ulcers were treated daily with aspirin-PC\textsuperscript{265}. A recently published clinical trial using a randomized cross-over design has provided evidence that aspirin-PC is significantly less toxic to the human gastric mucosa than unmodified aspirin over a 4-day study period\textsuperscript{266}, Further that the gastro protective activity did not alter the COX-inhibitory activity of the drug\textsuperscript{267}.

2.3.2.2.2. Membrane phospholipids

Exposure to aspirin has also been shown to induce a rapid and dose-dependent decrease in the surface hydrophobicity of the canine gastric mucosa mounted in Ussing chambers\textsuperscript{256, 257}. A similar effect was observed with other NSAIDs under both \textit{in vitro} and \textit{in vivo} conditions\textsuperscript{261, 262, 268}. The gastric and intestinal mucosa has also been shown to possess unusually high concentrations of surface active phospholipid DPPC\textsuperscript{269}. Work by Lichtenberger and colleagues using various biophysical techniques provided confirmatory evidence to support the distribution of the NSAID in PC bilayers\textsuperscript{270, 271}. Using Fourier transform infrared spectroscopy (FTIR) they identified that NSAIDs associates with PC in the membrane by electrostatic and hydrophobic interactions that are dependent upon the luminal pH. Salicylate inserted deep into
the bilayer undergoes hydrophobic interactions with the aliphatic fatty acid side chains of the phospholipid groups within the membrane. Using molecular dynamic simulations (MD), these workers further determined that anionic NSAIDs partition most favourably into the head group of the phospholipid bilayer by electrostatic interactions with the positively-charged quaternary ammonium component of choline. These non-covalent interactions were further characterized by biophysical techniques that characterize and identify chemical bonding between molecules such as Surface Plasmon Resonance and FTIR studies.

The incorporation salicylate into phospholipids, disturbed the lipid-lipid interactions to alter the packing shape of the lipid molecule within the membrane. The resultant increase in the lipid packing area, allowed the lipid acyl chains to move freely, thereby increasing the fluidity of the membrane. The increased fluidity consequently decreased the ability of the membrane to resist changes that resulted from forces that stretched or compressed the membrane. The decreased stiffness equated to a lower energy required to cause a deform in the membrane and increased the susceptibility of the membrane to form pores. In vitro work has shown that salicylate reduced the ability of the membrane to resist changes in a dose dependent manner, with 1 mM solute of salicylate decreasing membrane 'bending stiffness' by 40 %. Aspirin influenced the formation of pores in the membrane via its interaction with polar head groups of the membrane phospholipids in the ionized form and penetrated even deeper into the membrane in the unionized form. Additionally the incorporation of salicylate into the membrane further stabilized pores due to the decrease in energy required to 'bend' the lipids located around the pores. Changes in the lipid composition and bilayer thickness have also been shown to alter the function of embedded integral membrane proteins due to the conformational disturbance in the proteins' membrane-spanning domain. Hence it is likely that aspirin could influence the
lipid-protein interactions within the membrane to change protein conformation of the embedded proteins\textsuperscript{278, 280}.

### 2.3.2.3. Interaction with the mitochondrial membrane

The ability of aspirin to interact the epithelial membrane phospholipids is shown to be proportional to the rate of entry of the drug into the enterocytes\textsuperscript{281}. Aspirin during the formation of salicylate has been shown to increase mitochondrial membrane proton conductance by competing with protons to reduce ATP production\textsuperscript{282, 283} in the inner mitochondrial membrane\textsuperscript{284}. The H\textsuperscript{+} ions are necessary to uncouple the energy flow from the electron transport to the formation of adenosine tri phosphate (ATP). This has been shown in both \textit{in vitro}\textsuperscript{285} and \textit{in vivo}\textsuperscript{74, 286, 287} studies. In isolated mitochondria, the mitochondrial membrane proton conductance was shown to be increased more than fourfold at 1 mM salicylate with complete uncoupling exhibited between 2 and 4 mM salicylate\textsuperscript{159}. Studies in rodent models have revealed that the enterocyte mitochondrial damage could occur within an hr of treatment\textsuperscript{126}.

The reduction in cellular ATP production inhibits steps in cellular metabolic pathways such as the glycolytic pathway and the tricarboxylic acid cycle\textsuperscript{288}. Reduced ATP production also results in the loss of intercellular integrity since the intercellular TJ are under the control of ATP-dependent acto-myosin complexes\textsuperscript{74}. Decreased ATP production also causes the efflux of calcium out of the mitochondria into the cytosol to generate reactive oxygen species\textsuperscript{289} and stimulate calcium dependent enzymes that trigger apoptosis\textsuperscript{51}. Hence the mitochondrial damage induced by aspirin depletes intracellular energy and increases free radicals, both of which are known to disrupt the intercellular TJ\textsuperscript{37} to increase mucosal permeability\textsuperscript{289}.
2.3.3. Aspirin induced increase in intestinal permeability

A number of recent reports using the dual sugar absorption test\textsuperscript{114, 215, 290, 291} have indicated that concurrent dosage with aspirin increases permeability. Further, that dosage with aspirin may unmask latent inflammatory bowel diseases such as Crohn’s disease\textsuperscript{290} and augment changes in permeability from chronic alcohol ingestion\textsuperscript{114} presumably allowing them to be more readily detected in a clinical scenario. Such a method could allow clinicians to have greater understanding to identify relapse or of clinical improvement\textsuperscript{93}.

Hence, the aspirin provocation dual sugar absorption test could provide better discrimination by increasing the differences in groups\textsuperscript{292} and has led to its use in assessing permeability changes associated with Coeliac’s disease\textsuperscript{115}, Crohn’s disease\textsuperscript{93, 293}, ulcerative colitis\textsuperscript{294}, alcoholic liver disease\textsuperscript{114, 295}. Again, 10\% to 20\% of healthy relatives of patients with Crohn’s disease have been shown to have an abnormal increase in intestinal permeability\textsuperscript{93} which is markedly accentuated by pre-administering aspirin\textsuperscript{292}.

Most of these works on intestinal permeability have used different doses of aspirin (100 - 1300 mg) and different periods of urine collection (4 - 24 hrs)\textsuperscript{290, 292, 296, 297}. These report that the aspirin induced increase in permeability results in the increased absorption of the larger sugar probe and attribute it to the inhibition of COX\textsuperscript{254, 298-300}. Given the longer duration of sample collection that is used in current studies it has not been possible to separate the immediate from the long term effects of aspirin on permeability. The fact that aspirin can passively diffuse into the plasma membrane and interact with phospholipids could partly explain the prompt surface damage to the intestinal mucosa, independent of COX inhibition. Hence it is likely that the physical/topical effects of aspirin could alter the physical characteristics of the membrane to increase permeability.
2.3.4. Conclusion

The pharmacological effects of aspirin are well researched. Aspirin induced injury due to the GI tract is not entirely dependent on inhibition of mucosal COX activity, although a combination of COX-1 and COX-2 is known to induce enterocyte dysfunction.

Although aspirin has been previously used in tests of intestinal permeability a modification of the currently used study protocols could further our understanding of the short term effects of the drug on intestinal permeability. The response and recovery of the mucosa to a transient aspirin challenge could provide a measure of resilience and thus 'health' in the gut.


2.4. References


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Chapter 3

Development and calibration of an analytical method to quantify excreted urinary sugar probes

Various analytical methods have been used to assess intestinal permeability by quantifying probe molecules, such as lactulose and mannitol, in urine samples. This chapter describes the development and calibration of a convenient, robust, sensitive and reproducible method that detected trace quantities of urinary probe sugars that are commonly used to conduct the sugar absorption test. Following several methodological modifications to optimize the sensitivity and reproducibility, a final method was developed using high-performance liquid chromatography (HPLC) with refractive index detection.
3.1. Introduction

The dual sugar absorption test utilizing lactulose and mannitol has been successfully applied to the assessment of changes in intestinal permeability that accompany celiac disease\textsuperscript{1-3}, Crohn's disease\textsuperscript{4, 5}, and food allergies\textsuperscript{6-8}. Lactulose and mannitol are ideal probes as they are water soluble, non-charged, non-toxic molecules that are quantitatively cleared by the kidneys into urine\textsuperscript{9} within six hours of administration and are readily quantifiable\textsuperscript{10}.

Previous studies have employed diverse methodologies to quantify and determine urinary lactulose and mannitol concentrations. These include enzymatic assays\textsuperscript{11, 12}, colorimetric assays\textsuperscript{13} and thin layer chromatography (TLC)\textsuperscript{14}. Enzymatic and colorimetric assays do not allow simultaneous quantification of both sugars in urine samples and hence the two probes need to be quantified by separate assays making the process labour intensive. For example, studies by Benjamin\textsuperscript{15} and Dastych\textsuperscript{16} measured lactulose and mannitol in urine samples, each by spectrophotometry using two different assays. Lactulose was measured based on the hydrolysis of the sugar to fructose and galactose. Fructose was further converted to gluconate-6-phosphate and NADPH by three enzymes: hexokinase, glucose phosphate isomerase and 6-phosphate dehydrogenase. The NADPH produced in direct proportion to the lactulose concentration was measured at 340 nm. Mannitol was estimated by the conversion of the sugar to fructose and NADH by mannitol dehydrogenase. The NADH formed in proportion to the mannitol concentration was measured at 340 nm. An alternative method to estimate mannitol was based on the measurement of formaldehyde produced during the oxidation of periodic acid that was added to urine\textsuperscript{15}.

TLC overcomes the problem of using separate methods for measuring the sugars\textsuperscript{14}, however the process of TLC is technically demanding\textsuperscript{17}. For example, for the estimation of
rhamnose, sugar separation is achieved by multiple developments on half plates of F1500 plastic backed silica gel using butan-1-ol/ethyl acetate/pyridine/acetic acid/water. The layers need to be dried for half-hour between each run and then for an additional 4 hrs to remove pyridine prior to performing a 4-aminobenzoic/phosphoric acid colour reaction.

Accordingly, gas-liquid chromatography and high-performance liquid chromatography (HPLC) have been widely used. However gas chromatography is more labour intensive as it requires prior derivitization of urine samples. HPLC has high precision and sensitivity for quantifying probe sugars and is less labour intensive. A survey of the various studies showed that a range of detectors and column types were used (Table 3-1). Moreover, various sample preparation techniques have been employed even in studies using the same method of instrument for analysis. For example, in the studies by Parviainen et al. and Welcker et al., HPLC with pulsed amphometric detection was used however the probe sugar were eluted using the same column and flow rate but with different mobile phases. Again, sample preparation differed between both studies.

Mass spectrometers, pulsed amphometric detectors, light scattering detectors, and refractive index detectors have been frequently used to quantify the sugars in the mobile phase following injection in the HPLC system. Whilst mass spectrometry (MS) is highly sensitive and a reliable method, the detector is expensive and therefore not suitable for routine analysis. Lactulose and mannitol as they lack chromophore and fluorphore groups and hence the use of ultraviolet detection (UV) with photodiode array (PDA or DAD) often require additional equipment such as MS and extensive sample preparation. Pulsed amphomteric detection on the other hand, based on the redox potential of the sugar, is sensitive to quantify the sugars. Whilst it is easy to perform and economical it requires
additional mobile phases and a complex gradient elution\textsuperscript{39}. Refractive index detector is convenient and commonly used\textsuperscript{29} with an isocratic elution. The sensitivity of the detector however might be susceptible to baseline drifts caused by temperature and pressure fluctuations\textsuperscript{29}. Due to which it has been suggested that the HPLC system be positioned away from vents and that the detector be allowed to warm up for at least two hrs before use\textsuperscript{29}.

Depending on the eluent and the chosen detector system, a range of column types such as reverse phase (C18 and NH\textsubscript{2}), ion exchange (carbohydrate and monosaccharide H\textsuperscript{+}) and cation exchange (Aminex HPXC) columns were commonly applied (Table 3-1). Resin based columns, like the Aminex HPXC are beneficial as the resin modifies itself to optimize chromatographic conditions to be compatible with the compound structure. Hence, it allows the use of an isocratic HPLC system and simplifies the sample preparation method and hence prior derivatization of samples can be avoided.

The development of the methodology in this chapter is based on the use of an Aminex HPX87C cation exchange column (Biorad Laboratories) with refractive index detection as performed by Trehan \textit{et al.}\textsuperscript{37}, Akram \textit{et al.}\textsuperscript{25} and Shulman \textit{et al.}\textsuperscript{42}. We used a HPLC system - Shimadzu (Japan) equipped with Degasser Model DGU-20AS (Japan), Pump Model LC-20AT Prominence, Autosampler Model SIL-20AC and Column Oven CTO-20A. The bonded resin (packing material) of the column however retains components of the sample, which subsequently decreases its efficiency and selectivity. Therefore, to maintain and prolong the shelf-life of the the Aminex HPX87C 250 x 4.0-mm cation-exchange column, a micro guard pre-column (Bio-Rad Laboratories, Richmond, CA) was used and the column effluent was monitored with a refractive index detector (Shimadzu RID-10A).
Table 3-1: Summary of studies using HPLC

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method of analysis</th>
<th>Sample preparation</th>
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<tbody>
<tr>
<td>Camilleri et al., 2010&lt;sup&gt;34&lt;/sup&gt;</td>
<td>HPLC with mass spectrometer. Lactulose and mannitol were separated using NH&lt;sub&gt;2&lt;/sub&gt; 250 x 4.6 mm&lt;sup&gt;2&lt;/sup&gt; column (Phenomenex, USA).</td>
<td>50 µl of &lt;sup&gt;14&lt;/sup&gt;C-&lt;sub&gt;6&lt;/sub&gt;-Glucose (internal standard) was added to 50 µl of urine sample. Samples were diluted 1:40 with 2 ml water and treated with 4 ml dichloromethane and incubated for 30 min at room temperature. They were then vortex mixed for 1 min prior to centrifugation for 10 min at 3500 rpm. A final dilution of 1:400 was made by transferring 100 µl of supernatant to a test tube containing 1 ml of 85:15 acetonitrile-water and vortex mixed for 20 sec. LC mobile phase consisted of acetonitrile and water spiked with 0.1 % formic acid and sonicated to degas. Injection volume 50 µl.</td>
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<tr>
<td>Lostia et al., 2008&lt;sup&gt;31&lt;/sup&gt;</td>
<td>HPLC with mass spectrometer. Lactulose and mannitol were separated using NH&lt;sub&gt;2&lt;/sub&gt; column (Phenomenex, USA)</td>
<td>Urine samples were stirred for 1 min using a vortex mixer and centrifuged at 4500 g for 4 min. Each sample was then diluted 1:5 with acetonitrile. 50 µl aliquot of each sample was then transferred into a tube containing 450 µl internal standard. 200 µl was then transferred to a glass vial for the autosampler.</td>
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<tr>
<td>Nathavitharana et al., 1988&lt;sup&gt;40&lt;/sup&gt;</td>
<td>HPLC with a mass spectrometer Lactulose and mannitol were eluted using a Spherisorb-5 amino column (Phase Separations Ltd) or 250 x 4 mm LiChrosorb amino column (E Merck).</td>
<td>Urine samples were centrifuged and treated with Amberlite MB-1 in the acetate form. 2 µmol creatinine was added to the volume of the desalted sample and was further purified by preparative thin layer chromatography on foil backed plates. Areas of the plates corresponding to lactulose and mannitol standards were excised and eluted using acetonitrile:water and the eluent dried under vacuum. The residue was dissolved in 200 µl acetonitrile:water just before HPLC. Elution was carried out at 1.5 ml min&lt;sup&gt;-1&lt;/sup&gt; with acetonitrile:water.</td>
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<td>Trehan et al., 2005&lt;sup&gt;37&lt;/sup&gt;</td>
<td>HPLC with refractive index detector. Sucrose, lactulose and mannitol were estimated using an Aminex HPX87C 300 x 7.8 mm cation-exchange column with a Carbo C cartridge (Biorad Laboratories)</td>
<td>Urine samples were spun at 3000 rpm for 5 min and then filtered. 20 µl of sample was injected into the HPLC and eluted with degassed pure water at a flow rate of 0.6 ml min&lt;sup&gt;-1&lt;/sup&gt; at 85°C.</td>
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<tr>
<td>Ref.</td>
<td>Methodology</td>
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<tr>
<td>Vilela et al., 2008&lt;sup&gt;23&lt;/sup&gt;</td>
<td>HPLC with refractive index detector. Lactulose and mannitol were estimated using Rezek RH M monosaccharide H&lt;sup&gt;+&lt;/sup&gt; (8 %)&lt;sup&gt;19&lt;/sup&gt; column with a Supelcogel 33H&lt;sup&gt;®&lt;/sup&gt; pre-column (USA). Urine was filtered and passed through ion exchange resin following which 50 µl was injected into the HPLC. MilliQ water was used as the mobile phase at a flow rate of 0.6 ml min&lt;sup&gt;-1&lt;/sup&gt;.</td>
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<td>Akram et al., 1998&lt;sup&gt;25&lt;/sup&gt;</td>
<td>HPLC with refractive index detector. Lactulose and mannitol were estimated using Aminex HPX 87 C 300 x 7.8mm cation exchange column (Bio-Rad, Richmond, CA, USA) 20 µl of xylose (internal standard) was mixed with 1 ml of urine and centrifuged at 3000 rpm for 10 min and filtered. A 20 µl aliquot of the sample was injected into the HPLC system. The sugars were eluted with degassed water as the mobile phase at a flow rate of 0.6 ml min&lt;sup&gt;-1&lt;/sup&gt;.</td>
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<td>Miki et al., 1996&lt;sup&gt;22&lt;/sup&gt;</td>
<td>HPLC with refractive index detector. Lactulose, mannitol and rhamnose were estimated using Kromasil NH&lt;sub&gt;2&lt;/sub&gt; 250 x 4.6 mm column (Alltech) 2 ml urine was mixed with 0.5 g washed mixed ion exchange resin (Duolite). The mixture was vortex mixed for 10 sec and centrifuged for 10 min at 3000 g. The supernate was filtered and injected into the HPLC. Acetonitrile was used as a mobile phase at a flow rate of 1 ml min&lt;sup&gt;-1&lt;/sup&gt;.</td>
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<td>Parviainen et al., 2005&lt;sup&gt;36&lt;/sup&gt;</td>
<td>HPLC with pulsed amphometric detector. Lactulose and rhamnose were estimated using a Carbotoc PA100 column (Dionex, UK) Urine samples were diluted 50-250 fold in purified water containing 100 mg l&lt;sup&gt;-1&lt;/sup&gt; preservative. 25 µl of diluted urine was injected into the HPLC. Elution was carried out using NaOH during two isocratic periods separated by a steep gradient at a flow rate of 1 ml min&lt;sup&gt;-1&lt;/sup&gt;.</td>
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<td>Welcker et al., 2004&lt;sup&gt;24&lt;/sup&gt;</td>
<td>HPLC with pulsed amphometric detector. Lactulose, mannitol, rhamnose and cellobiose were eluted using an anion exchange column (Carbonpack PA1, Dionex) Protein was removed from urine samples with 0.5 ml periodic acid. The samples were then vortexed for 10 sec and centrifuged for 10 min at 2500 rpm. The supernatant was treated with Amberlite MB-3 resin in the acetate form, mixed for 10 min and centrifuged. An isocratic elution was carried out with 0.1 mol l&lt;sup&gt;-1&lt;/sup&gt; NaOH.</td>
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<td>Anderson et al., 2004&lt;sup&gt;41&lt;/sup&gt;</td>
<td>HPLC using pulsed amphoteric detector for lactulose and rhamnose and refractive index detector for sucrose. Separation was performed on a C18 column (Phenomenex, CA, USA). 100 µl phenyl-β-D-glucopyranoside (internal standard) was added to 10 ml of urine and filtered. 100 µl was injected into the HPLC. An isocratic mobile phase of 30 % methanol in water was used at a flow rate of 1 ml min&lt;sup&gt;-1&lt;/sup&gt;.</td>
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Fleming *et al.*, 1990

HPLC with pulsed amphometric detector. Lactulose and mannitol were estimated using 250 x 40 mm Dionex HPIC-AS6 anion exchange column (Dionex, UK)

Depending on collection volume, urine samples were diluted between 2.5 and 20 fold with de-ionized water. 1 ml of diluted urine was mixed with 1 ml of internal saccharide standards (arabinose 250 mg l\(^{-1}\) and cellobiose 25 mg l\(^{-1}\) in deionized water) and desalted with a mixture of washed 0.5 g ion exchange resin (Amberlite IR120 H and IRA400 CI in mass proportion of 1:1.5). The sample was then vortex mixed and centrifuged and the supernate filtered. 50 µl was injected into the HPLC. The sugars were eluted using 0.15 mol l\(^{-1}\) NaOH at a flow rate of 1 ml min\(^{-1}\) at 20°C.

Marsilio *et al.*, 1998

HPLC (Waters Associate) with light scattering detector. Lactulose, mannitol and cellobiose were estimated using 700 CH Carbohydrate analysis column with a Benson Carbohydrate BC-100 Ca\(^{2+}\) guard column (Alttech Associates)

C16 600 mg cartridges were conditioned with 5 ml methanol followed by 5 ml water. 2-3 ml urine was passed through the cartridge and the first ml of urine discarded. The residual volume was collected and diluted 1:1 with water. 200 µl of sample was diluted with 1.8 ml of water containing 75 ml l\(^{-1}\) of cellobiose and 400 g l\(^{-1}\) Amberlite IRA-400 resin. The mixture was vortex mixed for 10 sec and centrifuged for 2 min at 3000 g. 400 µl aliquot of the supernatant was centrifuged for 5 min at 3000 g. 50 µl aliquots were then analyzed by HPLC. Water was used as the mobile phase at a flow rate of 0.5 ml min\(^{-1}\) and column temperature maintained at 85 °C.
3.2. Methodological development

3.2.1. Reagents and Calibrators

Lactulose (L7877-25G), mannitol (M4125-500G), cellobiose (C7252-100G) and rhamnose (R3875-100G) were purchased from Sigma Aldrich, USA. Amberlite resin IR120 H and IRA410 Cl were obtained from Fluka, Sigma Aldrich, USA. 0.2 μm (pore size) cellulose acetate filters were supplied by Advantec, Toyo Roshi Kaisha Ltd, Japan. 1.5 ml HPLC vials were obtained from Fisher Scientific, UK. Sodium hydroxide was obtained from Sigma Aldrich and glacial acetic acid from Merck, Germany.

3.2.2. Sample preparation

Despite the use of a similar HPLC systems and detectors, the protocol for sample preparation varied in studies (Table 3-1). In most studies urine sample preparation involved the use of amberlite resin alone or with different proportions of buffers such as sodium hydroxide33, 43, 44 and glacial acetic acid45. Amberlite resin has been used previously to desalt urine samples, however the relative proportions and forms used varied between studies with either the chloride form, hydrogen form or both types of resin30, 32, 33, 46 being used. Hence the methodological development involved a series of experiments that were conducted to determine whether resin alone, or mixed with either sodium hydroxide or glacial acetic acid would be beneficial to treat urine samples. Urine samples were obtained from a colleague at the department following an overnight fast. The samples were obtained every morning over four days and frozen at -80 °C. Before the experiments the urines were thawed and bulked together.
In each experimental set samples were run in triplicate. An isocratic elution was carried out using water as the mobile phase at 84 °C (with the micro guard pre-column placed outside the column oven) at a flow rate of 0.3 ml min⁻¹. A 20 μl aliquot of the supernatant was then injected into the HPLC system. Each of the various sets of experiments described below was run concurrently with a 1 mg ml⁻¹ standard of lactulose and mannitol each. The position of the peaks in the standard solutions was compared to those in the treated samples using the data comparison mode of the LC solutions software (Shimadzu, Japan). In doing so, the effect of the treatment in removing or decreasing any interfering peaks at or near similar retention times as the sugars was assessed.

3.2.2.1. Addition of Amberlite resin to urine

The use of two forms, i.e. the chloride and hydrogen form, in mass proportion of 1:1 in three different amounts was investigated.

Procedure: 2 ml urine was diluted with 2 ml MilliQ water and treated with either 250 mg resin (125 mg Amberlite IR120 H and 125 mg of Amberlite IRA410 Cl), 500 mg resin (250 mg Amberlite IR120 H and 250 mg of Amberlite IRA410 Cl) or 1 g of resin (500 mg Amberlite IR120 H and 500 mg of Amberlite IRA410 Cl). Each sample was then vortex mixed for 15 sec and filtered through a 0.2 μm cellulose acetate filter into 1.5 ml HPLC vials.

Based on the chromatographic conditions, treatment of samples with 1 g resin was beneficial as it reduced the proximity of the peak from unknown substances in urine samples that was eluted near the retention time to that of the lactulose peak from the standard solution (Figure 3-1).
Figure 3-1: Sample treatment with Amberlite resin. Although only results from a single set of urine samples are shown, all samples were run in triplicate. The chromatogram, in the ‘post run comparison mode’ of the LC solutions software, depicts one set of urine samples treated with 250 mg resin (blue), 500 mg resin (brown) and 1000 mg resin (green) in mass proportion of 1:1 (Amberlite IR120 H: Amberlite IRA410 Cl) that was used in order to improve the resolution of the lactulose (L) peak indicated by the arrow.

The timing of peaks in urine (run in triplicate) was compared with that of lactulose (L-black) and mannitol (M-pink) standards run concurrently. The isocratic elution was carried out at 84 °C with the micro guard pre column placed outside the column oven at a flow rate of 0.3 ml min⁻¹.
3.2.2.2. Addition of a mixture of resin and sodium hydroxide to urine

25 μl sodium hydroxide\textsuperscript{33, 43, 44} was chosen to treat the urine samples along with 1 g of resin. The proportion of resin was chosen as it was shown to be the most effective in desalting samples as described in 3.2.2.1.

Procedure: 25 μl sodium hydroxide\textsuperscript{44} was added to 2.5 ml urine sample and 1 ml of the supernatant was diluted with 1 ml of water and treated with 1 g of Amberlite resin (500 mg Amberlite IR120 H and 500 mg of Amberlite IRA410 Cl). An undiluted urine sample was also run. This was prepared by mixing 2.5 ml of urine with 25 μl sodium hydroxide. The supernatant was filtered through 0.2 μm (pore size) cellulose acetate filter (\textit{Advantec, Dismic-13, Toyo Roshi Kaisha Ltd, Japan}). 1 ml of the supernatant was then treated with 500 mg Amberlite resin. A ‘blank’ or control sample containing 1 ml urine diluted with 1 ml of MilliQ water mixed with 1 g Amberlite resin (500 mg Amberlite IR120 H and 500 mg of Amberlite IRA410 Cl) was also run. Following treatment with the resin, all the samples were vortex mixed for 15 sec and filtered through a 0.2 μm cellulose acetate filter into 1.5 ml HPLC vials.

Based on the chromatograms obtained, it was concluded that diluting samples with water and treatment with sodium hydroxide and resin was beneficial in decreasing the proximity of the peak near the retention time to that of the lactulose peak from the standard solution in comparison to the undiluted sample (Figure 3-2). This reduction in the proximity of the peak was similar to that obtained following the ‘blank’ or control sample that was treated with 1 g resin only (Figure 3-2). Hence it was concluded that the additional step of buffering samples with sodium hydroxide was unnecessary.
Figure 3-2: Samples treated with Amberlite resin and buffered with sodium hydroxide. Although only results from a single set of urine samples are shown, all samples were run in triplicate. The chromatogram, in the ‘post run comparison mode’ of the LC solutions software, depicts one set of diluted (brown) and undiluted (green) urine samples treated with 25 μl sodium hydroxide and 1 g resin (Amberlite IR120 H: Amberlite IRA410 Cl) that was used in order to improve the resolution of the lactulose (L) peak indicated by the arrow. The additional benefit of the buffer was assessed by comparing them to the sample treated with only 1 g resin (blue).

The timing of peaks in urine (run in triplicate) was compared with that of lactulose (L-black) and mannitol (M-pink) standards run concurrently. The isocratic elution was carried out at 84°C with the micro guard pre-column placed outside the column oven at a flow rate of 0.3 ml min⁻¹. 
3.2.2.3. Addition of a mixture of resin and glacial acetic acid to urine

The addition of glacial acetic acid\textsuperscript{45} was also used to treat samples as a means of assessing if it could improve/remove the peak with a retention time close to that of lactulose. As conducted in section 3.2.2.2, urine samples were treated with a mixture of glacial acetic acid and 1 g Amberlite resin.

Procedure: 25 µl of glacial acetic acid was added to 500 µl urine and diluted with 500 µl MilliQ water and the treated with 1 g of Amberlite resin (500 mg Amberlite IR120 H and 500 mg of Amberlite IRA410 Cl). The supernatant was then filtered through 0.2 µm (pore size) cellulose acetate filter (Advantec, Dismic-13, Toyo Roshi Kaisha Ltd, Japan). An undiluted sample of urine was also run. This was prepared by adding 25 µl of glacial acetic acid to 1 ml of urine. The filtered supernatant was then treated with 1 g of Amberlite resin. The samples were run concurrently with a ‘blank’ or control sample containing 1 ml urine diluted with 1 ml of MilliQ water mixed with 1 g Amberlite resin. Following treatment with the resin, all the samples were vortex mixed for 15 sec and filtered through a 0.2 µm cellulose acetate filter into 1.5 ml HPLC vials.

Based on the chromatographic conditions, it was again concluded that diluting samples with an equal volume of water was beneficial decreasing the peak from unknown substances in the urine sample (Figure 3-3). However, treating samples with additional glacial acetic acid did not further reduce the proximity of the peak immediately before the lactulose peak in comparison to the ‘blank’ or control sample treated with resin only (Figure 3-3). This indicated that buffering samples with the acid would not have any added benefit to improve peak separation near the retention time of lactulose.
Figure 3-3: Samples treated with Amberlite resin and buffered with glacial acetic acid. Although only results from a single set of urine samples are shown, all samples were run in triplicate. The chromatogram, in the ‘post run comparison mode’ of the LC solutions software, depicts one set of diluted (brown) and undiluted (green) urine samples treated with 25 μl glacial acetic acid and 1 g resin (Amberlite IR120 H: Amberlite IRA410 Cl) that was used in order to improve the resolution of the lactulose (L) peak indicated by the arrow. The additional benefit of the buffer was assessed by comparing them to the sample treated with only 1 g resin (blue).

The timing of peaks in urine (run in triplicate) was compared with that of lactulose (L-black) and mannitol (M-pink) standards run concurrently. The isocratic elution was carried out at 84°C with the micro guard pre-column placed outside the column oven at a flow rate of 0.3 ml min⁻¹.
Based on these experiments the final method for preparation of urine sample was as follows: 1 ml of urine sample diluted with 1 ml of MilliQ and desalted with 1 g Amberlite resin (Amberlite IRA-410, Supelco Analytical and Amberlite IR120 hydrogen form, Fluka Sigma Aldrich in mass proportion of 1:1). The mixture was then vortex mixed for 15 sec and the resulting supernate filtered through 0.2 μm cellulose acetate disposable filter (Advantec, Dismic-13, Toyo Roshi Kaisha Ltd, Japan) into 1.5 ml HPLC vials (Fisherbrand). A 20 μl aliquot of the supernatant was injected into the HPLC system.

3.2.3. Optimization of column oven temperatures

The effect of column oven temperatures on peak separation and resolution was investigated over a range of temperatures (70 – 84 °C). The reason for doing these experiments were because the maximal temperature of the Aminex HPX87C column was 84 °C, which is higher than the recommended temperature of the micro guard pre-column (60 °C). Hence preliminary experiments were conducted to determine the optimal temperature at which both the column and micro guard pre-column could be heated.

Procedure: 1 ml of urine was each spiked with 1 ml of the lactulose standard or 1 ml of the mannitol standard. The samples were then treated with 1 g Amberlite resin (500 mg Amberlite IR120 H and 500 mg of Amberlite IRA410 Cl); vortex mixed for 15 seconds and filtered through a 0.2 μm (pore size) cellulose acetate filter into 1.5 ml HPLC vials. 20 μl aliquots of the supernatant were then injected into the HPLC system. Standard containing 1 mg ml⁻¹ lactulose and mannitol each were run concurrently with the samples. The samples and standards were injected in triplicate at a flow rate of 0.3 ml min⁻¹ using water as a mobile phase at
The following temperatures: (a) 60 °C with the micro guard pre-column placed inside the column oven, (b) 60 °C with the micro guard pre-column placed outside the column oven, (c) 70 °C with the micro guard pre-column placed outside the column oven, (d) 75 °C with the micro guard pre-column placed outside the column oven and (e) 84 °C with the micro guard pre-column placed outside the column oven.

It was concluded, on the basis of the chromatographic separation, when the column oven was heated to 60 °C with the micro guard pre-column placed inside the column oven (Figure 3-4) both lactulose and mannitol, in the urine samples, were each eluted at similar retention times as those in the standards. Heating the column and the pre-column at this temperature ensured better separation of the lactulose peak from a rider peak that was eluted in close proximity to the sugar.

Figure 3-4: Optimization of column oven temperature. The chromatogram, in the 'post run comparison mode' of the LC solutions software, depicts results from a single set of urines spiked with 1 mg ml⁻¹ of lactulose and mannitol standards that were treated with resin and eluted isocratically with the micro guard pre-column placed inside the column oven at 60 °C (a) and with the micro guard pre-column placed outside the column oven at 60 °C (b), 70 °C (c), 75 °C (d) and 84 °C (e). The peak separation and resolution was compared with lactulose (L) and mannitol (M) standards that were run under the same conditions concurrently with the samples.
3.3. Chromatographic conditions

Based on the set column oven temperature, the flow rate and run time for the separation of the probe sugars were determined in two parts based on planned experimental human studies. The first part was done to achieve chromatographic separation of lactulose and mannitol only, as we were to use the dual sugar absorption test in a pilot study. The second part was conducted to achieve chromatographic separation of lactulose, mannitol, cellobiose and rhamnose. These four sugars were to be used as a multiple sugar solution, administered to participants in a second human study. All samples were run in triplicate.

3.3.1. Chromatographic separation

(i) Lactulose and mannitol

Chromatographic separation was achieved at a flow rate of 0.3 ml min$^{-1}$. The analysis was performed at 60 °C, 858 psi with a run time of 12 min for the standards and 25 min for the samples (Figure 3-5a).

The retention times of lactulose and mannitol were 5.7 and 10.1 min respectively. No major interference was seen at the retention times corresponding to the peaks of the sugars. A small peak of constant area under curve (AUC) was eluted before mannitol was resolved (Figure 3-5b). The comparison and constancy of the peak with that of a ‘diluent’ or water sample run concurrently confirmed that the peak occurred due to the presence of some unknown substance in the diluent. However, the retention time of this peak was sufficiently different from that of mannitol (9.08 min) to allow mannitol to be well separated.
Figure 3-5: Chromatographic separation of lactulose and mannitol in standards. Although only results from a single set of urine samples are shown, all samples were run in triplicate. The chromatogram depict the (a) resolution and peak separation of standard solutions containing 250 $\mu$g ml$^{-1}$ lactulose and mannitol and (b) data comparison of a range of different concentrations of standard solutions containing 500 $\mu$g ml$^{-1}$ (black), 125 $\mu$g ml$^{-1}$ (pink), 31.3 $\mu$g ml$^{-1}$ (blue) and 3.9 $\mu$g ml$^{-1}$ (brown) lactulose and mannitol; with a consistent residual peak before mannitol from the diluent (water) shown as an asterisk. Lactulose (L) and mannitol (M) peaks were resolved at 5.7 min and 10.1 min respectively.
The chromatograms of the urine samples spiked with standard solutions containing lactulose and mannitol were well resolved within 25 min (Figure 3-6a) and gave similar peak retentions and resolutions as those of the standards. Spiked samples were corrected with a 'blank' un-spiked urine sample (Figure 3-6b) using the LC solutions software, in the post run mode. The area under curve of each sugar in each urine sample was then determined and compared these with those of a series of standards run concurrently.
Figure 3-6: Chromatographic separation of lactulose and mannitol in samples. Although only results from a single set of urine samples are shown, all samples were run in triplicate. The chromatogram depicts the (a) resolution and peak separation of urine spiked with standard solution containing 250 μg ml⁻¹ lactulose and mannitol (corrected with the 'blank' un-spiked urine sample) and (b) data comparison of urines spiked with a range of different concentrations of standard solutions containing 500 μg ml⁻¹ (blue), 250 μg ml⁻¹ (brown), 125 μg ml⁻¹ (green) and 62.5 μg ml⁻¹ (black) lactulose and mannitol; after correction with the 'blank' un-spiked urine sample. Lactulose (L) and mannitol (M) peaks were resolved at 5.8 min and 10.2 min respectively.
(ii) Lactulose, mannitol, cellobiose and rhamnose

At a flow rate of 0.1 ml min\(^{-1}\), using water as the mobile phase, there was well defined chromatographic separation of lactulose, mannitol, cellobiose and rhamnose. The analysis was performed at 60 °C, 288 psi with a run time of 35 min for the standards and 60 min for the samples.

Lactulose, mannitol, rhamnose and cellobiose were eluted at 14.89, 17.28, 20.48 and 30.87 min respectively (Figure 3-7a). Similar retention times were observed for each of the sugars in urine samples spiked with the known standard solutions (Figure 3-7b). No major interference was seen at the retention times corresponding to the peaks of the sugars. Again, a small peak (27 min) of unknown material from the water (diluent) was observed with a retention time close to that of mannitol. However, it did not interfere with the separation of the mannitol peak.
Figure 3-7: Chromatographic separation of lactulose, mannitol, cellobiose and rhamnose. Although only results from a single set of urine samples are shown, all samples were run in triplicate. The chromatogram depicts the (a) the resolution and peak separation of standard solutions containing 250 μg ml⁻¹ cellobiose, lactulose, rhamnose and mannitol and (b) a urine sample spiked with standard solution, containing cellobiose, lactulose, rhamnose and mannitol, having a final concentration of 250 μg ml⁻¹, and corrected with the baseline urine sample showing similar retention times as the standards. A consistent residual peak at 27 min from unknown substance in the diluent did not interfere with the elution of mannitol. All four sugars were well resolved within 35 min.
3.4. Calibration of the method

3.4.1. Calibration curves with reference solutions of probe sugars

(i) Lactulose and mannitol

Standard stock solutions (1 mg ml\(^{-1}\)) was prepared using 100 mg lactulose (Sigma Aldrich L7877-25G) and 100 mg mannitol (Sigma Aldrich M4125-500G), dissolved and made up to 100 ml with MilliQ water at room temperature.

A series of dilutions from 500 \(\mu\)g ml\(^{-1}\) to 3.9 \(\mu\)g ml\(^{-1}\) was prepared from the above stock solution. The detector response for lactulose and mannitol was checked with the various concentrations of standard test solutions (500 \(\mu\)g ml\(^{-1}\), 250 \(\mu\)g ml\(^{-1}\), 125 \(\mu\)g ml\(^{-1}\), 62.5 \(\mu\)g ml\(^{-1}\), 31.3 \(\mu\)g ml\(^{-1}\), 15.6 \(\mu\)g ml\(^{-1}\), 7.8 \(\mu\)g ml\(^{-1}\) and 3.9 \(\mu\)g ml\(^{-1}\) respectively) run \textit{in triplicate}. 20 \(\mu\)l of each standard was injected into the HPLC system.

The calibration curves for lactulose (Figure 3-8a) and mannitol (Figure 3-8b) were linear up to 500 \(\mu\)g ml\(^{-1}\) for both sugars and gave the following regression equations: lactulose, \(y = 563.88 \times\) and mannitol, \(y = 545.92 \times\) (expressed as the formula \(y = a + b \times\) where \(a = 0\) as the line intercepts axes at 0). The Limit of Detection (LOD), i.e. the lowest quantity of analyte that can be detected by the assay, was up to 4 \(\mu\)g ml\(^{-1}\) for both lactulose and mannitol in the standard respectively.
(ii) Lactulose, mannitol, cellobiose and rhamnose

Standard stock solution was prepared using analytical grade lactulose, mannitol, rhamnose and cellobiose. 1.25 g lactulose, 1.25 g mannitol, 1.25 g cellobiose and 1.25 g rhamnose was dissolved and made up to 250 ml using MilliQ water at room temperature to prepare a 5 mg ml\(^{-1}\) stock solution. One part of the stock was diluted to four parts of MilliQ water to prepare a 1 mg ml\(^{-1}\) stock solution.
A series dilution was prepared (500 μg ml\(^{-1}\) to 3.9 μg ml\(^{-1}\)) using the 1 mg ml\(^{-1}\) stock solution to assess the sensitivity of the method. The detector response for lactulose, mannitol, rhamnose and cellobiose in HPLC was checked using known concentrations of standard test solutions (500 μg ml\(^{-1}\), 250 μg ml\(^{-1}\), 125 μg ml\(^{-1}\), 62.5 μg ml\(^{-1}\), 31.3 μg ml\(^{-1}\), 15.6 μg ml\(^{-1}\), 7.8 μg ml\(^{-1}\) and 3.9 μg ml\(^{-1}\) of each sugar respectively) run *in triplicate*. 20 μl of each standard was injected into the HPLC system.

The calibration curves for lactulose, mannitol, cellobiose and rhamnose were each linear up to 500 μg ml\(^{-1}\) and gave the following regression equations: lactulose, $y = 1690.6x - 1823.7$; mannitol, $y = 1670.4x - 2230.3$; cellobiose, $y = 1658.8x - 1213.1$ and rhamnose, $y = 1479.2x + 3543.9$ respectively (Figure 3-9) with an $R^2 = 1$ for all sugars. The LOD for each of the four sugars in the standard was 4 μg ml\(^{-1}\) respectively.

*Figure 3-9: Calibration curves for lactulose (a), mannitol (b), cellobiose (c) and rhamnose (d) standard. The corresponding concentration of each sugar in each standard was 500 μg ml\(^{-1}\), 250 μg ml\(^{-1}\), 125 μg ml\(^{-1}\), 62.5 μg ml\(^{-1}\), 31.3 μg ml\(^{-1}\), 15.6 μg ml\(^{-1}\), 7.8 μg ml\(^{-1}\) and 3.9 μg ml\(^{-1}\) respectively.*
3.4.2. Calibration with spiked urine samples

3.4.2.1. Preparation of Urine Samples:

1 ml urine sample was spiked with 1 ml of each of the standards to obtain final concentrations of 500 $\mu$g ml$^{-1}$, 250 $\mu$g ml$^{-1}$, 125 $\mu$g ml$^{-1}$, 62.5 $\mu$g ml$^{-1}$ and 31.3 $\mu$g ml$^{-1}$ respectively (Table 3-2). All urine samples were treated with 500 mg Amberlite IR120 H and 500 mg of Amberlite IRA410 Cl and vortex mixed for 15 sec. The supernatant was filtered through 0.2 $\mu$m (pore size) cellulose acetate filter (Advantec, Dismic-13, Toyo Roshi Kaisha Ltd, Japan) into 1.5 ml HPLC vials (Fisher brand, Fisher Scientific, UK). 20 $\mu$l aliquot of the supernatant was then injected into the HPLC system.

Table 3-2: Preparation of urine spiked samples for calibration curves

<table>
<thead>
<tr>
<th>Initial Std conc ((\mu)g ml$^{-1}$)</th>
<th>1000</th>
<th>500</th>
<th>250</th>
<th>125</th>
<th>62.5</th>
<th>313.3</th>
<th>15.6</th>
<th>7.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std (ml)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Urine (ml)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Final Conc ((\mu)g ml$^{-1}$)</td>
<td>500.0</td>
<td>250.0</td>
<td>125.0</td>
<td>62.5.0</td>
<td>31.3</td>
<td>15.6</td>
<td>7.8</td>
<td>3.9</td>
</tr>
</tbody>
</table>

3.4.2.2. Linearity of the assay

The dilutional linearity (also termed as the dilutional parallelism or dilutional recovery) was used to validate the specificity and accuracy of the method. This was done with urine samples spiked with standards containing lactulose and mannitol to obtain concentration of 250 $\mu$g ml$^{-1}$, 190 $\mu$g ml$^{-1}$ and 125 $\mu$g ml$^{-1}$ and diluted in 1:2, 1:4 and 1:8 proportion with MilliQ water. Each dilution was run in triplicate and results expressed as observed to expected (O/E) ratios of recovery of each sugar.
(i) Lactulose and mannitol

The mean ratio of the observed to the expected recovery for lactulose and mannitol for the set of spiked urine samples injected in triplicate ranged between 90 % and 101 % across all dilutions suggested sufficient linearity of the assay (Table 3-3).

Table 3-3: Ratio of the observed/expected recovery of the two sugars using 3 spiked urine samples

<table>
<thead>
<tr>
<th>0/E Ratios (%)</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lac</td>
<td>Man</td>
<td>Lac</td>
</tr>
<tr>
<td>Mean(^a)</td>
<td>100.6±6.8</td>
<td>97.7±1.7</td>
<td>93.9±4.2</td>
</tr>
<tr>
<td>SE</td>
<td>0.87</td>
<td>0.44</td>
<td>0.686</td>
</tr>
<tr>
<td>CV(%)</td>
<td>6.8</td>
<td>1.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Min</td>
<td>90</td>
<td>95</td>
<td>91</td>
</tr>
<tr>
<td>Max</td>
<td>113</td>
<td>100</td>
<td>105</td>
</tr>
</tbody>
</table>

\(\text{a: Data in this row are presented as mean ± standard deviation of the O/E ratio in each of the dilutions as a composite for both the sugars in the three different urine samples (total data points 27, 9 data points per dilution). Urines were spiked with standards having concentrations of 250 µg ml\(^{-1}\), 125 µg ml\(^{-1}\) and 15.6 µg ml\(^{-1}\) were used neat and in 1:2, 1:4 and 1:8 dilutions. Min and Max value of percentage recoveries.\)

3.4.2.3. Accuracy of the assay

The accuracy of the assay expresses the degree of closeness between the reference value, i.e. the concentration in the standard, to that obtained from the found value, i.e. in the spiked sample. This was determined by evaluating the recovery of 3 different concentrations of urine samples spiked with standard solutions and results expressed as observed to expected (O/E) ratios.
(i) Lactulose and mannitol

Three urine samples having final concentrations of 250 μg ml⁻¹, 62.5 μg ml⁻¹ and 15.6 μg ml⁻¹ respectively were injected in ten separate injections each within the same run.

The mean recoveries of the sugars in the three different spiked samples ranged between 96 % and 110 % for lactulose and 102 % and 110 % for mannitol. Based on the recoveries of the sugars, the LOD for lactulose and mannitol in the spiked samples was 15 μg ml⁻¹ respectively (Table 3-4).

Table 3-4: Recovery of the two sugars from 3 different spiked urine samples

<table>
<thead>
<tr>
<th>O/E ratios (%)</th>
<th>Sugar addeda</th>
<th>Sample 1</th>
<th></th>
<th>Sample 2</th>
<th></th>
<th>Sample 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lac</td>
<td>Man</td>
<td></td>
<td>Lac</td>
<td>Man</td>
<td></td>
</tr>
<tr>
<td>Meanb</td>
<td></td>
<td>108.9±3.2</td>
<td>110.3±2.8</td>
<td>95.76±1.98</td>
<td>101.84±4.39</td>
<td>103.2±5.3</td>
<td>102.5±10.5</td>
</tr>
<tr>
<td>Min</td>
<td>105</td>
<td>106</td>
<td>93</td>
<td>97</td>
<td>95</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>113</td>
<td>116</td>
<td>99</td>
<td>111</td>
<td>113</td>
<td>130</td>
<td></td>
</tr>
</tbody>
</table>

a: lactulose + mannitol; b: Data expressed as mean± standard deviation of the O/E ratio for both the sugars in the three different urine samples (total data points 30, 10 data points each dilution). Urines were spiked with standards having concentrations, Sample 1 = 250 μg ml⁻¹, Sample 2 = 125 μg ml⁻¹, Sample 3 = 15.6 μg ml⁻¹. Min and max value of percentage recoveries.

(ii) Lactulose, mannitol, cellobiose and rhamnose

Three urine samples having final concentrations of 250 μg ml⁻¹, 125 μg ml⁻¹ and 62.5 μg ml⁻¹ were injected in four separate injections each within the same run.
The recoveries of lactulose ranged between 93 % and 109 %, mannitol between 96 % and 101 %, rhamnose between 94 % and 114 % and cellobiose between 101 % and 132 % respectively in the three concentrations (Table 3-5). Based on the recoveries of the sugars the LOD for all four sugars in the samples was 62.5 μg ml⁻¹.

**Table 3-5: Recovery of the two sugars from 3 different spiked urine samples**

<table>
<thead>
<tr>
<th>O/E ratios (%)</th>
<th>Sugar added*</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lac</td>
<td>Man</td>
<td>Cello</td>
<td>Rham</td>
</tr>
<tr>
<td>Mean¹</td>
<td>265.6±6.1</td>
<td>243.3±2.6</td>
<td>263.3±9.9</td>
<td>241.9±4.6</td>
</tr>
<tr>
<td>Min</td>
<td>104</td>
<td>96</td>
<td>101</td>
<td>94</td>
</tr>
<tr>
<td>Max</td>
<td>109</td>
<td>98</td>
<td>111</td>
<td>99</td>
</tr>
</tbody>
</table>

—a = lactulose + mannitol + cellobiose + rhamnose; b = data expressed as mean ± standard deviation of the concentration of the sugar. Urines spiked with standards having final concentrations, Sample 1 = 250 μg ml⁻¹, Sample 2 = 125 μg ml⁻¹ and Sample 3 = 62.5 μg ml⁻¹ respectively. Min and Max value of percentage recoveries.

3.4.2.4. Precision of the assay

The precision of the assay expresses the closeness of a series of individual measurements when the assay is applied repeatedly to multiple aliquots of a single homogenous volume of the sample. This was determined by evaluating the intra-assay variability by measuring 3 different concentrations of spiked urine samples injected within the
same run. The mean, standard deviation, coefficients of variation and standard errors for each sugar were calculated.

(i) Lactulose and mannitol

Three urine samples having final concentrations of 500 μg ml\(^{-1}\), 125 μg ml\(^{-1}\) and 31.25 μg ml\(^{-1}\) were injected 10 times within the same assay run.

The precisions of the assay for lactulose and mannitol examined as coefficients of variation for intra-assay variability in the three concentrations were between 2.0 % and 5.1 % for lactulose and 2.5 % and 4.4 % for mannitol (Table 3-6)

Table 3-6: Intra-assay variability of the concentration of the 2 sugars in 3 urine samples each repeated 10 times within the same run

<table>
<thead>
<tr>
<th>Urine Spike</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar</td>
<td>Mean(^a) (μg ml(^{-1}))</td>
<td>CV(^b) (%)</td>
<td>Mean(^a) (μg ml(^{-1}))</td>
</tr>
<tr>
<td>Lactulose</td>
<td>544.4±15.87</td>
<td>2.9</td>
<td>119.72±2.48</td>
</tr>
<tr>
<td>Mannitol</td>
<td>551.7±13.9</td>
<td>2.5</td>
<td>127.31±5.48</td>
</tr>
</tbody>
</table>

\(a\): mean ± standard deviation of the concentration of sugar in that urine sample from the 10 measurements within the same run; \(b\): the coefficients of variation for each sugar (CV = std dev/mean). Urines were spiked with standards having concentrations, Sample 1 = 500 μg ml\(^{-1}\), Sample 2 = 125 μg ml\(^{-1}\), Sample 3 = 31.3 μg ml\(^{-1}\)
(ii) **Lactulose, mannitol, cellobiose and rhamnose**

Three urine samples having final concentration of 250 μg ml⁻¹, 125 μg ml⁻¹ and 62.5 μg ml⁻¹ were injected 10 times within the same assay run.

The precisions of the assay for lactulose, mannitol, cellobiose and rhamnose examined as coefficients of variation for intra-assay variability in the three concentrations was 2.7 % for lactulose, between 2 % and 5.8 % for mannitol, between 3.3 % and 5.7 % for cellobiose and between 2.3 % and 3.6 % for rhamnose (Table 3-7).

**Table 3-7: Intra-assay variability of the concentration of the 4 sugars in 3 different spiked urine samples each repeated 10 times within the same run**

<table>
<thead>
<tr>
<th>O/E ratios (%)</th>
<th>Sugar added²</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lac</td>
<td>Man</td>
<td>Cello</td>
<td>Rham</td>
</tr>
<tr>
<td><strong>Mean</strong>³</td>
<td>255</td>
<td>235.8</td>
<td>283.9</td>
<td>238.5</td>
</tr>
<tr>
<td>±7.0</td>
<td>±4.8</td>
<td>±14.7</td>
<td>±6.2</td>
<td>±3.2</td>
</tr>
<tr>
<td><strong>CV%</strong></td>
<td>2.7</td>
<td>2.0</td>
<td>5.2</td>
<td>2.6</td>
</tr>
</tbody>
</table>

³ a = lactulose + mannitol + cellobiose + rhamnose; b = data expressed as mean ± standard deviation of the concentration of the sugar. Urines spiked with standards having final concentrations, Sample 1 = 250 μg ml⁻¹, Sample 2 = 125 μg ml⁻¹ and Sample 3 = 62.5 μg ml⁻¹ respectively
3.4.2.5. Reproducibility of the assay

The reproducibility of the assay or the repeatability of the assay was determined by evaluating inter-assay variability by measuring 3 concentrations of spiked urine samples injected in consecutive assay runs. The mean, standard deviation, coefficients of variation and standard errors for each sugar were calculated.

(i) Lactulose and mannitol

Urine samples having final concentration of 250 μg ml⁻¹, 125 μg ml⁻¹ and 31.25 μg ml⁻¹ were injected in 10 consecutive assay runs.

The reproducibility of the assay for lactulose and mannitol determined as the inter-assay coefficients of variation was between 2.0 % and 5.1 % for lactulose and 2.8 % and 3.9 % for mannitol in the three concentrations respectively (Table 3-8).

Table 3-8: Inter-assay variability of the concentration of 2 sugars in 3 urine samples each repeated 10 times in consecutive assays

<table>
<thead>
<tr>
<th>Urine Spike Sugar</th>
<th>Sample 1 Mean感官 (μg ml⁻¹)</th>
<th>CV感官 (%)</th>
<th>Sample 2 Mean感官 (μg ml⁻¹)</th>
<th>CV感官 (%)</th>
<th>Sample 3 Mean感官 (μg ml⁻¹)</th>
<th>CV感官 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactulose</td>
<td>251.0±5.0</td>
<td>2.0</td>
<td>126.4±4.0</td>
<td>3.1</td>
<td>31.6±1.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Mannitol</td>
<td>245.8±6.8</td>
<td>2.8</td>
<td>121.4±4.5</td>
<td>3.7</td>
<td>30.4±1.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>

a: mean ± standard deviation of the concentration of sugar in urine samples from the 10 consecutive assay runs; b: the coefficients of variation for each sugar (CV = std dev/mean). Urines were spiked with standards having concentrations, Sample 1 = 250 μg ml⁻¹, Sample 2 = 125 μg ml⁻¹, Sample 3 = 31.3 μg ml⁻¹.
(ii) Lactulose, mannitol, cellobiose and rhamnose

Urine samples having concentration of 250 μg ml⁻¹, 125 μg ml⁻¹ and 62.5 μg ml⁻¹ were injected in 10 consecutive assay runs.

The reproducibility of the assay for lactulose, mannitol and rhamnose determined as the inter-assay coefficients of variation in the three concentrations was between 2.7 % and 6.6 % for lactulose, 2.5 % and 6.2 % for mannitol, 6.5 % and 16.5 % for cellobiose and 3.3 % and 6.2 % for rhamnose (Table 3-9).

Table 3-9: Inter-assay variability of the concentration of 4 sugars in 3 different spiked urine samples each repeated 10 times in consecutive assays

<table>
<thead>
<tr>
<th>O/E ratios (%)</th>
<th>Sugar addeda</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lac</td>
<td>Man</td>
<td>Cello</td>
<td>Rham</td>
</tr>
<tr>
<td>Meanb</td>
<td>251.8</td>
<td>245.5</td>
<td>253.0</td>
<td>242.1</td>
</tr>
<tr>
<td>±6.9</td>
<td>±6.1</td>
<td>±16.5</td>
<td>±9.3</td>
<td>±4.7</td>
</tr>
<tr>
<td>CV%</td>
<td>2.7</td>
<td>2.5</td>
<td>6.5</td>
<td>3.8</td>
</tr>
</tbody>
</table>

a = lactulose + mannitol + cellobiose + rhamnose; b = data expressed as mean ± standard deviation of the concentration of the sugar. Urines spiked with standards having final concentrations, Sample 1 = 250 μg ml⁻¹, Sample 2 = 125 μg ml⁻¹ and Sample 3 = 62.5 μg ml⁻¹ respectively.
3.5. Testing the sensitivity of the method to detect sugars in urine samples collected in human studies

3.5.1. Calculating of the dose of lactulose and mannitol necessary to prepare the test solution

Studies of intestinal permeability have employed doses of mannitol between 2.0 - 5.0 g and of lactulose between 5 – 10 g administered in 100 – 120 ml water. In the present body of work, the test solution was formulated based on a commercial solution (Permagnost, Leavosan, Linz, Austria) containing 10 g lactulose and 5 g mannitol in 100 ml of water\(^4\)\(^6\).

Although analytical grade lactulose (Sigma Aldrich), was used for calibrating the HPLC methodology, in the human intervention studies Duphalac solution (Solvay Pharmaceuticals, Sydney, NSW, Australia), a pharmaceutical grade oral lactulose preparation, was used. The Duphalac information sheet provided by the manufacturer stated that every 5 ml of the solution contained 3.34 g lactulose. Hence 15 ml would contain the required 10 g of lactulose.

Duphalac solution was also stated to contain significant amounts of galactose and lactose. Due to the presence of these contaminants it was also determined whether Duphalac solution contained any significant quantities of mannitol in the solution. This was done by running the Duphalac samples with known standard solutions of lactulose and mannitol using the developed HPLC method. Calibration curves were obtained for each standard containing each sugar injected in triplicate.

The samples of Duphalac showed a peak at the same retention time as that of lactulose in the standard solutions that were run concurrently. Comparison with the standard curve \((R^2 = 1)\) (Figure 3-10) showed that, the concentration of lactulose recovered from the injected
Duphalac sample solution was 668 mg ml\(^{-1}\). Hence every 5 ml of the Duphalac solution contained 3.34 g of lactulose, in agreement with that stated on the product label.

![Graph showing calibration curve of standards of lactulose (0 - 1.22 mg ml\(^{-1}\)) and mannitol (0 - 1.27 mg ml\(^{-1}\)) run in triplicates concurrently with the Duphalac sample (1.5 mg l\(^{-1}\)).](image)

Again, the samples of Duphalac showed a peak at the same retention time as that of mannitol in the standard solutions that were run concurrently. By comparison with the standard curve (\(R^2 = 1\)) (Figure 3-10), the concentration of mannitol in the Duphalac sample solution was found to be 57 mg ml\(^{-1}\). Hence the Duphalac lactulose solution was contaminated with trace amounts of mannitol that needed to be adjusted when preparing the test solution to contain a total dose of 10 g lactulose and 5 g mannitol. Accordingly the test sugar solution for the human intervention study was prepared by weighing 20 g of Duphalac (Solvay Pharmaceuticals; Batch#336449), containing 10 g lactulose, 1.5 g galactose, 9.0 g lactose and
0.855 g mannitol (as calculated from the assay) and mixing with 4.143 g mannitol (*Sigma-Aldrich*; Batch#049K0016) and dissolving in 100 ml water.

### 3.5.2. Determining if the calculated dose of lactulose and mannitol is sufficient for them to be readily detected in human urine

Ethics approval was obtained from the Massey University Human Ethics Committee Southern A: (09/79). A single healthy female volunteer (aged 29 yr) fasted overnight. She refrained from consuming any prebiotic and probiotic supplements such as lactulose, from taking any NSAIDs a week prior to the test and from consuming alcohol for three days prior to the test and avoided exercise on the day before and the morning of the test.

A sample of her urine was collected on arrival at the laboratory for use as a ‘baseline’ sample. The participant consumed the solution containing the calculated dose of lactulose and mannitol. Urine sample were collected half-hourly over the six hr period. The volume of each of the half-hourly urine sample was recorded. The sample was centrifuged (*Heraeus Sepatech Megafuge 1.0R*) at 4500 rpm (3500 g), 14 °C for 10 min immediately following collection. 20 ml of supernatant was aliquoted and stored at -80 °C until it was thawed for HPLC analysis.

1 ml of sample was diluted with 1 ml of MilliQ and desalted with 1 gm Amberlite resin (*Amberlite IRA-410*, *Supelco Analytical* and *Amberlite IR120 hydrogen form, Fluka Sigma Aldrich* in mass proportion of 1:1). The mixture was vortex mixed for 15 sec and the resulting supernate was filtered through 0.2 μm cellulose acetate disposable filter (*Advantec, Dismic-13*) into 1.5 ml HPLC vials (*Fisherbrand*). 20 μl of the filtrate was injected into the HPLC system.
3.5.2.1. Method for resolving the peaks in urine samples

The first sample at 0 hr, i.e. ‘baseline’ (when the participant arrived at the laboratory) was used for the correction of subsequent half-hourly samples\textsuperscript{24}. Hence all thirteen samples collected following the ingestion of the sugar solution were corrected with the ‘baseline’ sample. For example, in the sample collected at 2.5 hr (Figure 3-11a i) was corrected with the ‘baseline’ sample using the LC solution software (Figure 3-11a ii). The sample collected at 5.5 hr (Figure 3-11b i) was similarly corrected using the LC software (Figure 3-11b ii).

In the event that the software was unable to detect the lactulose peak (from neighbouring noise, to correctly identify the underlying baseline, to maintain correct peak and baseline detection and to correctly handle rider peaks and other unresolved peaks\textsuperscript{29}) after the baseline correction, a manual method of integration was used. The manual integration is an acceptable method and is referred to as the process employed by the data user to integrate the peak height or area by manually setting the baseline using chromatographic LC software. The methods used are based on parameters described in literature\textsuperscript{29,47} and the LC troubleshooting guide and are described in Table 3-10.
Figure 3-11: Resolving peaks of lactulose and mannitol in urine samples. Chromatograms depict urine samples before (a(i) & b(i)) and after (a(ii) & b(ii)) they were corrected with the baseline 'blank' sample respectively. 20 μl of the samples were injected into the HPLC system and the sugars were eluted isocratically with MilliQ water at a flow rate of 0.3 ml min\(^{-1}\) for 25 min. Lactulose and mannitol peaks in samples appeared at 5.9 and 10.4 min respectively.
Table 3-10: Manual method of integration of urine samples following the baseline correction, using the LC solutions software, to accurately quantify the lactulose peak.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple overlapping peaks</td>
<td>In a situation when the lactulose peak was eluted with an unresolved peak group, the ‘drop’ method was used for determining the location of the chromatograms’ baseline profile. The drop method involved the addition of a vertical line from the valley between the peaks to the horizontal baseline. A vertical line is also drawn at the start and the stop points of the peak group.</td>
</tr>
<tr>
<td>Split peaks</td>
<td>When the peak lactulose appeared as a part of an unresolved peak group with a negative baseline, the ‘baseline to valley’ integration (baseline projection) was used. A baseline was drawn up, connecting the real baseline before and after the peak group. A perpendicular line from the valley between the peaks to this baseline was extended. The drop method was then used to add vertical lines from the valley between the peaks to the horizontal baseline.</td>
</tr>
<tr>
<td>Baseline ripple or negative</td>
<td>When the lactulose peak was resolved with a baseline drift at one end of the peak, the baseline was drawn up, connecting the real baseline before and after the peak group so that the calculated peak area was not excessively large.</td>
</tr>
</tbody>
</table>
3.5.2.2. Results

The peaks of lactulose and mannitol on HPLC appeared within 15 min with the lactulose and mannitol peak each appearing at 5.9 and 10.4 min respectively. These timings were close to that obtained from the standard solutions (5.7 min for lactulose and 10.1 min for mannitol). No major interference from any contaminants was observed at the retention time corresponding to the peak position of each sugar probe.

The baseline correction of samples was advantageous as it eliminated any traces of endogenous sugars present in urine prior to subjects being dosed with the sugar solution. Furthermore, the manual integration of the lactulose peak in some baseline corrected samples using the LC solutions software ensured that the lactulose peak was consistently, reproducibly and accurately detected.

3.5.2.3. Detection of cellobiose and rhamnose along with lactulose and mannitol

In a similar manner to that described previously in section 3.5.2, a single participant was dosed with a sugar solution containing 10 g lactulose, 5 g mannitol, 10 g cellobiose and 5 g rhamnose. Urine samples were collected half-hourly for six hrs. Urine sample preparation and HPLC analysis were similar to that described in section 3.5.2. Likewise, all urine samples collected over the six hr period were corrected with the ‘baseline’ sample. In the event that the software was unable to accurately detect the lactulose peak, after the baseline correction, the manual method of integration was applied (Table 3-10).

Cellobiose, lactulose, mannitol and rhamnose were each well resolved within 35 min with each of the peaks appearing at 14.2, 17.2, 20.4 and 30.3 min respectively. No major interference was observed at the retention times corresponding to the peak position
of each probe. However in samples collected during the latter period of urine collection, for example at 5.5 hr, following ‘baseline’ correction the cellobiose peak appeared below the baseline chromatograph (Figure 3-12) of the other component sugars.

![Figure 3-12: Resolving peaks of lactulose, mannitol, cellobiose and rhamnose in urine samples. Chromatogram depicting the urine sample collected at 5.5 hr after correction with the ‘baseline’ sample. 20 μl of sample was injected into the HPLC system and sugars were eluted isocratically with MilliQ water at a flow rate of 0.1 ml min⁻¹ at 60 ºC for 60 min.](image)

This made it difficult to quantify the sugar accurately. A concern with using cellobiose as a sugar probe is that there is reported break down of the sugar in the jejunum which could have interfered with the quantification of cellobiose excreted²⁴. Hence a limitation to the method is the inability to accurately and reproducibly quantify cellobiose when it is present at lower concentrations in urine samples. It is noteworthy that during the calibration of the method, there was greater variation in the recovery of cellobiose in the urine samples spiked with the lower concentration of the standard. Hence the developed method does not appear to be robust to detect and accurately quantify cellobiose in urine.
3.6. Conclusion

- The developed HPLC methodology is robust, sensitive, reproducible and easy to automate to accurately detect lactulose, mannitol and rhamnose in urine samples.

- Urine samples were chromatographed using a Shimadzu HPLC system equipped with an Aminex HPX87C, 250 x 4.0 mm cation-exchange column, protected by a micro guard cartridge pre-column. The use of the resin based column and RID allowed a simple isocratic elution with water as the mobile phase.

- Sample preparation was minimal and required no prior derivatization. The simplification of the methodology and HPLC equipment provided similar coefficients of variation to that reported by other workers in terms of the precision and reproducibility of the assay.

- Lactulose and mannitol were eluted isocratically at a flow rate of 0.3 ml min⁻¹, 858 psi at 60 °C in twenty two min. It is unlikely that the urine sample collection regime and subsequent spiking of the urine samples with lactulose and mannitol, for calibrating the method, had any effect on the results given that the timing and resolution of peak excretion of each sugar on HPLC was similar to that obtained in urine collected from a subject who had ingested the test sugar solution. Further, the concentration of the sugars in the half-hourly urine samples collected from a subject were within the upper and lower limits of detection based on the calibration curves and support the reproducibility of the method.

- For the detection of two additional sugars rhamnose and cellobiose, the HPLC conditions were modified to a flow rate of 0.1 ml min⁻¹, 288 psi at 60 °C in sixty min. However the method is limited in its ability to accurately and reproducibly quantify cellobiose in urine samples following dosage with the sugar solution in humans. It is noteworthy that the cellobiose peak is sufficiently resolved from that of lactulose so as
not to interfere with the peak in the excretion of the latter. Hence any addition of
cellobiose to the test solution containing lactulose, mannitol and rhamnose would not
interfere with the HPLC results. However, could provide some information regarding
the osmolar effects of the test solution (if any) on the excretion of lactulose and
mannitol over the six hour period of urine sample collection.

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate’s Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate’s contribution as indicated below in the Statement of Originality.

Name of Candidate: Ivana Roosevelt Sequeira

Name/Title of Principal Supervisor: Prof. Roger Graham Lente


In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

and / or

- Describe the contribution that the candidate has made to the Published Work:

__________________________
Ivana Sequeira

Candidate’s Signature

15.12.2014

Date

__________________________
[Signature]

Principal Supervisor’s signature

22.1.2015

Date
CHAPTER 4

The effect of aspirin and smoking on urinary excretion profiles of lactulose and mannitol in young females: Towards a dynamic, aspirin augmented, test of gut mucosal permeability.

Chapter 4 is a pilot study that evaluated if the standard clinical measure of intestinal permeability can be used to assess ‘gut health’ to determine wellness in healthy individuals. The chapter describes the first of a series of experiments that use aspirin as a reproducible, single, low potency, pro-inflammatory stimulus to augment the lactulose mannitol test.

This chapter has been published as: Sequeira IR, Lentle RG, Kruger MC, Hurst RD. The effect of aspirin and smoking on urinary excretion profiles of lactulose and mannitol in young women: toward a dynamic, aspirin augmented, test of gut mucosal permeability. *Neurogastroenterology and Motility* 2012; 24:e401-e11.
4.1. Abstract

**Background:** We explored the temporal dynamics of the lactulose mannitol test and the influence of a single dose of aspirin.

**Methods:** Twenty healthy female volunteers each received 600 mg aspirin or placebo in random sequence and were subsequently dosed with 10 g lactulose and 5 g mannitol, their urine collected every half-hour for six hrs.

**Results:** The lactulose:mannitol ratios (LMR) of urine samples collected over the entire six hr period were significantly higher than those collected in the first three hrs. Greater quantities of mannitol were excreted over the first than the subsequent three hrs. A similar pattern of temporal variation in mannitol excretion was found in smokers and non-smokers and was maintained following administration of a single 600 mg dose of aspirin. The rates at which lactulose was excreted were relatively constant over the entire 6 hr period of collection but mean levels were increased over the entire 6 hrs following the administration of aspirin. The effect of aspirin did not differ significantly between smokers and non-smokers.

**Conclusions:** Whilst the LMR test is sufficiently sensitive to reproducibly detect the increase in intestinal permeability resulting from a single 600 mg oral dose of aspirin, the temporal patterns of excretion of mannitol and lactulose differ both in the presence and absence of aspirin. Hence variation in sampling period and in method of dosage are likely to influence the result and it is preferable to examine the patterns of absorption of component sugars separately with due regard to the method of dosage.
4.2. Introduction

The permeability of the gut mucosa is known to alter with immune status\(^1\), \(^2\). Measurement of the change in the permeability of the gut mucosa following a standardized pro-inflammatory stimulus may provide information regarding its ability to respond to and recover from such a stimulus, i.e. ‘gut health’. There is continuing debate as to the relative proportions of nutrients that are absorbed by the transcellular and paracellular route in the healthy gut\(^3\), whether paracellular pore size varies along the length of the villus\(^4\), \(^5\); and whether the transit of nutrients such as glucose via the transcellular route can augment paracellular absorption\(^6\), \(^7\). However, there is unequivocal evidence that the transit of marker substances which are not actively transported across the mucosa varies with molecular size\(^8\) and that mucosal inflammation facilitates the transit of larger molecules\(^9\)-\(^11\). Indeed tests based on the relative quantities of two probes of differing molecular size are established as a means of identifying inflammatory relapses in diseases such as IBD and celiac disease\(^12\).

In earlier work the rate of absorption of a low molecular weight substance such as mannitol was assumed to be in proportion to mucosal area\(^5\), \(^11\) and used as a reference against the rate of absorption of a larger molecular weight probe such as lactulose (the LMR), which is normally low\(^13\) but augmented in inflammatory states\(^14\). Lately it has been recognized that the ability of the gut wall to absorb lactulose and mannitol varies regionally\(^15\). Hence for example the colonic mucosa is able to absorb proportionately greater quantities of lactulose than mannitol, when these probes are instilled directly into the empty colonic lumen, than does the small intestine following oral dosage with liquid probes\(^16\). Further, it is likely that the quantity of a given sugar that is absorbed varies with length of the gut that is in contact with the sugar during a given period. Hence the absorption of mannitol and lactulose are curtailed when the dose is delivered by
capsule\textsuperscript{13, 16}, the relatively localized zone of dispersal following the release of capsule contents in the lumen of the distal small\textsuperscript{17} or proximal large intestine providing a lesser area for mucosal absorption than that produced by ongoing fractional evacuation of a liquid probe from the gastric lumen\textsuperscript{18}. Thus the interpretation of the lactulose mannitol absorption test is complicated by the duration of the test and by the relative dispersal of the probe along the absorptive surface of the small and large intestine as well as any change in permeability resulting from disease or disorder.

There has been increasing interest in adapting permeability tests to enable assessment of mucosal response to a standardized perturbation. A number of recent reports have indicated that concurrent dosage with aspirin alters permeability as determined by permeability tests\textsuperscript{19-22}. Further there is evidence that such a regime may unmask latent inflammatory bowel diseases such as Crohn’s disease\textsuperscript{19} and augment changes in permeability from chronic alcohol ingestion\textsuperscript{22} presumably allowing them to be more readily detected in a clinical scenario. However this work used differing doses of aspirin (100 - 1300 mg aspirin) along with differing periods of bulking of urine samples (6 - 12 hrs) which precluded direct comparison and may in some cases have led to inconsistent results.

Before standardized perturbation tests can be developed it is necessary to obtain a more detailed quantitative description of the temporal profile of excretion of mannitol and lactulose in healthy subjects following a specific dosage regime\textsuperscript{13} and for comparison of these profiles with the temporal patterns of excretion after the same subjects have been dosed with an agent that is known to directly influence the permeability of the small intestinal mucosa e.g. aspirin\textsuperscript{23}. If dosage with aspirin directly effects the same principal parameters that form the basis of the normal permeability test, and does not cause some independent effect such as influencing urinary excretion or transit time, then the temporal profile of the excretion of the larger probe e.g. lactulose, though augmented, should be
similar to that in the normal test. Given recent evidence that mannitol absorption is augmented in certain medical conditions, e.g. irritable bowel syndrome with diarrhoea (IBS-D), it is important to establish whether the aspirin response is limited to the larger probe, i.e. that the timing and the magnitude of the excretion of the reference sugar, mannitol, that has been used to standardize for surface area is identical to that in the normal test. Again it is important to distinguish any effects that could arise from aspirin on residence time. Thus for example a reduction in the time of transit from the small to the large intestine of the column of digesta containing the two probes would cause the relative proportion of mannitol to be higher and the proportion of mannitol to be lower in a given time period.

The work reported here is the outcome of a study of the temporal urinary excretion profiles underlying clinical tests of intestinal permeability that employ mannitol and lactulose in healthy, carefully screened young women. We examine the temporal relationship between the urinary excretion of mannitol and lactulose in order to determine the effect of sampling time and assess the temporal responses of these same parameters following a single standardized pro-inflammatory stimulus, i.e. an oral dose of 600 mg of aspirin. We also explore the effect of smoking, a stimulus that is reported to decrease LMR in healthy volunteers\textsuperscript{24, 25} and has been claimed to benefit patients with ulcerative colitis\textsuperscript{26-29}, but to increase intestinal permeability and worsen the clinical status of persons with Crohn's disease\textsuperscript{30, 32}. 
4.3. Methods

4.3.1. General

The study was approved by the Massey University Human Ethics Committee: Southern A 09/79. We used a single 600 mg dose of aspirin to test the responsiveness of the lactulose/mannitol absorption test for intestinal permeability as a short-lived, low-level pro-inflammatory stimulus and the effect of smoking. Previous studies have indicated that aspirin can initiate oxidative stress\(^{33}\) and pro-inflammatory responses\(^{19}\) in gut mucosa that increases the ratio of absorbance of large to small molecular probes i.e. increased absorption of lactulose relative to mannitol\(^{20}\).

4.3.1.1. Study design

The half-hourly urinary excretion rates of either of the test sugars are likely to be inherently autocorrelated following an oral dosage regime given that they result from the sequential transit through the gut of a broad column of liquid containing the sugars that is the product of ongoing gastric\(^{18}\) and small intestinal\(^{17}\) mixing. The study was thus a pilot in this regard. Twenty participants successfully completed the experimental regime, a number that was broadly in line with the number of individuals per treatment in previous studies\(^{13,16,34,35}\). The order of the two treatments was randomized by random number tables and was blinded both to the subjects and to the researchers. Each sample for each treatment for each subject was assigned an individual code number by an ancillary worker, who was not otherwise involved in the study, and held by them pending completion of the analyses.

A urine sample was collected immediately after the administration of the treatment, for use as a baseline. Following an overnight fast, each participant received either a placebo
drink (100 ml of water) or 600 mg of soluble aspirin (*Dispirin®*; Reckitt Benkinser Healthcare, UK) in 100 ml of water at 0800 hr in randomized sequence, with a one-week interval between treatments. One hr later, each subject was given 10 g lactulose (*Duphalac®*, Solvay Pharmaceuticals, NSW, Australia) and 5 g mannitol (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 100 ml water¹⁹, followed immediately by 300 ml of water (this dosage was used as it mimicked that of Permagnost®, a commercial formulation that was widely used in clinical testing). Half an hr later, the subjects commenced half-hourly emptying of the bladder and urine collection was continued over 6 hrs (Table 4-1) with the first sample being discarded. This interval was chosen on the grounds that in previous studies, where samples were taken at longer intervals, the time at which peaking of excretion of the probes occurred had not been identified¹³. The participants were allowed no food during the entire urine collection period but were given a further 200 ml of water to drink three hrs after the ingestion of the lactulose and mannitol solution.

The volume of each half-hourly sample was determined immediately following collection, after which it was centrifuged at 4500 rpm, 4 °C for 10 min. Twenty ml of the supernatant from each sample was stored at -20 °C pending HPLC analysis.
Table 4-1: Variation in urine volumes between smokers and non-smokers receiving either placebo and aspirin over the period of sampling

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Subjects</th>
<th>Urine Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First 3 hr</td>
<td>Placebo</td>
<td>Smokers</td>
<td>179.44 ± 2.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>278.27 ± 2.74</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>Smokers</td>
<td>192.67 ± 2.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>277.45 ± 3.18</td>
</tr>
<tr>
<td>Second 3 hr</td>
<td>Placebo</td>
<td>Smokers</td>
<td>284.72 ± 4.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>311.45 ± 2.66</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>Smokers</td>
<td>276.50 ± 3.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>365.27 ± 3.48</td>
</tr>
<tr>
<td>Entire 6 hr</td>
<td>Placebo</td>
<td>Smokers</td>
<td>586.56 ± 6.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>774.82 ± 4.86</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>Smokers</td>
<td>641.67 ± 4.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>810.45 ± 4.90</td>
</tr>
</tbody>
</table>

Results expressed as Mean ± SEM

4.3.1.2. Protocol

The selection process was designed to recruit healthy subjects aged between 20 and 40. We also endeavoured to minimize variance whilst maximizing potential for recruitment of smokers by selecting only female subjects, men being more likely to be current alcohol drinkers\(^{36}\) women being more likely to be smokers\(^{37}\). Prior to an interview with a clinician, subjects were required to complete a questionnaire (Appendix 1) which excluded persons with any GI disorders (and family history) including recent gut infection evidenced by a recent history of abdominal pain, nausea, vomiting, diarrhea, passage of blood and mucus in stools; those taking ongoing prescription or OTC medication or multivitamins except for contraceptive pills. The number of standard alcohol drinks
consumed was calculated from results of alcohol consumption in the questionnaire to ensure that participants did not exceed a moderate alcohol intake. Only subjects who had no history of or current urinary tract infections, vaginal conditions that caused discharge, aspirin sensitivity or the consumption of prebiotic and probiotic supplements such as lactulose were accepted into the trial. All participants were then reviewed by a clinician to validate their responses to the questions.

Each participant was asked to refrain from taking any NSAIDs for at least a week prior to the test, to refrain from consuming alcohol for three days prior to the test\(^{38}\) and to avoid exercise\(^{39, 40}\) on the day before and on the morning of the test.

### 4.3.2. HPLC

#### 4.3.2.1. Equipment and Calibration

Urinary sugar concentrations were determined by HPLC using a method similar to that of Trehan et al\(^{41}\). This was carried out with a Shimadzu (Japan) HPLC system equipped with Degasser Model DGU-20AS (Japan), Pump Model LC-20AT Prominence, Autosampler Model SIL-20AC, Column Oven CTO-20A, 250 x 4.0-mm cation-exchange column (Aminex HPX87C, Bio-Rad laboratories, Richmond, CA) protected by a micro guard cartridge pre-column (Bio-Rad Laboratories, Richmond, CA). Lactulose and mannitol were then eluted isocratically with MilliQ water as a mobile phase, at flow rate of 0.3 ml min\(^{-1}\), 858 psi at 60 °C in twenty two min. The component sugars were detected with a refractive index detector (Shimadzu RID-10A, Japan).

The instrument was calibrated with doses of lactulose and mannitol using solutions of known concentrations from 3.9 \(\mu\)g ml\(^{-1}\) to 500 \(\mu\)g ml\(^{-1}\), each prepared from 1 mg ml\(^{-1}\) stock solutions. The stock solutions were prepared with analytical grade lactulose (Sigma
Aldrich L7877-25G, St. Louis, MO, USA) and mannitol (Sigma Aldrich M4125-500G, St. Louis, MO, USA) dissolved in MilliQ water at room temperature.

The sensitivity of the HPLC assay was determined with baseline urine samples that were each spiked with the various standards. The linearity of the assay was determined by evaluating the dilutional parallelism of the urine samples measured neat and diluted 1:2, 1:4 and 1:8 in MilliQ water. Each dilution was injected in triplicate and results expressed as observed to expected (O/E) ratios of recovery of each sugar. The accuracy of the assay was determined by evaluating the recovery of three different spiked urine samples (250 μg ml⁻¹, 62.5 μg ml⁻¹ and 15.6 μg ml⁻¹). Ten separate preparations of each of these were injected within the same run and results expressed as O/E ratios. The precision of the assay was determined by evaluating intra-assay variability by measuring three different urine samples (500 μg ml⁻¹, 125 μg ml⁻¹ and 31.25 μg ml⁻¹) injected 10 times within the same assay run and calculating the mean, standard deviation, coefficients of variation and standard errors for both sugars. The reproducibility of the assay was determined by evaluating inter-assay variability by measuring three different urine samples (250 μg ml⁻¹, 62.5 μg ml⁻¹ and 15.6 μg ml⁻¹) in 10 consecutive assay runs and calculating the mean, standard deviation, coefficients of variation and standard errors for both sugars.

4.3.2.2. Urinary Analysis

A 1 ml aliquot of each urine sample was diluted with 1 ml of MilliQ water and desalted with 1 g Amberlite resin (Amberlite IRA-410, Supelco Analytical and Amberlite IR120 hydrogen form, Fluka Sigma Aldrich) in mass proportion of 1:1. The mixture was then vortexed for 15 sec and the supernatant was filtered with 0.2 μm (pore size) cellulose acetate filter (Advantec, Dismic-13, Toyo Rashi Kaisha Ltd, Japan) into 1.5 ml HPLC vials (Fisherbrand,
A 20 μl aliquot of filtrate from each sample was then injected into the HPLC system.

### 4.3.3. Data processing and statistical analysis

The concentrations (mg ml⁻¹) of lactulose and mannitol in each of the urine samples were determined from the areas under the curve (AUC) relative to those of a series of standards that were run concurrently. The quantity of each probe excreted over each time period was then determined by multiplication with the sample volume. The quantity of each probe excreted per half-hour period was plotted over the six hr period to evaluate consistency of excretion rate and to determine the general temporal pattern of excretion of each sugar. The real time lactulose:mannitol ratio (LMR) was calculated for each half-hour period expressed as the percentage of the quantity excreted, consecutive values being plotted to track variation over time after dosage. Two ‘clinical’ values of intestinal permeability were also derived by summing the component half-hourly lactulose and mannitol excretion (mg) over three hr and six hr periods and calculating the ratio of appropriate summed quantities.

Statistical analyses were conducted using the SYSTAT statistical software package version 13 (Systat Software Inc., Chicago, IL). The distribution of the data was examined using the Kolmogorov-Smirnov-Lilliefors test. Differences in cumulative LMR and cumulative excretion of lactulose and mannitol over the first three hrs and the second three hrs, the first three hours and the entire six hrs and the first three hrs and the second three hrs were each assessed by a doubly repeated measure of ANOVA with probabilities of post hoc comparisons corrected with a Bonferroni algorithm (Appendix 2). The algorithm used in this analysis also detected and reported any outliers that had unduly influenced the
outcome. Hence the effects of the duration of the period over which urine results were bulked (excretion during the first three hrs compared with that during the second three hrs and the entire six hrs) and of treatment (aspirin or placebo) were assessed as a variation within subjects and smoking status as variation between subjects. It is noteworthy that the first three hr, second three hr and six hr cumulative data for LMR, lactulose excretion and mannitol excretion are inherently autocorrelated, but the analysis was nevertheless considered useful as it provided a comparison for the effectiveness of two commonly used time periods in clinical testing. The extent to which there was correlation between the total amount of lactulose or mannitol excreted during the six hr period of collection without aspirin and the total volume of urine produced during the same period was explored in all twenty subjects by determining Pearson correlation coefficients and the significance of the Bartlett chi squared statistic.

4.4. Results

4.4.1. General

Of the 28 participants, 8 subjects were excluded: two owing to the presence of vaginal secretions containing mannitol in the initial and subsequent early urine samples of their test series; two due to intercurrent illness; and four who failed to fully comply with conditions of the test. Hence 20 participants completed the test protocol satisfactorily. Of the twenty healthy female participants 11 were non-smokers and were aged between 22 and 35 (mean 26.2 yr), the other 9 subjects were smokers who consumed between 10 and 20 cigarettes per day, and were aged between 21 and 35 (mean 25.5 yr). Eight of the subjects were teetotalers (2 in the smoking group and 6 in the non-smoking group).
The LMRs as well as the lactulose and mannitol excretion data required log conversion to render them amenable to parametric analysis. Hence the distribution of the log converted data did not differ significantly from normal on Kolmogorov Smirnov-Lilliefors test.

4.4.2. Reliability of the assay

There were no effects of order in which the samples were analyzed by HPLC with either sugar within or between runs (samples were selected randomly for each HPLC run). Similarly there were no general trends in the overall order in which samples were taken during the experiment.

Calibration curves for lactulose and mannitol were linear up to 500 μg ml⁻¹ for both sugars and were fitted by the following linear regression equations where \( a = 0 \) as the line intercepts axes at 0; lactulose: \( y = 563.88 \, x \); mannitol: \( y = 545.92 \, x \).

The limits of detection (LODs) for lactulose and mannitol in standard solutions were both 4 μg ml⁻¹. The ratio of the observed to expected recovery for mannitol ranged between 84 and 100 % and for lactulose ranged between 90 and 113 % across all dilutions, suggesting there was sufficient linearity of the assay. The accuracy of the assay lay between 97 - 116 % for mannitol and 93 – 113 % for lactulose, respectively. Based on the recoveries of the sugars, the LOD for lactulose and mannitol in the urine samples was 15 μg ml⁻¹. The precision and reproducibility of the assay for intra-assay and inter-assay variability (CV %) for lactulose and mannitol was between 2.0 and 5.1 %, and 2.5 and 4.4 % respectively.
4.4.3. Variation in urinary excretion of lactulose, mannitol and LMR with placebo

There was no significant correlation between either the total quantity of mannitol or the total quantity of lactulose excreted and the total volume of urine produced. The LMR of urines increased in a curvilinear manner over time with incremental half-hourly increase tending to decrease after five hrs. The overall LMRs of the urines collected during the first three hrs (mean: 0.04 ± 0.03) were significantly lower on repeated measures ANOVA (d.f 1,19; F = 48.74; P < 0.001) than those collected over the second three hrs (d.f 1,19; F = 34.33; P < 0.001) (mean: 0.1 ± 0.06) and those collected over the entire six hrs (mean: 0.06 ± 0.05) (Figure 4-1). The LMRs of urines collected from smokers did not differ significantly from those of non-smokers in urine samples collected over any of the periods of collection (Table 4-2).
Figure 4-1: Comparison of the effect of the duration of urine collection (first 3 hr, second 3 hr and 6 hr) on the urinary lactulose:mannitol ratio (LMR) in 20 healthy female subjects following consumption of either 600 mg aspirin or a placebo. The overall LMR of the urine collected over the first 3 hr was significantly lower on repeated measures ANOVA (P < 0.001) than urine collected over the entire 6 hr and the second 3 hr. The administration of 600 mg of aspirin caused a significant (P < 0.001) overall increase in the LMR in urine over all periods of collection relative to the placebo. (Mean ± SEM)
Table 4-2: Differences in the quantities of lactulose, mannitol and cumulative LMR between smokers and non-smokers after receiving either placebo and aspirin in urines collected over the first three hrs, the second three hrs and the entire six hr period.

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Subjects</th>
<th>LMR</th>
<th>Lactulose (mg)</th>
<th>Mannitol (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First 3 hr</td>
<td>Placebo</td>
<td>Smokers</td>
<td>0.03</td>
<td>19.86 ± 1.47</td>
<td>329.18 ± 3.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>0.05</td>
<td>27.28 ± 1.18</td>
<td>315.52 ± 2.60</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>Smokers</td>
<td>0.16</td>
<td>36.09 ± 1.95</td>
<td>355.87 ± 4.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>0.10</td>
<td>74.30 ± 2.73</td>
<td>258.47 ± 2.86</td>
</tr>
<tr>
<td>Second 3 hr</td>
<td>Placebo</td>
<td>Smokers</td>
<td>0.10</td>
<td>27.60 ± 1.63</td>
<td>154.24 ± 2.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>0.12</td>
<td>34.01 ± 1.42</td>
<td>178.57 ± 2.50</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>Smokers</td>
<td>0.24</td>
<td>67.69 ± 2.60</td>
<td>165.61 ± 3.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>0.31</td>
<td>85.99 ± 2.88</td>
<td>162.72 ± 2.70</td>
</tr>
<tr>
<td>Entire 6 hr</td>
<td>Placebo</td>
<td>Smokers</td>
<td>0.05</td>
<td>47.46 ± 2.19</td>
<td>483.42 ± 3.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>0.07</td>
<td>61.28 ± 1.75</td>
<td>494.09 ± 3.00</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>Smokers</td>
<td>0.20</td>
<td>103.79 ± 3.12</td>
<td>521.48 ± 5.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non smokers</td>
<td>0.15</td>
<td>160.30 ± 3.94</td>
<td>421.19 ± 3.40</td>
</tr>
</tbody>
</table>

Results expressed as Mean ± SEM.
4.4.3.1. Temporal variation in excretion of mannitol

There was a (predictable) significant (d.f 1,19; $F = 141.39; P < 0.001$) increase in the excretion of mannitol with the duration of collection (mean 0 - 3 hr: $322 \pm 2.07$ mg; mean 0 - 6 hr: $486.6 \pm 2.40$ mg). The quantities of mannitol in the urine samples collected during the first three hours were significantly greater than in those collected during the second three hours (mean 3 - 6 hr: $179 \pm 2.50$ mg) (d.f 1,19; $F = 48.88; P < 0.001$). Plots of half-hourly mannitol excretion showed that excretion rate peaked in all subjects between 1.5 to 2 hrs post-dosage (Figure 4-2A), so that half-hourly concentrations were consistently higher over the first three hrs and consistently lower over the succeeding three hrs of collection. The excretion pattern of mannitol did not differ between smokers and non-smokers (Table 4-2).
Figure 4-2: Variation of excretion of mannitol and lactulose (mg) in urine samples from 20 healthy female smokers and non-smokers at half-hourly intervals over a six-hr period following the administration of placebo and 600 mg aspirin. (A) Half-hourly mannitol excretion peaked between 1.5 and 2 hr after dosage. This pattern did not change following the consumption of aspirin in smokers and non-smokers. (B) Half-hourly lactulose excretion increased following dosage with aspirin, the quantity excreted during the first 3 hr did not differ significantly from that collected over the second 3 hr. (Mean ± SEM)
4.4.3.2. Temporal variation in excretion of lactulose

There was an overall (predictable) significant increase (d.f 1,19; F = 202.55; P < 0.001) in the excretion of lactulose with increase in the duration of urine collection (mean 0 - 3 hr: 24 ± 0.92 mg, mean 0 - 6 hr: 57 ± 1.38 mg). The quantities of lactulose in the urine samples collected during the first three hrs did not differ significantly from that in those collected over the second three hrs (mean 3 - 6 hr: 31 ± 1.06 mg). Plots of half-hourly lactulose excretion (Figure 4-2B) showed no consistent pattern of variation with time. Again, the pattern of excretion of lactulose did not differ between smokers and non-smokers during the entire period of collection (Table 4-2). However, though not significantly different, the excretion of lactulose appeared to be lower in smokers than in non-smokers.

4.4.4. Variation in LMR and excretion of lactulose and mannitol with aspirin

The administration of 600 mg of aspirin one hour before the administration of the two probes caused the LMRs of urines collected over the entire six-hr period of collection (mean: 0.17 ± 0.10) to increase significantly (d.f 1,19; F = 23.95; P < 0.001) relative to that following administration of the placebo (Figure 4-1), the LMR of urines collected over the second three hrs (mean: 0.27 ± 0.10) being significantly greater (d.f 1,19; F = 12.41; P = 0.002) than in those collected over the first three hrs (mean: 0.12 ± 0.10) (Figure 4-1 and Figure 4-3). The LMRs of urines collected from smokers and non-smokers over the entire period of collection did not differ significantly from each other following dosage with aspirin (Table 4-2).
Figure 4-3: Variation of lactulose:mannitol ratio (LMR) in the successive half-hourly urine samples from 20 healthy female subjects following consumption of either 600 mg aspirin or placebo. The LMRs of the urines collected after dosage with aspirin were significantly (P < 0.001) greater than in those collected in urine following dosage with placebo. (Mean ± SEM)

4.4.4.1. Variation in mannitol excretion after aspirin

There was a significant increase (d.f 1,19; F = 109.29; P < 0.001) in the excretion of mannitol with the duration of collection following dosage with aspirin (mean 0 - 6 hr: 466 ± 3.1 mg). Hence there was a similar pattern of excretion to that in un-dosed individuals where the quantities of mannitol collected over the first three hrs (mean: 302 ± 2.68 mg) was significantly greater (d.f 1,19; F = 36.67; P < 0.001) than those collected during the second three hrs (mean: 164 ± 2.05 mg). In particular, neither the quantity of mannitol excreted during the first three hrs nor that excreted over the second three hrs nor that excreted over the entire six hrs changed significantly following the
consumption of aspirin relative to that collected over the same period following consumption of the placebo (Figure 4-2A and Figure 4-4). Hence the dynamics of absorption and excretion of mannitol appeared to not be influenced by the acute pro-inflammatory stimulus.

Figure 4-4: Comparison of the effect of the duration of urine sample collection on the cumulative excretion of mannitol following consumption of either 600 mg aspirin or placebo in 20 healthy female subjects. Although mannitol excretion increased with the duration of collection, it did not change significantly following the consumption of aspirin. Similarly, there were no significant differences between smokers and non-smokers either before or after consumption of aspirin. (Mean ± SEM)
4.4.4.2. Variation in lactulose excretion after aspirin

Dosage with aspirin resulted in a significant (d.f 1,18; F = 16.83; P = 0.001) and sustained increase in lactulose excretion over the entire six hr period of collection (mean 0 - 6 hr: 135 ± 2.60 mg) (Figure 4-2B & Figure 4-5) relative to that after consumption of the placebo.

Figure 4-5: Comparison of the effect of duration of urine sample collection on the cumulative excretion of lactulose following consumption of either 600 mg aspirin or placebo in 20 healthy female subjects. The overall excretion of lactulose was significantly greater (P < 0.001) following consumption of aspirin with smokers demonstrating a lower excretion of lactulose in comparison to non-smokers. (Mean ± SEM)
Hence though there was a predictable significant increase in the excretion of lactulose with the duration of collection (d.f 1,19; F = 63.67; P < 0.001) there was no difference in the pattern of excretion between the first three hrs (mean: 57 ± 1.82 mg) and the second three hrs (mean: 78 ± 1.97 mg) of collection. Smoking status had no significant effect on overall lactulose excretion following the administration of aspirin although the excretion of lactulose appeared to be lower in smokers than in non-smokers (Figure 4-2B and Table 4-2).

4.5. Discussion

Our results show that the temporal excretion profiles of the component sugars in the LM test are similar in smoking and non-smoking subjects. Further that this similarity is maintained following dosage with 600 mg of aspirin whilst the mean level of lactulose excretion (and hence LMR) is increased. These results fit in with findings that aspirin directly augments small intestinal permeability as assessed by LMR\textsuperscript{20-22}. It is noteworthy that the difference in LMR induced by aspirin was identifiable in samples taken throughout the six hr period subsequent to dosage. Also that the LMR rose monotonically for six hrs after dosing, at the end of which period much of the dose would have entered the colon where lactulose absorption is reported to be augmented relative to mannitol and the LMR correspondingly increased\textsuperscript{16}. Hence, the monotonic increase resulted in the LMRs of urines collected during the second three hr period of collection being consistently and significantly greater than in those collected over the first three hrs regardless of aspirin dosage and caused the LMRs of urines collected over the entire six hrs to be greater than that in those collected over the first three hrs. These differences were maintained in subjects who smoked, indicating a consistent sensitivity of the test results to
the duration over which urine is collected. Similar broad trends have been recently reported by other workers\textsuperscript{13} although these differences became apparent over longer time periods (2 - 4 hrs compared with 8 - 24 hrs).

The broad monotonic increase in LMRs in successive half-hourly urines suggest that a diagnosis of increased permeability based simply on a cut-off value for LMR\textsuperscript{43} may be confounded by slight changes in the timing of urinary sampling even when these times are within the range that is used in clinical testing. Further, that the change in LMR with time precludes the meaningful comparison of published clinical permeability tests in which the timings of urinary collection differ.

Our findings regarding the temporal patterns of excretion of the two probes add considerable detail to the broader trends reported hitherto\textsuperscript{13}, showing how differences in their rates of appearance in the urine contribute to the temporal variation and longstanding changes in LMR. Urinary excretion of mannitol peaked consistently around two hrs after dosage at levels around the previously reported range\textsuperscript{44, 45}, whilst the urinary excretion of lactulose remained relatively constant throughout the six hr period of collection. Hence the peaking and subsequent decline in the uptake and excretion of mannitol over the first four hrs appears to be responsible for the variation in LMR between urines bulked over the three and six hr collection period. This finding fits with the conclusions of Rao et al. that the proportion of mannitol that is absorbed in the small intestine is greater than that absorbed in the colon\textsuperscript{16}.

However the fact that the peak in mannitol absorption spans a four hr period suggests that the column of digesta created by exponential emptying of the stomach and subsequent ongoing intestinal mixing takes a considerable time to clear the small intestine creating a corresponding lag and damping effect on any rise in LMR as the sugars enter the colon. Such a hypothesis requires an assumption that small intestinal residence time is more
than 2 hrs and conflicts with suggestions of Rao et al.\textsuperscript{16} that it varies between 0 and 120 min.

However our conclusion is supported by a large body of work comprising over 100 studies summarized by Yu et al.\textsuperscript{46} that the mean small intestinal transit time is 199 min with a 95\% confidence interval of 7 min; these statistics indicating that up to 20\% of the dose remained in the small intestine at 240 min. Further that small intestinal transit time was independent of ‘gender, age, body weight and the presence of food in the stomach’\textsuperscript{46}. Again it is supported by analyses of scintigraphic data\textsuperscript{47} that used a deconvolutional method developed by Malagelada et al.\textsuperscript{48} to determine transit times that were not based on the less accurate method of departure of a particular fraction of tracer from the stomach and its arrival at the intestine. Correspondingly, it seems likely that any shift in the length of, or transit time of the column of digesta containing the probes through the gut lumen such as could be brought about by a change in the dosage regime (for example from a liquid to an encapsulated dose) could cause the gradient of the LMR slope and hence the ‘optimal’ sampling time to change.

The lack of difference in the temporal patterns of mannitol excretion with and without aspirin suggest the substance has little effect on the capacity of the mucosa to absorb mannitol and thus that mannitol may be used as a reference substance in lieu of the mucosal surface area as was enunciated in early work\textsuperscript{9, 14}. This observation fits in with reports indicating that mannitol is absorbed via the apices of enterocytes\textsuperscript{5, 49} via a mechanism that is not reported to be influenced by aspirin. However the evanescent nature of the peak and its probable underlying dynamics discussed hitherto indicate that careful standardization of sampling time would be required.
The lack of any correlation of urinary excretion of lactulose (d.f 1,18; $R^2 = 0.011$; $p = 0.651$) or mannitol (d.f 1,18; $R^2 = 0.131$; $p = 0.117$) with urinary volume appears to conflict with the findings of Mattioli et al. However, these workers did not restrict nutrient and fluid intake beyond the first two hrs of a five hr sampling period whereas intake was controlled throughout the six hr sampling period of our study. Hence it is probable that hydration status and glomerular filtration rate did not vary greatly between subjects in our study.

The prompt and sustained elevation of lactulose after various doses (100 - 325 and 1300 mg) of aspirin has been described in previous studies and fits in with reports of the effects of aspirin on the cellular actin cytoskeleton and tight junction integrity. The relative invariance of this response over the six hr experimental period suggests it is less likely to be affected by minor variations in sampling period although the general increase in excretion rate with increase in sampling time indicates a similar ongoing change to that reported by Rao et al. as the column of digesta containing the probe moves from the small intestine into the colon. Given the high degree of temporal variance in the excretion of mannitol it seems that an assessment of intestinal permeability by lactulose concentration alone may be more robust. This, along with the finding that the absorption of mannitol is augmented in subjects with IBS-D and that both mannitol and lactulose absorption are augmented in subjects with colitis, leads us to concur with the conclusion of these workers that the quantities rather than the ratios of probes be used as a basis for assessing changes in permeability. Whilst a range of probes could be used in this regard they would need to be of a physiologically relevant range of sizes. Were two probes of similar sizes to be used, the results could be confounded by competition for similar sized passive absorptive sites. Ideally some form of allometrically derived compensation to allow for
differences in subject size/mucosal surface area would need to be incorporated so as to provide a basis for clinical comparison.

The failure of the lactulose level to increase significantly after 3 hrs suggests that the rate of uptake of lactulose from the colonic mucosa is lower than that obtained after direct instillation into the colonic lumen following lavage. As noted by these workers the process of lavage may have influenced the permeability of the mucosa, moreover it is likely that a higher ambient concentration of lactulose would be maintained under these experimental conditions than when the lactulose was a constituent of viscous pseudoplastic digesta that normally occupies the colonic lumen.

The lack of significant difference between the lactulose levels (and LMRs) of smokers and non-smokers is in line with previous reports. Similarly the lack of difference in the LMRs of smokers and non-smokers following dosage with aspirin is in line with recent reports that short term dosage with nicotine does not alter NSAID induced compromise of gut barrier function. It is noteworthy however that baseline intestinal permeability of smokers is reported to differ from that of non-smokers when permeability is assessed by other probes e.g. 51Cr-EDTA.

Given the somewhat lower excretion of lactulose by smokers than by non-smokers, it is possible that a study with larger sample size may identify significant differences between the two populations. Similarly the somewhat greater increase in lactulose secretion of smokers following aspirin challenge than that of non-smokers after dosage with aspirin does suggest that aspirin augmented tests may unmask different components of latent inflammatory diseases of the bowel that affect intestinal permeability.

In conclusion, our results have demonstrated that the dynamics of the absorption and excretion of the probes in the lactulose mannitol test differ over time and that this
difference is sufficient to cause the magnitude of the LMR to vary significantly with the duration and timing of the sampling period. Our results also suggest that the lactulose mannitol test is sufficiently sensitive to detect an increase in permeability from a single 600 mg dose of aspirin for collection periods of up to six hrs. Hence the use of an aspirin-augmented lactulose mannitol test with a standardized protocol may be of use in identifying subclinical inflammatory disorders of the bowel. The augmented test may also have application in exploring the effects of foods and therapeutic agents in minimizing pro-inflammatory responses (preventative effect) or their effects in hastening recovery from a pro-inflammatory disturbance (restorative effect).

4.6. Afterword

Standardizing the lactulose mannitol test protocol and augmenting the test with a single 600 mg dose of aspirin appears to be a robust method for measuring ‘health’ in the gut. The temporal patterns of absorption and timing in peak excretion suggest that the ratio of the probes sugars will vary based on the duration of sampling. The conclusions of the study raised debate among the scientific community with a letter published in Neurogastroenterology and Motility (Appendix 3). The letter addressed the fact that we recommend the use of the absolute values of each sugar instead of only the ratio as has been traditionally used. We published a response to the Letter (Appendix 4) that justified our claim on the grounds of reproducible results obtained in the following chapter.
4.7. References

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MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate’s contribution as indicated below in the Statement of Originality.

Name of Candidate: Ivan Roosevelt Sequeira

Name/Title of Principal Supervisor: Prof. Roger Graham Lente


In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80 %

and/or

- Describe the contribution that the candidate has made to the Published Work:

Ivana Sequeira

Candidate’s Signature: ____________________________

15.12.2014

Date

Principal Supervisor’s Signature: ____________________________

Date: 22.1.2016

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CHAPTER 5

Differential trafficking of saccharidic probes following aspirin in clinical tests of intestinal permeability in young healthy female subjects.

Chapter 5 evaluated the effects of co-administration of two types of disaccharide probes and two types of monosaccharide probes; each group having identical molecular radii. It was reasoned that the use of similar sized probes would further elucidate the effect of the physicochemical properties of probe sugars on permeation across the intestinal mucosa. This is especially in the case of similar sized probe sugars as they might compete for similar absorption sites, i.e. pores. The study would also provide any additional information regarding the influence that aspirin may have on the physiochemical properties of the probes to consequently affect their absorption across the mucosa.

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5.1. Abstract

**Background:** The effect of inflammatory change on the absorption of different sized probes and their permeability ratios are poorly understood. We aimed to determine the effects of a pharmacological agent on permeability of the gut mucosa to saccharidic probes of larger and smaller molecular weight.

**Methods:** Permeability was assessed by half-hourly urinary excretion of a combined dose of D-mannitol, L-rhamnose and lactulose following consumption of a single 600 mg dose of aspirin and compared with a placebo in a cross over study in twenty healthy female volunteers.

**Results:** The temporal patterns of excretion of all probes were bimodal being best fitted by polynomial functions. The relatively small early peak was evident for at least four hrs for smaller sugars but was less evident with lactulose, being overshadowed by a larger second peak. These conclusions were further supported by separate analyses of the segments of the temporal plots between 2.5 and 4 hrs and between 4.5 and 6 hrs. The forms of these curves did not change significantly following dosage with aspirin. A greater proportion of the total dose of mannitol than rhamnose was excreted over the collection period. Following the consumption of aspirin the cumulative rate of excretion of these smaller sugars was significantly reduced whilst that of lactulose was increased over the six hr collection period.

**Conclusions:** Aspirin exhibits contrary effects on absorption of larger and smaller probes influencing the outcome of the test. These results have important consequences for the design and comparison of clinical tests of permeability.
5.2. Introduction

The sugar absorption test for measuring intestinal permeability is well researched and generally accepted for use in the surveillance of recovery from inflammatory and autoimmune diseases such as coeliac disease. This non-invasive and reliable method is well established in the detection of intestinal and extra-intestinal conditions however the dynamics that govern the recovery of the probes are less clear.

It is generally agreed that, in healthy individuals, permeability of the gut to probes of smaller molecular mass i.e. monosaccharides is greater than to those of higher molecular mass such as lactulose, chromium-labelled ethylenediamine tetra-acetic acid (Cr-EDTA) and polyethylene glycols (PEG) and that permeability to larger probes is selectively increased in persons with disorders such as Crohn’s disease and Coeliac disease. Typically the dual sugar absorption test provides the opportunity to acquire site-specific information by exploiting the differences in digestibility and degradation of component sugars. However, there is on-going debate regarding the physicochemical mechanisms and sites of transit of these probes through the intestinal mucosa.

The rate of absorption of the probes does not vary simply as a consequence of their molecular mass, radius or hydrated volume. Calculations based on the Einstein-Stokes diffusion relationship predict that the ratio of lactulose to mannitol diffusing across the mucosa will be between 0.71 - 0.80, a value that is considerably higher than that found in the urine i.e. around 0.25. Whilst calculations predict that the diffusivity of solutes of lower molecular weight will be impaired to a greater extent than those of higher molecular weight by solvent drag, the rates of recovery of monosaccharide probes are generally greater than those of higher molecular mass.
The finding that hyperosmolar stress of the intestinal mucosa (osmolarity of lumen contents >1500 mosmol kg\(^{-1}\))\(^{27}\) caused the urinary excretion of lactulose and Cr-EDTA to increase but did not influence that of L-rhamnose\(^{28}\) led to a hypothesis that larger probes are absorbed at different sites to smaller probes\(^{24}\). Larger probes were postulated to be absorbed via a paracellular route whilst the smaller probes were absorbed transcellularly via the apical membrane\(^1\). However such a hypothesis is problematic with regard to the passive transcellular permeation of the smaller sugars. Whilst the deoxysugar L-rhamnose (MW 164 Da; hydrated molecular volume 0.139 nm\(^3\)) is known to permeate erythrocytes\(^{24}\) and hence can traverse the plasma membrane, the sugar alcohol D-mannitol, a probe of slightly larger molecular weight (MW 182 Da; hydrated molecular volume 0.154 nm\(^3\))\(^{23}\), is able to induce osmotic shrinkage in cells and membrane vesicles\(^{29}\) and is therefore unlikely to passively transit the apical membrane of the enterocyte in significant quantities.

A contrary hypothesis that to some extent circumvents this difficulty is that all probes transit via paracellular routes regardless of their size, and that tight junction occlusion, and hence the diameter of paracellular ‘pores’, decreases as enterocytes mature during their transit from the germ cell layer at the bases of the villi to the their apices\(^{20, 30} \). Hence, greater numbers of paracellular sites would always be available for transit of smaller probes than for those of larger molecular dimensions.

The principal difference between these two hypotheses lies in the nature of the apical and paracellular plasma membranes. Were the second hypothesis to be correct, assuming that the numbers of embedded pore proteins were low in the regions of tight junctions, then the application of an agent that altered the properties of the lipid membrane, for example by stabilizing or creating defects\(^{31}\), would enhance the transit of both small and large molecular weight probes\(^{31}\). Conversely, were smaller probes to be absorbed transcellularly via pores in the apical membrane that are associated with pore
proteins\textsuperscript{32}, then the patterns of absorption at the two sites could differ. Hence, the administration of an agent that influenced both the properties of the lipid membrane and the pore proteins embedded in the apical membrane could for example decrease the rate of transcellular absorption of a smaller probe whilst augmenting paracellular absorption of the larger probe via membranous defects.

Evaluation of the effects of such an agent is complicated by regional variation in the permeability of the mucosa to larger and smaller probes in successive segments of the gut\textsuperscript{16}. The relative proportion of the probes recovered is known to vary with time and with the rate at which they pass into the large intestine\textsuperscript{3}. Such variation could result either from relative differences in the mucosal surface area or from variation in absorptive physiology in successive segments\textsuperscript{33}. Hence, for example, the greater rate at which water is absorbed in the colon and the consequent increase in solvent drag could augment the absorption of larger probes. Any test that seeks to elucidate differences in mucosal sites of absorption of smaller and larger probes should therefore restrict observations to periods when they are likely to be contained within the lumen of the small intestine, by appropriate limitation of the times during which urine samples are collected\textsuperscript{3}.

The current work compared the absorption characteristics of four simultaneously administered saccharidic probes in the small intestine before and following the administration of a single dose of aspirin, an agent which is known to influence both the lipid\textsuperscript{31} and proteinous\textsuperscript{32} components of the enterocyte cell membrane. The periods of residence of the probes in the small and large intestine were evinced by fitting curves to the pooled temporal data for absorption and excretion of the two sugars in the experimental subjects over the entire period of urine collection and assuming that differences in the rates of absorption in colonic and small intestinal lumen would engender differing temporal
profiles of absorption and excretion\(^3\). We anticipated that we would then be able to define
the optimal period for urine sample collection according to this curve.

5.3. Methods

5.3.1. Ethical Approval

The study was approved by the Massey University Human Ethics Committee:

5.3.2. Subjects and screening procedure

The procedure for selection and screening of subjects endeavoured to minimize the
variance and to maintain the comparability of the results of the current study with a those
from a previous study\(^34\). Given that gastric emptying in women is slower than that in
men\(^35, 36\) and could engender differences in the magnitude and timing of the peaks of the
component sugars used in the test, we recruited only female subjects.

Twenty healthy female participants between 20 – 40 years of age completed the
study. This number was chosen as it is broadly in line with the number of individuals in
previous studies that used the sugar absorption test\(^14, 37, 38\).

Subjects were screened by a health questionnaire (Appendix 5) and a clinical
interview to exclude participants with GI disorders, those taking ongoing prescription or
OTC medication, prebiotic or probiotic or vitamin supplements and those who had more
than moderate alcohol intake. Similarly, to exclude subjects with any history of current
urinary tract infections, vaginal discharge and aspirin sensitivity.
Participants were instructed to refrain from taking any NSAIDs for at least a week prior to the test, to refrain from consuming alcohol for three days prior to the test, to avoid exercise on the day prior and the morning of the test and to fast overnight before attending the test.

5.3.3. Experimental protocol

Each experimental session commenced at 8.00 am when a baseline urine sample was collected from each subject. The order of treatments (aspirin or placebo) was randomized between the two sessions. Hence each subject received either a placebo drink (100 ml of water) or 600 mg of soluble aspirin (*Dispirin*®; Reckitt Benkinser Healthcare, Berkshire, UK) in 100 ml of water at the first session. One hr after receiving the treatment, each subject consumed a solution containing 10 g lactulose (*Duphalac*®, Solvay Pharmaceuticals, Sydney, NSW, Australia), 10 g cellobiose (*Sigma-Aldrich, St. Louis, MO, USA*), 5 g D-mannitol (*Sigma-Aldrich, St. Louis, MO, USA*) and 5 g L-rhamnose (*Sigma-Aldrich, St. Louis, MO, USA*) dissolved in 250 ml water, followed immediately by 150 ml of water. Half-hourly urine collections commenced after half an hr and continued for a period of 6 hrs, the first sample being discarded. No food was consumed during the entire urine collection period but a further 200 ml of water was given three hrs after the ingestion of the sugar solution. The volume of each half-hourly urine sample was determined and the urine then centrifuged at 4500 rpm, 4 °C for 10 min before being decanted and stored at -20 °C pending HPLC analysis.
5.3.4. **HPLC**

5.3.4.1. **Equipment and Calibration**

Urinary sugar concentrations were determined by HPLC using a method similar to that described previously by us\textsuperscript{34} using a Shimadzu (Japan) HPLC system with a 250 x 4.0 mm cation-exchange column (Aminex HPX87C, Bio-Rad laboratories, Richmond, CA). Lactulose, cellobiose, D-mannitol and L-rhamnose were eluted isocratically with MilliQ water as the mobile phase, at a flow rate of 0.1 ml min\textsuperscript{-1}, 288 psi at 60 °C in sixty min and detected with a refractive index detector (Shimadzu RID-10A, Japan).

The instrument was calibrated using reference solutions prepared with analytical grade lactulose (Sigma Aldrich L7877-25G, St. Louis, MO, USA), mannitol (Sigma Aldrich M4125-500G, St. Louis, MO, USA), D-cellobiose (Sigma Aldrich C7252-100G, St. Louis, MO, USA) or L-rhamnose monohydrate (Sigma Aldrich R3875-100G, St. Louis, MO, USA) dissolved in MilliQ water at room temperature.

The sensitivity of the HPLC assay was determined using baseline urine samples spiked with the various standards. The accuracy, precision and reproducibility of the assay were each determined by evaluating the recovery of ten replicates of three different spiked urine samples (250 μg ml\textsuperscript{-1}, 125 μg ml\textsuperscript{-1} and 62.5 μg ml\textsuperscript{-1}).

Experimental urine samples were selected randomly for each HPLC run to eliminate any effects of order for each sugar either within runs or between runs. Each sample for each treatment for each subject was assigned an individual code number by an ancillary worker, who was not otherwise involved in the study, and held by them pending completion of the analyses.
5.3.5. Data processing and statistical analysis

The concentrations (mg ml\(^{-1}\)) of lactulose, mannitol, rhamnose and cellobiose in each of the urine samples were determined from the areas under the curve (AUC) relative to those of a series of concurrent standards. The quantity of each probe excreted during each time period was calculated by multiplication with the sample volume and the result expressed as a percentage recovery of the ingested dose.

Statistical analyses were conducted in the SYSTAT statistical software package version 13 (Systat Software Inc., Chicago, IL)\(^{40}\). Parametricity of log converted cumulative data was examined by Lilliefors test. Differences in the cumulative lactulose, mannitol and rhamnose excretion were evaluated by doubly repeated measure of ANOVA with probabilities of post hoc comparisons subject to Bonferroni correction. Although the cumulative data was inherently autocorrelated, the analysis was considered useful as it provided a comparison of the variation in patterns of excretion of the sugars.

5.3.5.1. Temporal patterns of recovery: curve fitting of raw data

5.3.5.1.1. Absorption of smaller sugars

The overall trends in the temporal patterns of variation of the raw half-hourly recoveries of mannitol and rhamnose of each of the 20 participants were each determined firstly by direct examination of temporal plots. The times at which the peaks in excretion of each of the sugars occurred, i.e. 1.5 – 2 hr for the smaller sugars and 3.5 – 4 hr for lactulose, were determined for each subject and the results compared by ANOVA.

To obtain the profile of excretion of each sugar and establish trends in the data, the general forms of the curves obtained from the pooled data for each subject were
determined by curve fitting in Curve Expert Basic 1.40 in ‘curve finder’ mode. This method ensured that the forms of the curves were not subject to any bias. Differences between the curves obtained for mannitol and rhamnose, and after concurrent dosage with aspirin, were assessed by relevant comparisons of the terms/coefficients and their associated standard errors for each of the derived best-fit mathematical models using Students $t$. The latter was calculated as the difference between the two slopes divided by the standard error of the difference in the two slopes.

The components of the composite curve, i.e. the portions delineated by the time of the initial mannitol peak (2 hr) and the subsequent lactulose peak (4 hr) were considered as a sequence of separate relationships and the overall changes in the percentage recoveries of the various sugars evaluated in each, both with and without concurrent dosage with aspirin. Hence the values in the $2\frac{1}{2}$ - 4 hr and the $4\frac{1}{2}$ - 6 hr periods were each regressed against time and the slopes obtained compared by Students $t$-test using a $p$ value of $< 0.05$ with respect to curve component, to sugar and to treatment. Although it is reasonable to do a comparison of the curves by covariate analysis, this was not performed due to the lack of any correlation between data on height, weight and age of the subjects (using the Pearson's correlation) and the excretion of lactulose and mannitol over the six hr period.

5.3.5.1.2. Direct comparisons of absorption of smaller sugars

Correlations between the amounts of mannitol and those of rhamnose excreted half-hourly were explored by simple linear regression of data obtained from all subjects during the six hr period and compared with a unitary slope representing a null hypothesis that there were no differences in their rates of absorption.
The effects of aspirin on half-hourly excretions of each of the probes were explored by regressing pooled data obtained from all subjects during the first four hrs of collection obtained after consumption of placebo/control (water) against that following consumption of aspirin. The corresponding slopes and their associated standard errors were compared by Students $t$-test with a value of 1 representing the null case, that the rate of absorption of the probe was not influenced by aspirin. A probability of $< 0.05$ was considered significant. We reasoned that an increase or decrease in slope would occur were aspirin to augment or decrease excretion.

Given that estimates of slopes of $Y$ on $X$ and $X$ on $Y$ are biased even when the variables that are being compared have the same units (as was the case in this study) and that, sensu strictu, the geometric mean of the linear regression coefficient of $Y$ on $X$ and of $X$ on $Y$ gives a less biased estimate of slope$^{41}$, we supplemented our comparisons of slopes from simple linear regression against assumed values for the null hypothesis (see below) with comparisons of slopes estimated as the standard major axis$^{42}$, i.e. the ratio of standard deviations ($Sy/Sx$) using the standard error obtained in linear regression in $t$-test comparisons with a unitary slope$^{41}$.

5.3.5.1.3. Direct comparison of the half-hourly rates of absorption of lactulose

The effect of aspirin on the quantity of lactulose recovered in the half-hourly urine samples over the first four hrs of collection was similarly explored by regressing raw data obtained from all subjects after consumption of placebo/control (water) against that obtained after consumption of aspirin. A doubly repeated measure ANOVA for the effect of treatment and time was performed using arcsine transformed data obtained from the Johnson algorhythm in the Minitab 16 statistical package.
5.4. Results

5.4.1. Participants

Twenty one healthy subjects (mean age: 30.5 yr) completed the trial. The results of one subject were excluded from the subsequent analysis owing to failure in dietary compliance. Hence, twenty participants completed the study successfully. No untoward gastrointestinal side effects were experienced by any of the subjects following consumption of the probe solution or aspirin during any of the experimental sessions.

5.4.2. Reliability of the assay

Calibration curves obtained for all 4 sugars were linear up to 500 μg ml\(^{-1}\) and were described by the following equations:

- Lactulose: \(y = 1860 \pm 39.83 x - 12902.5 \pm 8130, R^2 = 1\)
- Mannitol: \(y = 1609.7 \pm 3.19 x - 2202.7 \pm 650, R^2 = 1\)
- Rhamnose: \(y = 1415.2 \pm 6.39 x + 1081.8 \pm 1303.9, R^2 = 1\)
- Cellobiose: \(y = 1710.1 \pm 20.21 x + 7447.9 \pm 4125, R^2 = 0.99\)

The minimum detectable concentration in the standard solution for each sugar was 8 μg ml\(^{-1}\). The limit of detection for the sugars was 62.5 μg ml\(^{-1}\) in baseline spiked urine samples. The accuracy of the assay lay between 93 - 109 % for lactulose, 96 - 101 % for mannitol, 101 – 132 % for cellobiose and 94 - 114 % for rhamnose respectively. The precision and reproducibility of the assay for intra-assay and inter-assay variability (CV %) for lactulose was between 2.7 % and 6.6 %, mannitol was between 2.0 % and 6.2 %, that
for rhamnose was between 2.3 % and 6.2 % and that for cellobiose was between 3.3 % and
16.2 % respectively. The results obtained for cellobiose indicated that the sugar could not
be quantified with a sufficient degree of accuracy using the HPLC system that was
employed for the other sugars hence precluding a meaningful comparison with the other
sugars.

5.4.3. Temporal variation in the recovery of the two smaller sugars,
mannitol and rhamnose and the larger sugar lactulose

The best-fit 6 hr curves selected by the Curve fit algorhythm for temporal variation
of the raw half-hourly percentage recovery data of mannitol and rhamnose after
consumption of either placebo (Figure 5-1a,c) or aspirin (Figure 5-1b,d) were all fourth
order polynomials. Similarly, the best fit curves describing lactulose recovery after
consumption of either placebo (Figure 5-1e) or aspirin (Figure 5-1f), whilst differing from
those of the smaller sugars, were fourth-order polynomials.
Chapter 5

(a) S = 0.27770185  
   r = 0.77106051

(b) S = 0.24416250  
   r = 0.77953721

(c) S = 0.19985366  
   r = 0.72396602

(d) S = 0.17048281  
   r = 0.69700200

(e) S = 0.03606383  
   r = 0.27171151

(f) S = 0.05202189  
   r = 0.25752015
The temporal patterns of probe excretion described by each of these polynomials were bimodal, the later peak being smaller with smaller sugars and larger with lactulose. Hence maximal absorption of the smaller sugars occurred between 1 and 2 hr after dosing (Figure 5-2 a-d) the peak being evident for at least four hrs after dosing. In the case of lactulose the second peak was larger than the first and occurred between 3.5 and 4.5 hr after dosing (Figure 5-2 e,f). The curves of individual subjects were of broadly similar form (Figure 5-1, Figure 5-2, insets). Neither the overall forms of the curves nor the timing of the peaks (Figure 5-1) changed significantly on ANOVA (Table 5-1 and Table 5-2) following dosing with aspirin. Whilst the curves were broadly similar in form, there were significant differences in the coefficients of polynomials between the smaller sugars and following dosage with aspirin which precluded meaningful statistical comparison of the magnitude and timing of the component peaks and the trough between them.

Figure 5-1: Polynomial curve fitted to temporal patterns of variation in percentage recovery of saccharidic probes in half-hourly urine samples from 20 healthy female volunteers after administration of placebo (a,c,e) and aspirin (b,d,f). Insets depict the bimodal pattern of variation for each sugar for one participant. Following administration of placebo, the curves can be described by the following equations: (a) for mannitol: \( y = -(7.18 \pm 0.51)x + (1.69 \pm 0.7)x^2 + (7.54 \pm 0.34)x^3 - (6.17 \pm 0.005)x^4 \) (R\(^2\) = 0.63); (c) for rhamnose: \( y = +(5.97 \pm 0.38)x + (1.01 \pm 0.53)x^2 + (8.99 \pm 0.25)x^3 + (8.59 \pm 0.05)x^4 - (5.08 \pm 0.003)x^5 \) (R\(^2\) = 0.54); and (e) for lactulose: \( y = +(1.93 \pm 1.87)x - (2.09 \pm 2.64)x^2 + (8.51 \pm 1.26)x^3 + (1.30 \pm 0.25)x^4 + (6.48 \pm 0.02)x^5 \) (R\(^2\) = 0.32). Following administration of aspirin, the curves can be described by the following equations: (b) for mannitol: \( y = -(5.86 \pm 0.45)x + (1.52 \pm 0.64)x^2 - (7.19 \pm 0.30)x^3 + (1.18 \pm 0.06)x^4 - (6.67 \pm 0.004)x^5 \) (R\(^2\) = 0.63); (d) for rhamnose: \( y = +(7.38 \pm 0.32)x + (8.29 \pm 0.45)x^2 - (4.12 \pm 0.22)x^3 + (7.22 \pm 0.04)x^4 - (4.36 \pm 0.003)x^5 \) (R\(^2\) = 0.50); and (f) for lactulose: \( y = +(7.17 \pm 1.90)x + (1.27 \pm 2.68)x^2 + (8.87 \pm 0.25)x^3 - (9.09 \pm 0.02)x^4 \) (R\(^2\) = 0.25). S, sample standard deviation.
The temporal profiles of the 2½ - 4 hr and 4½ - 6 hr segments of the plots for the excretion of both mannitol and rhamnose were of broadly linear configuration each with a similar pattern of decrease in rates of excretion with (Figure 5-2 b,d) and without aspirin (Figure 5-2 a,c). The excretion rates in the period 1 - 2 hr showed no such tendency of linear decline. The excretion rates in the period from 4½ - 6 hr declined at a significantly lower rate (Table 5-1) than in the 2½ - 4 hr segments.
Table 5-1: Comparison of the peak defined segments of the temporal pattern of variation of excretion of the three sugars in 20 healthy female volunteers, showing variation of slopes of segments on simple linear regression

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Segment/Time</th>
<th>df</th>
<th>t</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>2.5 - 4</td>
<td>18</td>
<td>0.94</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4.5 - 6</td>
<td>18</td>
<td>0.83</td>
<td>NS</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>2.5 - 4</td>
<td>18</td>
<td>0.78</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4.5 - 6</td>
<td>18</td>
<td>0.71</td>
<td>NS</td>
</tr>
<tr>
<td>Lactulose</td>
<td>2.5 - 4</td>
<td>18</td>
<td>1.43</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4.5 - 6</td>
<td>18</td>
<td>1.43</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Treatment</th>
<th>df</th>
<th>t</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>Placebo</td>
<td>18</td>
<td>3.73</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>18</td>
<td>3.17</td>
<td>0.005</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>Placebo</td>
<td>18</td>
<td>2.22</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>18</td>
<td>2</td>
<td>0.06</td>
</tr>
<tr>
<td>Lactulose</td>
<td>Placebo</td>
<td>18</td>
<td>2.85</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>18</td>
<td>5.71</td>
<td>0</td>
</tr>
</tbody>
</table>

Conversely, the excretion rates in the 2½ - 4 hr and 4½ - 6 hr segments of the plots for the excretion of lactulose (Figure 5-2 e,f) differed in configuration from those of mannitol and rhamnose. Hence in the period from 2½ to 4 hr after dosing, lactulose excretion rates showed a tendency to increase, this increase being more marked after dosing with aspirin. Although lactulose excretion rates showed a tendency to decline in the period from 4½ to 6 hr, the rate of decline was similar after dosing with aspirin (Table 5-1). Hence the mean slopes of lactulose excretion over the periods 2½ - 4 hr and 4½ - 6 hr

Table 5-2: Comparison of the peak defined segments of the temporal pattern of variation of excretion of the three sugars by ANOVA in 20 healthy female volunteers, showing variation in peak mean values between treatments (i.e. aspirin and placebo).

<table>
<thead>
<tr>
<th>Sugar</th>
<th>df</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol (1.5 - 2 hr)</td>
<td>38</td>
<td>1.11</td>
<td>NS</td>
</tr>
<tr>
<td>Rhamnose (1.5 – 2 hr)</td>
<td>38</td>
<td>2.89</td>
<td>NS</td>
</tr>
<tr>
<td>Lactulose (3.5 – 4 hr)</td>
<td>38</td>
<td>0.0</td>
<td>NS</td>
</tr>
</tbody>
</table>
after the consumption of placebo were of opposite sign and each differed significantly from those obtained with mannitol (2½ - 4 hr: d.f 1,18; \( t = 6.56; P < 0.001 \), 4½ - 6 hr: d.f. 1,18; \( t = 3.44; P = 0.003 \) respectively) and rhamnose (2½ - 4 hr: d.f 1,18; \( t = 4.31; P < 0.001 \), 4½ - 6 hr: d.f 1,18; \( t = 2.73; P <0.05 \) respectively). Again, after dosage with aspirin, the mean slopes of lactulose excretion differed significantly from those of mannitol (2½ - 4 hr: d.f 1,18; \( t = 3.93; P = 0.001 \), 4½ - 6 hr: d.f 1,18; \( t = 2.73; P < 0.05 \) respectively) and rhamnose, this only during the 2½ - 4 hr period (d.f 1,18; \( t = 2.18; P < 0.05 \)). It is however noteworthy that the slope of the SLR for the decline in lactulose excretion during the period 4½ - 6 hr following consumption of aspirin was not significant (Figure 5-2).

5.4.4. Cumulative recoveries of probes

5.4.4.1. Following consumption of placebo

The quantities of mannitol (d.f 1,19; \( F = 181.76; P < 0.001 \)) and rhamnose (d.f 1,19; \( F = 94.82; P < 0.001 \)) recovered were significantly greater in urine samples collected over the first three hrs than over the second 3 hr (Table 5-3). Conversely, the quantities of lactulose recovered in the urine samples collected over the first three hrs were significantly (d.f 1,19; \( F = 17.37; P < 0.001 \)) lower than those collected over the second 3 hrs.
Table 5-3: Cumulative recoveries of lactulose, mannitol and rhamnose in urine samples expressed as the percentage recovery of the ingested dose

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Mannitol</th>
<th>Rhamnose</th>
<th>Lactulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>First 3 hr</td>
<td>Placebo</td>
<td>5.20 ± 0.26§ *</td>
<td>3.25 ± 0.22*</td>
<td>0.16 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>4.64 ± 0.24‡ †</td>
<td>2.73 ± 0.21‡</td>
<td>0.29 ± 0.10‡</td>
</tr>
<tr>
<td>Second 3 hr</td>
<td>Placebo</td>
<td>2.65 ± 0.20* †</td>
<td>1.64 ± 0.19*</td>
<td>0.27 ± 0.09†</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>2.23 ± 0.19‡ †</td>
<td>1.50 ± 0.17‡</td>
<td>0.40 ± 0.10‡</td>
</tr>
<tr>
<td>Entire 6 hr</td>
<td>Placebo</td>
<td>8.09 ± 0.32* †</td>
<td>5.07 ± 0.30‡</td>
<td>0.48 ± 0.10†</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>7.09 ± 0.30‡ †</td>
<td>4.40 ± 0.27‡</td>
<td>0.75 ± 0.15‡</td>
</tr>
</tbody>
</table>

Results expressed as Mean ± SEM. * P < 0.001 for placebo 0-3 h versus 3.5-6 hr; † P < 0.001 for aspirin 0-3 hr versus 3.5-6 h; ‡ P < 0.05 for placebo versus aspirin

5.4.4.2. Following consumption of aspirin

Although significantly greater quantities of mannitol (d.f 1,19; F = 175.86; P < 0.001) and rhamnose (d.f 1,19; F = 78.58; P < 0.001) continued to be recovered in urines collected over the first 3 hr than the second 3 hr, the total quantities of mannitol recovered were generally lower after dosing with aspirin (Table 5-3). Hence, the quantities of mannitol recovered during the first 3 hr (d.f 1,19; F = 8.70; P < 0.05), the quantities recovered during the second 3 hr (d.f 1,19; F = 7.14; P < 0.05) and the total quantities recovered over the entire 6 hr (d.f 1,19; F = 9.26; P < 0.001) were all significantly lower than those over the corresponding period after consumption of placebo. Similarly the quantities of rhamnose recovered during the first 3 hr (d.f 1,19; F = 15.79; P < 0.05) and the total quantities recovered over the entire 6 hr (d.f 1,19; F = 5.19, P < 0.05) were significantly lower after consumption of aspirin than after consumption of placebo whilst the quantities recovered in the second 3 hr were not (Table 5-3).
Significantly greater quantities (d.f 1,19; F = 16.55; P < 0.001) of lactulose continued to be recovered from urines collected over the second 3 hr than from those collected over the first 3 hr (Table 5-3). There were significant (d.f 1,19; F = 10.85; P < 0.001) increases in the total quantities of recoveries of lactulose recovered from urines collected over the entire 6 hr period following dosage with aspirin in comparison to those following consumption of the placebo.

These results fit in with the changes in ratios of large to small sugars according to collection period (Table 5-4). The estimated hourly excretion rates of the probes, varied with the duration of the collection periods both with and without aspirin (Table 5-4). Hence, the estimated hourly rates of urinary excretion for mannitol, rhamnose and lactulose both with placebo and with aspirin were similar with collection periods of 2 and 3 hr, although the estimated mean hourly rates of excretion in the 3 hr period fell consistently below those in the 2 hr period of collection. Further, with a 4 hr collection period, the estimated hourly rates of excretion of mannitol and rhamnose fell whilst those of lactulose increased considerably. These changes are reflected in the ratios of small to large sugars, those based on 4 hr collections being around three times larger than those based on 2 hr collections (Table 5-4).
Table 5-4: Variation in the percentage excretion of urinary lactulose, mannitol and rhamnose over collection periods of differing duration in 20 healthy female participants

<table>
<thead>
<tr>
<th>Duration</th>
<th>Mannitol Placebo</th>
<th>Mannitol Aspirin</th>
<th>Rhamnose Placebo</th>
<th>Rhamnose Aspirin</th>
<th>Lactulose Placebo</th>
<th>Lactulose Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hr Ratio Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.26 ± 0.16</td>
<td>2.05 ± 0.21</td>
<td>1.41 ± 0.10</td>
<td>1.18 ± 0.09</td>
<td>0.06 ± 0.007</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.025-0.028</td>
<td>0.049-0.066</td>
<td>0.040-0.044</td>
<td>0.082-0.118</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 hr Ratio Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.93 ± 0.14</td>
<td>1.72 ± 0.12</td>
<td>1.15 ± 0.10</td>
<td>0.99 ± 0.09</td>
<td>0.05 ± 0.007</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.024-0.028</td>
<td>0.048-0.056</td>
<td>0.038-0.049</td>
<td>0.086-0.095</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 hr Ratio Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.27 ± 0.11</td>
<td>1.09 ± 0.09</td>
<td>0.81 ± 0.10</td>
<td>0.69 ± 0.07</td>
<td>0.10 ± 0.017</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.072-0.085</td>
<td>0.170-0.178</td>
<td>0.117-0.129</td>
<td>0.274-0.276</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as Mean ± SEM. *The ratio range is for both lactulose:mannitol and lactulose:rhamnose

5.4.5. Analysis of the half-hourly rates of recoveries of the probes

5.4.5.1. Comparisons of the rates of excretion and effects of aspirin on smaller sugars

There was a significant correlation ($R^2 = 0.828$) and a significant linear regression (d.f 1,218; $F = 1046; P < 0.001$) of the half-hourly rates of excretion of mannitol (abscissa) with those of rhamnose (ordinate) after consumption of placebo (Figure 5-3). The slope of the regression line was significantly lower than unity (d.f 1,18; $F = 32.34; P < 0.001$) whilst the intercept did not differ from zero. The slope of the reduced major axis (RMA) (0.67 ± 0.02) did not differ from that of the linear regression and was similarly below unity (Figure 5-3). Hence the amount of rhamnose excreted in a given half-hour was generally lower than that of mannitol when any differences in the errors of measurement in the two treatments for probe were factored out by the RMA procedure.
The data points of the regression of the half-hourly rates of excretion of mannitol obtained over the first 4 hr following consumption of aspirin against those following consumption of the placebo all lay below the line of direct equivalence (slope = 1) (Figure 5-4a) as did those for regression of the half-hourly rates of excretion of rhamnose over the same time period following consumption of aspirin against those without aspirin (Figure 5-4b).

Further, the slopes obtained from this regression for mannitol (Figure 5-4a) and for rhamnose (Figure 5-4b) were both significantly less than unity on t-test (d.f. 1,18; \( t = 7.33; P < 0.05 \) and d.f 1,18; \( t = 8.34; P < 0.05 \)) respectively. The slopes from the reduced major axis regression for mannitol (0.90 ± 0.06) and rhamnose (0.83 ± 0.06) were similarly below unity, that for rhamnose being significantly lower on \( t \) test (d.f 1,18; \( t = 2.83; P < 0.05 \)).
Hence, the amount of rhamnose or mannitol excreted in a given half-hour was generally lower after the consumption of aspirin when any differences in the error of measurement in the two treatments were factored out by the RMA procedure.

![Figure 5-4](image)

**Figure 5-4:** Relationship between half-hourly urinary excretion of (a) mannitol and (b) rhamnose after administration of the placebo to that after aspirin (600 mg) over a 4 hr period. Data pooled from 20 healthy female volunteers. Solid lines indicate slope obtained on simple linear regression (SLR). The reduced major axis (RMA) is also indicated. For (a) mannitol, $y = 0.556 \pm 0.06x + 15.02 \pm 3.03$, $R^2 = 0.38$; for (b) rhamnose, $y = 0.499 \pm 0.06x + 10.05 \pm 1.79$, $R^2 = 0.36$
5.4.5.2. Rates of excretion of lactulose and effects of aspirin

There was no significant correlation of the half-hourly rates of excretion either of mannitol or of rhamnose with those of lactulose either with or without aspirin dosage. Similarly there was no significant correlation of the excretion rates of lactulose with and without aspirin. However the bulk of the data points on the graph relating recovery with and without aspirin (Figure 5-5) lay above the line of direct equivalence indicating that rates of excretion were generally higher following dosage with aspirin. Comparison of the two sets of arcsine transformed \((y = -2.71 + 1.04 \times \text{Asinh}(\frac{x-0.18}{0.51}))\) values by doubly repeated measures ANOVA showed significant (d.f 1,18; \(F = 13.68; P = 0.002\)) elevation of excretion of lactulose after dosage with aspirin with no significant variation in this elevation between subjects.

Figure 5-5: Relationship between half-hourly urinary excretion of lactulose after administration of placebo to that after administration of aspirin (600 mg) over a 4 hr period. Data pooled from 20 healthy female volunteers. Although there was no significant linear relationship, the arcsine-transformed half-hourly excretion rates of lactulose were significantly (d.f 1,18; \(F = 13.68; P = 0.002\)) higher on doubly repeated measures ANOVA following dosage with aspirin than those following consumption of the placebo.
5.5. Discussion

The consistent bimodal variation in the rate of excretion of each probe over time fits in with reports of previous workers indicating that the permeability of the gut mucosa, and hence rates at which larger and smaller sugar probes are absorbed, varies between the large and the small intestine\textsuperscript{3, 16}. The lack of a clear separation between the component peaks, evidenced by the significant variation between the constants for the terms of the polynomials, is presumably in part due to differences in the relative size and digestive capacity of the gut in the different subjects influencing the mixing and dilution of the column of digesta containing the probes as it traverses the gut. Nevertheless, the differences in the patterns of excretion between the 2½ - 4 hr and 4½ - 6 hr components of the temporal plots for smaller sugars and lactulose are consistent with differences in the relative permeabilities of segments of the gut to the different sized probes. Hence the consistently declining excretion of mannitol and rhamnose during the 2½ - 4 hr and 4½ - 6 hr periods and the significantly lower rate of decline in the 4½ - 6 hr than in the 2½ - 4 hr indicate that the column of digesta from which these sugars are absorbed moves during this time from a segment that is more ‘permeable’ to one that is less permeable to these probes (Figure 5-2). This fits in with the work by Rao \textit{et al.} demonstrating that a greater proportion of mannitol is absorbed in the small intestine than the colon\textsuperscript{3}. Similarly, the rising rate of lactulose excretion during the period 2½ - 4 hr and the subsequent decline in the 4½ - 6 hr period, together indicate that the column of digesta from which these sugars are absorbed moves during this time from a segment that is less ‘permeable’ to traverse one that is more permeable to this probe. Again this work fits in with that reported by Rao \textit{et al.}, however it is noteworthy that the rate of excretion of lactulose in our study was lower than that following direct instillation into the colon\textsuperscript{3}. 
These temporal, and likely segmental, differences in absorption rates of different probes are reflected in the variations of the cumulative rates of excretion, and of the ratios of larger to smaller sugars, with the duration of the sampling period. Hence it is important to consider the duration of the sampling period in undertaking, or comparing the results from, clinical tests of permeability for example in the quantification of primary or secondary disaccharidase deficiencies when urine samples are being bulked over a 3 or a 4 hr period. It may also be useful to standardize the timing of the test to reduce any chronobiological variance and to use fasted subjects so as to minimize the confounding effect of the differing transit time and consequent differences in contribution from colonic absorption.

Lactulose, mannitol and rhamnose are small intestinal permeability probes and once enter the colon undergo degradation by colonic bacteria. The total recoveries obtained for each sugar in the study are within the reported ranges in previous published literature. However, the differences in the rates and relative quantities of smaller sugars excreted raise questions regarding the comparability of studies that use these probes that were previously considered interchangeable. The significantly greater cumulative excretion of mannitol than rhamnose, particularly during the first 3 hr, with correspondingly greater half-hourly rates of excretion of mannitol throughout the period of collection, together indicate a generally greater permeability of the mucosa to mannitol. This conclusion is supported by the direct regression of half-hourly excretion rates of rhamnose against those of mannitol also indicating that the overall rate of excretion of rhamnose is lower than that of mannitol. Given that the doses of the two sugars were administered on a gram for gram basis, the higher molecular weight of mannitol should cause its molar concentration to be slightly lower in the gut lumen which would decrease its rate of absorption. However the rate at which mannitol was absorbed was higher despite this disproportion. Whilst previous
reports indicate that absolute rates of absorption of the probes cannot be predicted from their molecular characteristics\textsuperscript{21-23} the result does fit in with their relative molecular geometries in that the hydrated molecular volume of mannitol is greater\textsuperscript{23, 30} than that of rhamnose. Hence whilst its diffusion rate is lower, the quantities absorbed will be correspondingly augmented by solvent drag\textsuperscript{44, 45}.

This study is the first to report that the rates of excretion and total amounts of the smaller sugars excreted are both reduced at the same time as those of lactulose are increased following dosage with aspirin. Previous studies have reported that the excretion of mannitol and of rhamnose is reduced in subjects with active coeliac\textsuperscript{46-48} and inflammatory disease of the small intestine\textsuperscript{49, 50} and attributed this to a reduction in absorptive surface area\textsuperscript{9} for example by villous atrophy\textsuperscript{51}. Given that such a reduction is unlikely in healthy subjects following the consumption of a single 600 mg dose of aspirin, this suggests that aspirin has a specific influence on absorption via the apical membrane. Hence a general effect of aspirin on the structure of the phospholipid membrane\textsuperscript{31} could lead to changes in the configuration and function of embedded apical pore proteins\textsuperscript{32}. Alternatively a similar change in the configuration of pore proteins could result from the intracellular effects of absorbed aspirin, i.e. the local inhibition of cyclooxygenase with consequent depletion of prostaglandins\textsuperscript{52}. Again the molecular size of aspirin (180 Da) is similar to that of mannitol (182 Da) and is slightly greater than that of rhamnose (164 Da). It is also possible that aspirin competes physically with mannitol and rhamnose for entry into the pore as it is of similar molecular size.

In contrast with the cumulative excretion of the smaller sugars, which is more pronounced in the first than in the second 3 hr, the cumulative quantity of lactulose excreted was consistently elevated after dosage with aspirin and the half-hourly rates of excretion are correspondingly elevated. Hence it seems likely that the absorption of
lactulose was enhanced by the creation of and or stabilization of longstanding defects in the lipid membrane along the entire length of the tubiform intestine, perhaps at or near the tight junctions\textsuperscript{31}. Although the excretion of lactulose was uniformly increased in the subjects in our study and we found no subjects in whom there was an unduly large response, we acknowledge that sensitivity to aspirin may be genetically predetermined\textsuperscript{50} and thus that subjects may on occasion be more sensitive to it. Indeed this latter variation may be clinically important as it could indicate mucosal disturbance in latent inflammatory conditions\textsuperscript{50}. It is also possible, when subjects with pre-existing inflammatory conditions are dosed with aspirin, that this could reduce inflammation, augment mucosal integrity and have a restorative effect on permeability.

In conclusion, the results of the current work provide some insight as to the limitations of using ratios of the large and small sugars in clinical tests. Our results support the findings of previous workers that the pattern of absorption of larger and smaller saccharidic probes is complicated by differences in the dynamics of absorption in the proximal and distal tubiform gut which are exemplified by biphasic excretion curves. Further that absorption dynamics can be more complex in that the administration of aspirin, a drug that is known to augment the absorption of larger probes\textsuperscript{50}, also reduces the absorption of smaller probes. The latter finding could explain differences in previously published permeability ratios following dosage with aspirin\textsuperscript{50, 53}. Our findings also indicate the need for standardization of the test sugars that are used in clinical testing as their absorption appears to some extent to be influenced by their physicochemical properties.

It is important to note a limitation of this study in respect of gender. Whilst the few studies that document variation in the excretion of the sugars, and hence intestinal permeability\textsuperscript{54-56}, with gender are inconclusive, it is possible that such differences\textsuperscript{35, 56} could
influence the timing and magnitude of the temporal GI absorption profiles of these sugars. Hence more work is needed to verify that similar dynamics apply in male subjects.

5.6. Afterword

The manner in which the above study was conducted and the use of appropriate statistical methods for analyzing the data, received accolades in an Editorial by Klaus and Zarogiannis (Appendix 6) in Clinical and Experimental Pharmacology and Physiology. The sensitivity and reproducibility of the simple non-invasive standardized lactulose mannitol aspirin augmented test helped elucidate that the absorption of smaller similar sized sugars across the intestinal mucosa is physiologically governed by their physicochemical properties. Further that the temporal patterns of absorption could provide a basis for identifying the residence time of the column of digesta containing each of the sugars as it moves distally along the different segments of the GI tract.
Chapter 5

5.7. References


MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the Statement of Originality.

Name of Candidate: Ivana Roosevelt Sequeira

Name/Title of Principal Supervisor: Prof. Roger Graham Lentle

Name of Published Research Output and full reference: Sequeira IR, Lentle RG, Kruger MC, Hurst RD. Assessment of the effect of lactulose and mannitol and other liquids on digesta residence times in various segments of the gut using a proprietary wireless motility capsule.

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%
  and / or
- Describe the contribution that the candidate has made to the Published Work:

__________________________  
Ivana Sequeira  
Candidate's Signature

15.12.2014  
Date

__________________________  
Principal Supervisor’s signature

22/1/2015  
Date

GGS Version 3– 16 September 2013

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CHAPTER 6

Assessment of the effect of intestinal permeability probes (lactulose and mannitol) and other liquids on digesta residence times in various segments of the gut determined by wireless motility capsule: a randomised control trial

This Chapter assessed the use of the SmartPill to determine GI motility patterns in response to the drinks used in the expanded standardized lactulose mannitol test. The determination of segmental residence times would help improve the understanding of the relationship between the absorption of the probe sugars across the mucosa and their passage through the various segments of the gut.

This chapter is in final review for publication as: Sequeira IR, Lentle RG, Kruger MC, Hurst RD. Assessment of the effect of intestinal permeability probes (lactulose and mannitol) and other liquids on digesta residence times in various segments of the gut using a proprietary wireless motility capsule: a randomized control trial. 2015 PloS one
6.1. Abstract

**Background:** Whilst the use of the mannitol/lactulose test for intestinal permeability has been long established it is not known whether the doses of these sugars modify transit time. Similarly it is not known whether substances such as aspirin that are known to increase intestinal permeability to lactulose and mannitol and those such as ascorbic acid which are stated to be beneficial to gastrointestinal health also influence intestinal transit time.

**Methods:** Gastric and intestinal transit times were determined with a SmartPill following consumption of either a lactulose mannitol solution, a solution containing 600 mg aspirin, a solution containing 500 mg of ascorbic acid or an extract of blackcurrant, and compared by doubly repeated measures ANOVA with those following consumption of the same volume of a control in a cross-over study in six healthy female volunteers. The dominant frequencies of cyclic variations in gastric pressure recorded by the Smartpill were determined by Fast Fourier transforms.

**Results:** The gastric transit times of lactulose mannitol solutions, of aspirin solutions and of blackcurrant juice did not differ from those of the control. The gastric transit times of the ascorbic acid solutions were significantly shorter than those of the other solutions. There were no significant differences between the various solutions either in the total small intestinal or colonic transit times. The intraluminal pHs during the initial quartiles of the small intestinal transit times were lower than those in the succeeding quartiles. This pattern did not vary with the solution that was consumed.

The power of the frequencies of cyclic variation in intragastric pressure recorded by the Smartpill declined exponentially with increase in frequency and did not peak at the reported physiological frequencies of gastric contractile activity.
Conclusions: Whilst the segmental residence times were broadly similar to those using other methods, the high degree of variation between subjects generally precluded the identification of all but gross variation between treatments. The lack of any differences between treatments in either total small or large intestinal transit times indicates that the solutions administered in the lactulose mannitol test of permeability had no consistent influence on the temporal pattern of absorption. The negatively exponential profile and lack of any peaks in the frequency spectra of cyclic variation in gastric intraluminal pressure that were consistent with reported physiological frequencies of contractile activity profile suggests that the principal source of this variation is stochastic likely resulting from the effects of external events occasioned by normal daily activities on intra-abdominal pressure.

6.2. Introduction

The lactulose mannitol (LM) test is well established in the clinical investigation of intestinal permeability\(^1\text{3}\). Latterly it has been demonstrated that the rates of absorption and excretion of the probe sugars vary significantly with time from dosage\(^4\text{, 5}\). A body of evidence indicates that there are segmental differences in permeability of the mucosa to lactulose and mannitol\(^6\). However, little work has been done to directly assess whether the osmolarities of these two sugars or the presence of substances such as aspirin and ascorbic acid, which are used in a recently developed LM test that is adapted to assess gut health, influence gut segment transit times.

The lack of work in this field is largely due to the lack of suitable techniques. Several methods have been used to assess gut segment transit times notably radiographic techniques, dosage with metabolizable markers that can be detected in the breath tests\(^7\) and gamma scintigraphy\(^8\text{, 9}\). Thus gastric transit time can be determined indirectly by quantifying
carbon dioxide in expired air after consumption of $^{13}$C labelled octanoic acid$^{10}$ and small intestinal transit time similarly from excretion of $^{13}$C labelled glycosyl ureides$^{11}$. However these methods do not directly assess the physical passage of the gut contents$^{12}$. Again, whilst the use of scintigraphy may be well suited to the sequential determination of small intestinal$^{13}$, large intestinal and whole gut$^{14}$ transit times of pharmaceuticals, the technique requires dosage with isotopes such as Technetium 99 m$^{15}$.

Recent developments in wireless telemetry have made it possible to assess compartmental and total residence times by identifying changes in ambient luminal pH and pressure recorded during the transit of a wireless motility capsule (WMC)$^{15}$. These capsules have recently become commercially available (SmartPill Corporation, Buffalo, NY, USA), are approved for use in determination of these parameters by the American and European Neurogastroenterology and Motility Societies$^{15}$ and have minimal harmful effects$^{16}$. The use of the WMC to check compartmental transit times in relation to passage of permeability probes is thus convenient and non-invasive$^{17}$.

Some technical difficulties remain, thus for example the WMC may empty relatively promptly from the stomach if ingested during the intermeal interval, i.e. phase III of the migrating motor complex cycle (MMC) compared with that ingested during the postprandial period$^{18}$. Hence the manufacturers advise that concurrent administration of a small amount of nutrient, such as glucose, with the WMC may prevent this occurring. Given that no mechanisms operate within the human small or large intestine to selectively retain either solid or liquid matter$^{19}$, it is likely that the WMC will transit at a similar rate to other particulate matter and in synchrony with the liquid phase, especially during the inter meal interval. This hypothesis is supported by data showing strong correlation between the gastric emptying of a radiolabelled meal and that of the WMC$^{20}$ and between colonic transit times determined using radio opaque markers and those determined using the WMC$^{21}$.
Hence it appears that transit times derived directly from the transit of the WMC may be as clinically relevant as those derived indirectly by other clinically accepted methods\textsuperscript{22}.

The purposes of the current work were firstly to identify whether the doses of lactulose and mannitol that are used in the LM test of intestinal permeability had any effect on the transit times of a proprietary WMC, the SmartPill\textsuperscript{®}. Secondly, to determine whether a 600 mg dose of soluble aspirin that is used to promote intestinal permeability in a recent modification of the LM test designed to assess gut health\textsuperscript{4} had any effect on Smartpill transit times. Thirdly, to determine whether ascorbic acid (a weak organic acid) or blackcurrant extract, substances that have been shown to promote gastrointestinal wellbeing; by ameliorating oxidative damage to the mucosa\textsuperscript{23, 24, 25, 26} and by influencing the growth of beneficial groups of microflora\textsuperscript{27, 28} respectively, had any effect on Smartpill transit times. These comparisons were necessary as any alteration in the timings of the peaks in absorption of the sugar probes could influence assessments of gut permeability.

6.3. Method

6.3.1. Ethical Approval

Ethics approval was obtained for the study from the Massey University Human Ethics Committee: Southern A 12/42. Before they commenced the trial subjects were required to give written informed consent. Participants were recruited from October 2012 to February 2013. The trial was successfully registered with the Australian New Zealand Clinical Trials Registry (ANZCTR): ACTRN12615000596505.
6.3.2. Selection and screening of subjects

Six healthy female participants between 20 – 40 years of age (mean age: 30 yr) were recruited. Female participants were used as prior work in developing a more accurate methodology for the LM test\textsuperscript{29} and work modifying the test to include dosage with an agent that increased intestinal permeability\textsuperscript{4, 5} had used only female participants. Further, that other work had identified significant variation in intestinal permeability with gender\textsuperscript{30, 31}. There is no data available regarding variation of gastric and intestinal motility and residence time using the SmartPill and the drinks used in the expanded lactulose mannitol test. Hence data reporting variance between normal healthy subjects which could provide a meaningful test of statistical power were not available. Six subjects were chosen based on previous studies using the SmartPill that have used a similar sample size and obtained statistically significant results\textsuperscript{32, 33}.

Whilst the likelihood of chronic gastric retention of the SmartPill is known to be low\textsuperscript{15}, we restricted participants to those of medium body size on the assumption that they would have a larger gastric volume and pyloric diameter\textsuperscript{34}. Again subjects were restricted to those with a body mass index (BMI) of $< 35 \text{ kg m}^{-2}$ so as to avoid any attenuation of the WMC signal by overlying abdominal adipose tissue and interference with detection by the data logger\textsuperscript{35}. Hence the recruited participants were 162 – 172 cm tall (mean: 167 cm) and weighed 60 – 73 kg (mean: 66 kg).

All subjects were screened with a health questionnaire (Appendix 7) to exclude those with a personal or strong family history of chronic gastrointestinal (GI) disorders or recent abdominal pain, nausea, vomiting, diarrhea, passage of blood and mucus in stools. Similarly subjects with a history of dysphagia, gastric bezoars, strictures, fistulas, bowel obstructions, diverticulitis, or previous GI surgery\textsuperscript{33} were excluded. Subjects with an implanted electromechanical medical device, smokers, those with an intake of alcohol in
excess of one standard drink per day, those with aspirin sensitivity and those taking regular
prescription or over the counter medications (OTC) were also excluded. Similarly those
subjects who consumed drugs that were likely to influence GI transit time, those
consuming vitamins, prebiotics or probiotic supplements such as lactulose. All participants
were reviewed by a clinician to validate their medical history and their responses to the
questionnaire before they were admitted to the study.

Subjects consumed self-selected diets during the study period but were asked to
avoid consuming foods and fruits that contained high levels of ascorbic acid or
anthocyanins (Appendix 8). They also were asked to avoid consuming NSAIDs or alcohol
for three days prior to and during the study period22, 35 and to avoid performing any
vigorous exercise during this time32.

6.3.3. Experimental protocol

Each participant received each of the five treatments in a randomized sequence at
weekly intervals, the latter calculated from the day when the SmartPill was voided. The
randomization procedure for the order in which each subject received the various
treatments was generated by a computer program. On the evening prior to each
experimental session, each participant was required to consume a plain meal that was low
in fat and fibre and to subsequently fast overnight. Upon arrival at the Human Nutrition
Unit (*Massey University, Palmerston North, New Zealand*) each participant was given 250 ml of
water to drink immediately. Fifteen min later they were fitted with a data-logger which was
attached to a lanyard and maintained in close proximity to the stomach. They then
swallowed the activated and calibrated WMC with 250 ml of one of five treatment
solutions that also contained 5 g of glucose. Hence the five treatment solutions were water
(placebo); 10 g lactulose (Duphalac®, Solvay Pharmaceuticals, NSW, Australia) and 5 g mannitol (Sigma-Aldrich, St. Louis, MO, USA) mixture; 600 mg aspirin (Disprin®; Reckitt Benckiser Healthcare, UK), 500 mg ascorbic acid (Hawkins Watts, New Zealand) and a blackcurrant extract containing 1167 mg total anthocyanins (Just the Berries Ltd, New Zealand). A brief description of the drinks is detailed in Table 6-1.

Table 6-1: Characteristics of the various treatment solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Content</th>
<th>Amount (ml)</th>
<th>Osmolarity (mOsmol/L)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>Water + 5 g glucose*</td>
<td>250</td>
<td>103</td>
<td>7.54</td>
</tr>
<tr>
<td>Lactulose mannitol</td>
<td>Water + 10 g lactulose + 5g mannitol +5 g glucose*</td>
<td>250</td>
<td>372</td>
<td>6.59</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Water + 600 mg soluble aspirin + 5 g glucose*</td>
<td>250</td>
<td>129</td>
<td>5.30</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Water + 500 mg Ascorbic acid + 5 g glucose*</td>
<td>250</td>
<td>113</td>
<td>3.45</td>
</tr>
<tr>
<td>Blackcurrant extract</td>
<td>Water + 1167 mg anthocyanin + 5 g glucose*</td>
<td>250</td>
<td>116</td>
<td>3.85</td>
</tr>
</tbody>
</table>

*=5 g glucose added to avoid prompt gastric emptying on advice of the Smartpill manufacturer

Each participant was subsequently monitored over seven hrs in the human nutrition suite. The participants were not allowed to eat during the study period; however they consumed 200 ml water four hrs after commencement of the session. Participants were provided with a standardized meal at the conclusion of the experimental session before their departure. They were asked to keep the data logger/receiver near their abdomen at all times. Hence during activities such as taking a shower, they were instructed to place it in a dry area, no less the 5 feet from them. They were also required to carry documentation with them at all times which would inform
any medical personnel that they had ingested the SmartPill. Each participant was asked to complete an activity diary (Appendix 11) noting the timing of meals, the passage of stools, and the occurrence of gastrointestinal symptoms such as pain/discomfort, nausea, vomiting etc. and to press the ‘event’ button on the data recorder to time mark each of these events. Participants were asked to attend the laboratory on subsequent days, after collecting (Appendix 12) and bringing with them all stools they had voided. Each stool was collected into a time labelled container and held for three min after voiding to allow any decrease in ambient temperature to be detected by the data logger. This procedure was repeated until the capsule had been voided and identified. The data logger was subsequently downloaded via the software provided by the manufacturer (MotiliGI software, Given Imaging Corp).

6.3.4. Data processing and statistical analysis

Gastric emptying time (GET), small bowel transit time (SBTT) and colonic transit time (CBTT) were determined on a basis of change in pH, pressure and temperature profiles in a similar manner to that described by previous workers\textsuperscript{15, 32, 33, 36} (Figure 6-1).
Chapter 6

Strong pyloric contractions associated with the capsule moving into the duodenum

Proximity to pancreatic duct increases the pH

Exit from the small intestine

Lower pH due to SFAs. Gradual increase in the pH in the proximal and middle colon as the SFAs are absorbed.

**Entire Test**

Gradually falling pH in the stomach

GET

SBTT

CBTT
Hence gastric residence times were determined as the interval from the time of ingestion of the capsule to that of a rapid rise in pH (> 3 pH units) subsequent to the asymptotic intragastric decline in pH, that was synchronous with the time of cessation of pressure waves in excess of > 200 mmHg pressure. It is noteworthy that the retention times of the WMCs was likely to have been somewhat greater than the maximum residence times of the ingested liquid as residual particulate solids of greater than 1 mm in linear dimension are known to exit the stomach after concurrently ingested liquid on commencement of phase III of the MMC cycle.

The times at which the intragastric pHs stabilized after the initial exponential decline were determined by visual inspection of a plot of Log pH vs time \((\text{OriginLab, Northampton, MA})\). This enabled the half times \((T_{1/2})\) of the exponential intragastric decline in pH for each of the five treatments to be determined. The mean \(T_{1/2}\) values following each of the five treatments in the six participants required transformation in the Johnson algorithm of the Minitab 16 statistical package to render them amenable to parametric analysis. This software fits a functional transformation with outcome that best fits a normal distribution. Variation in transformed \(T_{1/2}\) values with treatment was assessed by repeated measures ANOVA.

Small intestinal residence times were taken from the time when the phasic generation of pressures in excess of 200 mmHg ceased to the time when there was a
sustained fall in pH of at least 1.3 pH units below the mean small intestinal pH, i.e. pH > 4. This fall in pH was presumed to result from the production of short chain fatty acids (SCFA) from bulk fermentation of oligosaccharides by colonic microbiota. The variation in pH during small intestinal transit was examined over four equal consecutive time periods, i.e. quartiles. The variations in pH during the four quartiles within each of the five treatments were compared by doubly repeated measures ANOVA.

Colonic transit times were determined from the time of the commencement of the fall in pH approximately by 1.3 pH units that marked the commencement of colonic fermentation until the time when the temperature dipped sharply below body temperature as a result of expulsion of the WMC. The colonic trace was divided into two periods on the basis that the capsule was more likely to be in the proximal colon during the initial period and in the distal colon in the latter period. Hence differences in the pH levels during the two periods were compared by doubly repeated measures ANOVA.

We determined the range of frequencies in the pressure recordings during the time when the WMC was in the gastric lumen by Fast Fourier Transform and then assessed the relative proportion of the frequency spectrum that was close to reported frequencies of antral contractions in the stomach, i.e. around 3 cycles per minute (cpm). The time series data for the pressure recordings were extracted from the MotiliGI software (Given Imaging Corp) and spectral density functions produced by Fourier transform in Matlab R2012a (The Mathworks, Inc., Massachusetts, US) for each participant following the consumption of the placebo.

Any data that was not normally distributed was transformed in the Johnson algorithm of the Minitab 16 statistical package. This software fitted a function that best transformed the data set to one with a normal distribution. Statistical analyses were conducted in the SYSTAT statistical software package version 13 (Systat Software Inc., Chicago, IL).
Differences in the transit times within segment following the consumption of aspirin, ascorbic acid, blackcurrant extract, lactulose mannitol solution and the placebo drink were assessed by repeated measures ANOVA of appropriately transformed data with the probabilities of post hoc comparisons corrected by the Bonferroni algorithm. In order to provide statistical statements that are suitable for inclusion in future comparisons degrees of freedom (d.f) and F values were included in all statements in addition to probabilities.

Covariance between the total transit times of the three major gut segments within subjects was also explored by multivariate analysis (principal component analyses) in the Minitab 16 statistical suite. Analyses were conducted using both the raw pooled data from the three segments and pooled data that were together transformed with the Johnson algorithm.

6.4. Results

All six participants successfully completed the trial. No untoward gastrointestinal side effects, GI disturbances or retention of the pill were reported by any of the subjects during any of the experimental sessions following the intake of the SmartPill.

The gastric (-0.31 + 0.47*Asinh((X -1.29)/0.09), small bowel (0.22+0.57*Asinh ((X-4.90)/0.32) and colonic (0.46+0.82*Ln((X+1.49)/(133.20-X)) total transit times of all six participants each required Johnson transformation to render them suitable for comparisons between treatments within segments by ANOVA. All gastric half-time data also required arcsine transformation (1.42+1.61*Asinh((X-0.38)/0.11) to render them amenable to ANOVA.
The quartile values for small intestinal pH also required arcsine transformation (1.93+0.73*Asinh((X-0.73)/0.08)) to render them amenable to ANOVA.

All colonic pH data for the proximal and distal halves of colonic residence time were normally distributed and amenable to ANOVA.

The raw pooled untransformed transit times for each segment from each subject were used for multivariate analysis as well as the pooled Johnson transformed data (1.03+0.46*Ln((X-0.43)/(117.24-X)).

6.4.1. Stomach

The overall mean gastric emptying time taken over all subjects for all treatments was 1.72 ± 0.2 hr with large variation between subjects. Hence analysis of transformed values showed that the overall gastric emptying times for the placebo drinks (plain glucose solution) (mean: 1.36 ± 0.23 hr), did not differ significantly from those containing glucose, lactulose and mannitol (mean: 1.79 ± 0.31 hr), those containing aspirin and glucose (mean: 2.08 ± 0.83 hr) or blackcurrant juice and glucose (mean: 2.30 ± 0.47 hr) (Figure 6-2). However the transformed overall gastric emptying times for the ascorbic acid and glucose drink (mean: 1.07 ± 0.19 hr) were significantly shorter (d.f 1,4; F = 30.88; P = 0.005) than those following consumption of solutions containing glucose, lactulose and mannitol.
Figure 6-2: Variation with treatment in overall gastric emptying times determined by the wireless motility capsule in six healthy females. There were no significant differences in gastric emptying times between placebo (glucose solution) and solutions containing either lactulose and mannitol, aspirin or blackcurrant juice with the transformed data. However the solution containing ascorbic acid emptied significantly more slowly \((P = 0.005)\) on ANOVA than that containing lactulose and mannitol.

The transformed half times based on the exponential decline in intragastric pH up to the point of stabilization differed significantly between the various treatments \((d.f \ 4,20; \ F = 4.57; \ P = 0.009)\). Hence the gastric half times for the placebo drink were significantly shorter (mean: 0.16 ± 0.03 hr) than those for either the aspirin drink (mean: 0.31 ± 0.04 hr) \((d.f \ 1,5; \ F = 10.21; \ P < 0.05)\), the lactulose mannitol drink (mean: 0.33 ± 0.04 hr) \((d.f \ 1,5; \ F =12.59; \ P < 0.05)\) or the blackcurrant drink
(mean: 0.32 ± 0.03 hr) (d.f 1,5; F = 11.31; P < 0.05). Again, the half-time for the ascorbic acid drink (mean: 0.22 ± 0.03 hr) did not differ significantly from that for the placebo drink but was significantly lower than that for the aspirin (d.f 1,5 ; F = 6.9; P = 0.05) and blackcurrant drinks (d.f 1,5; F = 6.25; P = 0.05).

The spectral densities of the pressure recordings obtained when the WMC was in the stomach, following the consumption of the placebo solution, were all negative exponentials with no peaks visible at or around the reported frequencies of antral contractions (Figure 6-3).

Figure 6-3: Spectral density of a fast Fourier transform (FFT) of variation in gastric pressure determined by wireless motility capsule in a single healthy female after consumption of the placebo solution.
6.4.2. Small Intestine

The overall mean small intestinal emptying time for all subjects over all treatments was $4.63 \pm 0.22$ hr but there was large variation between subjects. Analysis of transformed values showed that there were no significant differences between the overall small intestinal transit times for the placebo solution (mean: $4.38 \pm 0.35$ hr), and those for either the lactulose mannitol solution (mean: $4.64 \pm 0.68$ hr), the aspirin solution (mean: $4.03 \pm 0.36$ hr), the blackcurrant solution (mean: $5.12 \pm 0.63$ hr) or the ascorbic acid solution (mean: $4.98 \pm 0.34$ hr) (Figure 6-4).

Figure 6-4: Variation with treatment in small bowel emptying times determined by wireless motility capsule in six healthy females.
The ambient pHs during successive quartiles of the total time taken for the SmartPill to traverse the small intestine following consumption of the placebo solution differed significantly (d.f 3,12; F = 12.67; P < 0.001). Hence, the pH levels during the first quartile were lower than those in the subsequent quartiles (Table 6-2) and generally rose progressively in succeeding quartiles.

Table 6-2: Variation in pH over four equal consecutive quartiles of small intestinal residence time

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>5.23 ± 0.27†</td>
<td>6.61 ± 0.15‡</td>
<td>7.35 ± 0.10* •</td>
<td>7.48 ± 0.04‡ •</td>
</tr>
<tr>
<td>LM</td>
<td>4.49 ± 0.65‡</td>
<td>6.60 ± 0.15‡</td>
<td>7.42 ± 0.05* •</td>
<td>7.52 ± 0.06‡ •</td>
</tr>
<tr>
<td>BC</td>
<td>5.37 ± 0.25‡</td>
<td>6.85 ± 0.19‡</td>
<td>7.19 ± 0.08‡</td>
<td>7.18 ± 0.09‡</td>
</tr>
<tr>
<td>AA</td>
<td>4.96 ± 0.27‡</td>
<td>6.37 ± 0.13‡</td>
<td>7.21 ± 0.10‡</td>
<td>7.33 ±0.06*</td>
</tr>
<tr>
<td>Aspirin</td>
<td>5.19 ± 0.21‡</td>
<td>6.80 ± 0.10‡</td>
<td>7.34 ± 0.07‡</td>
<td>7.50 ± 0.06‡</td>
</tr>
<tr>
<td>Overall</td>
<td>5.06 ± 0.17‡</td>
<td>6.64 ± 0.07‡</td>
<td>7.30 ± 0.04*</td>
<td>7.40 ± 0.04‡</td>
</tr>
</tbody>
</table>

Results expressed as Mean ± SEM; LM = lactulose mannitol, BC = blackcurrant extract, AA = ascorbic acid

Q1, Q2, Q3, Q4 = quartiles of the small intestine residence time

Differences in superscripts within rows (‡, †, *, †, * ) indicate significant differences (P < 0.05) between quartiles

Similarly the ambient pHs during successive quartiles for the lactulose mannitol solution (d.f 3,12; F = 23.18; P < 0.05), the ascorbic acid solution (d.f 3,12; F = 28.62; P < 0.001) and the blackcurrant drink (d.f 3,12; F = 5.61; P < 0.05) all differed significantly, with the patterns of difference generally similar to those for the placebo a with progressive rise in pH over the first two quartiles (Table 6-2).
6.4.3. Colon

The mean overall colonic transit time was 53.6 ± 5.58 hr. The overall colonic transit times following the consumption of the placebo solution (mean: 55.67 ± 10.61 hr) did not differ significantly from those for either lactulose mannitol solution (mean: 60.27 ± 15.80 hr), the aspirin solution (mean: 57.81 ± 10.57 hr), the blackcurrant solution (mean: 39.03 ± 8.71 hr) or the ascorbic acid solution (mean: 55.16 ± 17.25 hr) (Figure 6-5).

The overall pH levels in the initial half of the total colonic residence time were significantly lower (d.f 1,5; $F = 11.81; P < 0.05$) than those during the latter half (Table 6-3). There were no significant differences with treatment in the patterns of mean pHs during the initial and the subsequent half of the total colonic residence (Table 6-3).

![Figure 6-5: Variation with treatment in colonic emptying times determined by the wireless motility capsule in six healthy females.](image-url)
Table 6-3: Variation in pH in the initial and latter half of the colonic residence time with various treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial period (proximal colon)</th>
<th>Latter period (distal colon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>6.22 ± 0.24</td>
<td>7.20 ± 0.09</td>
</tr>
<tr>
<td>LM</td>
<td>6.12 ± 0.14</td>
<td>7.36 ± 0.27</td>
</tr>
<tr>
<td>BC</td>
<td>6.20 ± 0.28</td>
<td>7.08 ± 0.14</td>
</tr>
<tr>
<td>AA</td>
<td>5.98 ± 0.17</td>
<td>7.26 ± 0.34</td>
</tr>
<tr>
<td>Aspirin</td>
<td>6.35 ± 0.37</td>
<td>7.32 ± 0.14</td>
</tr>
<tr>
<td>Overall</td>
<td>6.17 ± 0.11†</td>
<td>7.25 ± 0.09†</td>
</tr>
</tbody>
</table>

Results expressed as Mean ± SEM; LM = lactulose mannitol, BC = blackcurrant extract, AA = ascorbic acid

Differences in superscripts within rows (‡, †) indicate significant differences (P < 0.05) in pH between the proximal and distal colon.

### 6.4.4. Multivariate comparison of segment residence times

The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.48. Using raw data the first component, PC1, represented 43% of the variance in the residence time of the three components of the gut, with successive components representing 33.3% and 23% respectively. Retention of the first 2 components yielded a model that accounted for 76.3% of variance (Table 6-3). The weightings of the first component indicated the total residence times in the large intestine (-0.71) were inversely correlated with those in the stomach (0.47) and small intestine (0.53). Similar results were obtained when pooled then transformed data were used.
Table 6-4: Results of Principle component analysis of gastric, small and large intestinal transit times

<table>
<thead>
<tr>
<th>Component</th>
<th>Initial Eigenvalues</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>% of Variance</td>
<td>Cumulative %</td>
</tr>
<tr>
<td>1</td>
<td>1.292</td>
<td>43.064</td>
<td>43.064</td>
</tr>
<tr>
<td>2</td>
<td>0.999</td>
<td>33.31</td>
<td>76.374</td>
</tr>
<tr>
<td>3</td>
<td>0.709</td>
<td>23.626</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Weighting of 1st Component</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>GET</td>
<td>47</td>
</tr>
<tr>
<td>SBTT</td>
<td>53</td>
</tr>
<tr>
<td>CTT</td>
<td>-71</td>
</tr>
</tbody>
</table>

6.5. Discussion

Broadly speaking, the determination of compartment residence times from changes in pH appears to give values that fit in with reports based on other methods of determination\(^{13, 48}\). The lack of any significant variation in the overall gastric, small intestinal and large intestinal transit times obtained with the different drinks indicated that none of their components greatly influenced the passage of digesta through the various segments. Moreover the mean gastric transit time of the placebo solution (1.36 ± 0.23 hr) in the present study was close to that reported in another study using the SmartPill (1.53 ± 0.73 hr)\(^{18}\) in which 200 ml radiolabelled water was given to fasted subjects. The volume of water given in the latter study was reported to be insufficient to halt periodic phase III MMC induced contractions\(^{18}\). Hence it is likely that the glucose that was added to each drink in our study did not abolish phase III of the MMC cycle. Apart from the
ascorbic acid drink; there were no differences between the gastric residence times of the various drinks used in our study. In this respect it is noteworthy that the ascorbic acid drink had the lowest pH (Table 6-1) and induced the lowest intra gastric pH. Given that the lactulose and mannitol solution contained the same concentration of the two sugars that are used in the improved Lactulose Mannitol test for intestinal permeability⁴,⁵, it is evident that the dose levels of the sugars will not influence transit times.

The mean small bowel transit times over all treatments was 4.63 hr, a value that is well within the overall range (3 – 6 hr) reported by other workers⁴⁸ for healthy individuals, as was that of the lactulose mannitol solution (4.64 ± 0.68 hr). Both values were close to the mean transit time determined on meta-analysis¹³ of 201 gamma scintigraphic assays of the passage of various pharmaceutical dosage forms in healthy human subjects. The lack of any significant differences between the overall transit times of the various solutions through the small intestine indicates that lactulose and mannitol did not alter the transit of digesta through the small intestine during phase III of the MMC cycle. Hence, whilst the administration of lactulose has been reported to accelerate small intestinal transit⁴⁹,⁵⁰, this did not occur with the concentrations that were used in the study and thus would be unlikely to occur with the standardized test of intestinal permeability⁴,⁵.

The overall pattern of rise in ambient pH in consecutive quartiles of total small intestinal transit time following ingestion of the placebo fits in with reports that the capacity for the buffering in chyme increases distally along the small intestine⁵¹. Hence the lower pH in the proximal small intestine likely resulted from chyme having recently exited the stomach; whilst the higher pHs in the later quartiles presumably result from admixture with alkaline pancreatic and brush border secretions. This pattern of increase in intraluminal pH, and hence of buffering, appears robust as similar patterns of pH were obtained with all five drinks.
The mean overall colonic transit time (53.59 hr) was within the range reported for healthy individuals, i.e. between 24 - 60 hr\(^4\). The differences in lumen pH during the initial half of the total colonic transit time from those in the later half supports the hypothesis that increasing absorption of SCFAs generated by fermentation progressively lowers the pH of the colonic content\(^41,42\).

The spectral density profile of pressure recordings taken whilst the WMC was in the cavity of the stomach, did not peak at or around the known frequencies of antral slow wave associated contractions\(^44,45\). The regular exponential form of the spectral density curve that was found in this study is thus likely to result from a series of random fluctuations in extraneous pressure over a wide range of frequencies rather than physiological events associated with the lumen. Hence the use of the motility index to detect peristalsis (calculated from the software as \(\log_e(\text{sum of amplitude x number of contractions + 1})/\text{time}\)) may be inappropriate in ambulant or mobile subjects. We suggest that the use of the capsule to detect and analyse gastrointestinal motility may lack fidelity and needs further investigation\(^52\).

The similarity of the gastric residence time to that reported during phase III MMC\(^18\) indicates that the dose of glucose was insufficient to terminate this activity. Hence, under the conditions of the standardized lactulose mannitol test\(^4,5,29\), none of the agents in the drinks is likely to influence transit time through a particular segment of gut. The finding also raises questions as to whether glucose-mediated negative feedback to gastric and other on flow\(^53\) operates efficiently during phase III MMC.

Together the data indicate that the administration of the solutions in the expanded lactulose mannitol test does not change the temporal characteristics of transit through any particular segment. Hence, it is likely that the administration of any of these solutions will not result in significant delay or hurry of the passage of chyme and thus will not displace
the peak time of excretion so as to influence the absorption of lactulose or mannitol at a
given time after dosage.

6.6. References


Chapter 7

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate’s Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate’s contribution as indicated below in the Statement of Originality.

Name of Candidate: Ivana Roosevelt Sequeira

Name/Title of Principal Supervisor: Prof. Roger Graham Lente

Name of Published Research Output and full reference: Sequeira IR, Lente RG, Kruger MC, Hunt RD. Standardizing the Lactulose Mannitol Test of Gut Permeability to Minimize Error and Promote Comparability. PLOS one 2014; 9(6):e92256

In which Chapter is the Published Work: Chapter 7

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

and/or

- Describe the contribution that the candidate has made to the Published Work:

Ivana Sequeira

Candidate’s Signature

15.12.2014

Date

Principal Supervisor’s signature

Date

GRS Version 3–16 September 2011
Standardizing the lactulose mannitol test of gut permeability to minimise error and promote comparability

Chapter 7 addresses the lack of standardization in test protocols with respect to the timing at which urine samples are collected to accurately reflect small intestinal permeability. Given the results from the previous studies, it is evident that the temporal and segmental patterns of absorption of lactulose and mannitol can be used as a basis for identifying residence time within the different segments of the GI tract. The lactulose mannitol data from study cohorts in Chapter 4 and Chapter 5 were pooled, to obtain a larger data set for analysis, as a consistent reproducible response to both sugars was obtained with and without the aspirin challenge. It was reasoned that the data could be pooled for analysis based on the lack of any significant difference in the urinary excretion of lactulose and mannitol, in the two study cohorts, by ANOVA.

This chapter has been published as: Sequeira IR, Lentle RG, Kruger MC, Hurst RD. Standardising the Lactulose Mannitol Test of Gut Permeability to Minimise Error and Promote Comparability. PloS one 2014;9(6):e99256.
7.1. Abstract

**Background:** Lactulose mannitol ratio tests are clinically useful for assessing disorders characterized by changes in gut permeability and for assessing mixing in the intestinal lumen. Variations between currently used test protocols preclude meaningful comparisons between studies. We determined the optimal sampling period and related this to intestinal residence.

**Methods:** Half-hourly lactulose and mannitol urinary excretions were determined over 6 hrs in 40 healthy female volunteers after administration of either 600 mg aspirin or placebo, in randomized order at weekly intervals. Gastric and small intestinal transit times were assessed by the SmartPill in 6 subjects from the same population. Half-hourly percentage recoveries of lactulose and mannitol were grouped on a basis of compartment transit time. The rate of increase or decrease of each sugar within each group was explored by simple linear regression to assess the optimal period of sampling.

**Results:** The between subject standard errors for each half-hourly lactulose and mannitol excretion were lowest, the correlation of the quantity of each sugar excreted with time was optimal and the difference between the two sugars in this temporal relationship maximal during the period from 2½ - 4 hr after ingestion. Half-hourly lactulose excretions were generally increased after dosage with aspirin whilst those of mannitol were unchanged as was the temporal pattern and period of lowest between subject standard error for both sugars.

**Conclusions:** The results indicate that between subject variation in the percentage excretion of the two sugars would be minimized and the differences in the temporal patterns of excretion would be maximized if the period of collection of urine used in clinical tests of small intestinal permeability were restricted to 2½ - 4 hr post dosage. This
period corresponds to a period when the column of digesta containing the probes is passing from the small to the large intestine.

### 7.2. Introduction

A number of tests based on the passive absorption of simple sugars have been used to assess gut permeability as an index of recovery from inflammatory bowel disease (IBD)\(^1,\ 2\) and from autoimmune diseases such as coeliac disease\(^3,\ 4\) The ratio of the quantities of urinary lactulose and mannitol excreted during a given period has been used most frequently\(^5,\ 6\), although a number of workers have used rhamnose rather than mannitol\(^7,\ 8\). All three probe sugars are passively absorbed from the intestine, not extensively metabolized, and excreted unchanged in urine in proportion to the quantities absorbed\(^9\).

The division of the quantity of lactulose excreted by the quantity of mannitol excreted, i.e. the use of ratios, was postulated to compensate for any differences in the surface area of the intestinal mucosa between subjects\(^10,\ 11\). However subsequent work has demonstrated a number of factors that may confound the basis of such comparisons as they render the result dependent upon the type of sugar used and the time period over which the cumulative urinary excretion was determined. Firstly, the rate of excretion of the two ‘reference’ sugars that are assumed to reflect surface area, i.e. mannitol or rhamnose, is relatively reduced in a number of inflammatory conditions, a phenomenon that has been attributed to villus atrophy\(^12\). Secondly, the overall rates of excretion of the two reference sugars differ, that of mannitol being significantly higher than that of rhamnose\(^13\). Thirdly, the rates of excretion of lactulose and mannitol vary with time from dosage due to the differing permeabilities of the small intestine and colon to these sugars\(^14,\ 15\). These
differences cause the ratio of the quantities of the two sugars to vary with the period of
time over which their excretion is determined. Thus, the early peak in mannitol excretion
causes the LMR to be lower when cumulative excretion is assessed over 2 hrs whilst the
later peak in lactulose excretion and lower rate of mannitol excretion 4 hrs after dosage will
cause the LMR to increase when cumulative excretion is assessed over 4 or more hrs\textsuperscript{13}. These effects may be magnified in the presence of a pro-inflammatory condition\textsuperscript{16-18} or a
pro-inflammatory stimulus such as a single 600 mg dose of aspirin\textsuperscript{19, 20} as these will cause
the overall excretion of lactulose to increase\textsuperscript{4, 21} and that of mannitol to be reduced\textsuperscript{13}. These
various problems have led researchers to suggest that temporal profiles of absorption of
the two sugars should be considered separately as well as in a ratio\textsuperscript{22}.

In view of these complexities there is a need for the test protocol to be
standardized to facilitate meaningful comparisons between populations. Further, to adopt a
time at which urine samples should be collected during which the rates of excretion of the
probe sugars are broadly similar between subjects so as to the maximize sensitivity of the
test.

Hence the purpose of the current work was to assess the temporal profiles of
absorption of lactulose and mannitol in a large sample of fit subjects following
administration of either placebo or aspirin in a randomized sequence. Thereby to determine
the optimal period over which urine samples should be collected, so as to minimize
between subject experimental error with due regard to the maximum retention times of the
sugar probes in the stomach and in the small intestine as determined with the SmartPill\textsuperscript{14}. 
7.3. Materials and Methods

7.3.1. Use of aspirin

Aspirin was used as a pro-inflammatory stimulus on the grounds that it induces a reproducible temporary elevation in permeability as determined by lactose mannitol permeability tests\textsuperscript{23-26}. The rapid increase in permeability is thought to be due to the topical effect of the drug\textsuperscript{27} that involves the partitioning of aspirin into the lipid membrane to alter biophysical properties\textsuperscript{28}.

7.3.2. Screening and experimental protocol

All participants were recruited from a fit, healthy female population of postgraduate students in Palmerton North, New Zealand of mixed ethnicity. Female subjects were chosen so as to avoid any gender differences that could influence the recovery of the sugars due to differences in gastric emptying times\textsuperscript{29}. The subjects were screened by a health questionnaire and a clinical interview to exclude participants with GI disorders, those taking ongoing prescriptions or over the counter (OTC) medication, prebiotic, probiotic or vitamin supplements and those who had more than a moderate alcohol intake. Similarly, the screening procedure excluded subjects with any history of current urinary tract infections, vaginal discharge and aspirin sensitivity. Written consent was obtained from all participants before the start of the study as outlined by the Massey University Human Ethics Committee approval procedure.

Although the menstrual cycle has not been shown to have any effect on the recovery of the sugars\textsuperscript{30} or on gastric emptying\textsuperscript{31}, all experimental sessions in both studies were conducted during periods when the participants were not menstruating. This was done to avoid contamination of the urine samples with blood. Participants were instructed
to refrain from taking any NSAIDs for at least a week prior to the test, to refrain from consuming alcohol for three days prior to the test, to avoid exercise on the day prior to and on the morning of the test and to fast overnight before attending the test. Each experimental session commenced at 8.00 am when a baseline urine sample was collected from each subject. The order of administration of the treatments (aspirin or placebo) was randomized and double blinded between the two sessions. Each participant received either a placebo drink (100 ml of water) or 600 mg of soluble aspirin (Dispirin®; Reckitt Benkinser Healthcare, UK) in 100 ml of water at each session. One hr after receiving the treatment, each subject consumed a solution containing 10 g lactulose (Duphalac®, Solvay Pharmaceuticals, NSW, Australia) and 5 g D-mannitol (Sigma-Aldrich, St. Louis, MO, USA) followed immediately by a drink of water. A total of 500 ml of fluid (including the treatment and sugar solution) was therefore consumed by each participant over the first hr. Half-hourly urine collections were commenced after the ingestion of the sugar solution and continued for a period of 6 hrs. No food was consumed during the entire urine collection period but a further 200 ml of water was given three hrs after the ingestion of the sugar solution. Hence the total amount of fluid administered over the seven hr period was 700 ml. This regime was adopted so as to facilitate half-hourly urine sample collection.

Ethics approval for this part of the study was granted by the Massey University Human Ethics Committee Southern A: 09/79, 11/37. Urinary lactulose and mannitol were quantified with an HPLC system (Shimadzu, Japan) with a refractive index detector using a method described and validated in a previous trial\textsuperscript{13,15}. 
7.3.3. Determination of transit time of the sugars solutions through the stomach and small intestine

Gastric and small intestinal transit time were determined using a wireless pressure and pH sensitive capsule, the SmartPill (SmartPill Corporation, Buffalo, NY)\textsuperscript{14} (see below) in six healthy female subjects between 20 – 40 years of age (mean age: 30 yr) who were recruited from the same population and who had been similarly screened. As in the permeability study, each participant attended the laboratory at 8.00 am following an overnight fast. On arrival at the laboratory, each participant consumed a 250 ml drink of water. Fifteen min later they were fitted with the SmartPill receiver/data-logger which was attached to a lanyard that maintained it in close proximity to the stomach. They then swallowed the activated and calibrated SmartPill device along with a 250 ml drink containing 10 g lactulose, 5 g mannitol and 5 g glucose. Hence the volume of water consumed was identical to that used in the first study. Each participant was subsequently monitored for seven hrs\textsuperscript{32}. As in the permeability study, the participants were not allowed to eat or drink during the study period but were given a further 200 ml water mid-way through the session. Ethics approval for this part of the study was obtained from the Massey University Human Ethics Committee Southern A 12/42.

A body of work has been published describing the use of the SmartPill in determination of gastric and small intestinal transit times\textsuperscript{33-35} to solids and liquids and the device has been approved for the determination of these parameters in a clinical context\textsuperscript{14}. The use of the motility capsule is considered a viable alternative to scintigraphy in the assessment of whole gut transit time\textsuperscript{36}. The transit times are calculated from changes in the temporal profile of pH and temperature from the data-logger using the MotiliGI\textsuperscript{TM} software supplied\textsuperscript{14, 32, 33, 36}. Hence gastric residence time was determined as the interval taken from the time of ingestion of the capsule to that of the subsequent rise in pH above
that of the asymptotic intra gastric decline on entry into the duodenum\textsuperscript{36} (> 3 pH units). It is noteworthy that such retention time was likely to have been at or above the maximum residence time of the ingested liquid as a body of evidence indicates that particulate solids of greater than 1 mm in linear dimension exited the stomach after concurrently ingested liquid\textsuperscript{35}, i.e. during phase III of the MMC cycle\textsuperscript{37, 38}. The small bowel residence time was taken from the time at which the capsule entered the duodenum to that on entry into the caecum when the pH dropped by at least 1 pH unit\textsuperscript{33} below the mean small intestinal pH, i.e. pH > 4\textsuperscript{32} on entry into the caecum.

### 7.3.4. Data analysis

For the first part of the study, the quantities of lactulose and mannitol in each half-hourly urine sample were determined by multiplication of the relevant concentration (mg ml\textsuperscript{-1}) and sample volume and the result expressed as a percentage recovery of the ingested dose. Data analyses were conducted in the SYSTAT statistical software package version 13 (Systat Software Inc., Chicago, IL)\textsuperscript{39}.

The mean gastric and small intestinal residence times determined by passage of the SmartPill were used as reference points for the partitioning of the temporal profiles of urinary excretion according to the times of transit of the column of digesta containing the probes into and out of particular segments of intestine.

The temporal patterns of half-hourly excretions were considered as a sequence of separate relationships and the overall changes in the percentage recoveries of the various sugars were evaluated in each, both with and without concurrent dosage with aspirin. Hence the values in the 2½ - 4 hr and the 4½ - 6 hr groups were each regressed against time and the slopes obtained on simple linear regression compared by Students \(t\)-test with
respect to group, to sugar and to treatment, with a probability of < 0.05 (one tail) being considered as significant.

Mean hourly percentage recoveries for each sugar were derived from the relevant cumulative recoveries over the various time intervals, i.e. 2 hr and 4 hr. Log converted mean hourly percentage recoveries of each sugar were checked for parametricity by the Lilliefors test prior to analysis by doubly repeated measure ANOVA for the effect of treatment and time.

To assess whether any differences in the cumulative recoveries of each sugar could influence the LMR in healthy individuals and in patients with IBDs, we compared the results of our work with those of published studies on subjects with Coeliac or Crohn’s disease\textsuperscript{40-42} that employed similar protocols and doses of lactulose and mannitol. We determined cumulative recoveries over a 5 hr period for each sugar, both after dosage with placebo and after dosage with aspirin, to allow meaningful comparisons with the five hr recoveries reported in these studies. Where possible, the percentage recoveries and associated standard errors were calculated from the reported values in the studies.

Comparisons of the temporal pattern of excretion of mannitol with that of published blood glucose values after standard glucose tolerance test were made by assuming that a blood glucose value in the latter represented the outcome of the instantaneous rate at which glucose was absorbed from the gut vs. that at which it was absorbed by the tissues from the blood. Hence the published temporal sequence of blood glucose values could be converted to quantities of glucose required to maintain the recorded glucose levels above fasting levels over a sequence of half-hour periods by calculating the requisite area under the curve according to the method of Tai \textit{et al.}\textsuperscript{43}. 
7.4. Results

None of the forty sedentary, non-smoking, female participants who completed the first part of the study (mean age: 28.2 yr) experienced any untoward gastrointestinal effects following consumption of the sugar solutions or aspirin. No subjects complained of thirst during the period of urine collection. The overall mean half-hourly urine volumes were $50.9 \pm 5.3$ ml and $50.9 \pm 4.6$ ml following the consumption of the placebo drink and the aspirin drink respectively. The mean pooled six hourly urine volumes were $878.23 \pm 63.33$ ml and $826.76 \pm 50.46$ ml following the consumption of the placebo drink and the aspirin drink respectively.

None of the six sedentary, non-smoking, female participants who participated in the second part of the study (mean age: 30 yr) experienced any untoward effects following dosage with the sugar solutions or the SmartPill and voided the latter within 27 – 120 hrs.

7.4.1. Trends in the percentage recovery of the two probe sugars

The temporal curves of percentage excretion of mannitol and lactulose each exhibited an early and a later peak, the latter being relatively small for mannitol and large for lactulose (Figure 7-1 A & C). The rise in mannitol excretion, which first peaked at around two hrs after dosage, was evident for at least four hrs after dosage. It is noteworthy that the position of the initial peak in mannitol excretion varied from 1 to 2 hrs post dosage between subjects, both after placebo and aspirin (Figure 7-1 A & B, represented by the horizontal bars). The initial peak in lactulose excretion was less evident and was overshadowed by a subsequent larger peak which occurred at around 4 hr. The position of the larger peak in lactulose excretion varied between subjects from $3\frac{1}{2}$ to $4\frac{1}{2}$ hr post
dosage both after the consumption of placebo and after the consumption of the aspirin (Figure 7-1 C & D, represented by the horizontal bars).

Hence the bimodal temporal patterns of the percentage excretion of the two sugars could be categorized into three distinct periods (Figure 7-1). During the first period, which occurred in the interval between 1 and 2 hr after dosage, the percentage recovery of mannitol tended to increase whilst that of lactulose was more variable. During the second period, which occurred in the interval between 2½ and 4 hr after dosage, the percentage recovery of mannitol tended to decline whilst that of lactulose tended to increase. During the third period, which occurred in the interval between 4½ and 6 hr after dosage, the percentage recoveries of both mannitol and lactulose tended to decrease, the decline in lactulose being more rapid.

### 7.4.2. Determination of retention time

The mean gastric emptying time of the six subjects determined from the SmartPill data concurrent with dosage with the lactulose mannitol solution was 1.79 ± 0.31 hr. This was close to the duration of the first period of the temporal curve and the mean time at which the main peak in mannitol excretion occurred (1.79 ± 0.08 hr). Similarly the mean small intestinal emptying time (4.64 ± 0.68 hr) was close to that of the duration of the second phase in the temporal curve and the mean time at which the main peak in lactulose excretion occurred (4.30 ± 0.07 hr).
Figure 7-1: Half-hourly percentage urinary recovery of ingested dose of mannitol (A & B) and lactulose (C & D) in 40 healthy female volunteers following dosage with placebo (A & C) or aspirin (B & D). The classification of the data into periods (I, 0 – 2 hr, corresponding to the passage of probes from the stomach to the SI; II, 2½ - 4 hr, corresponding to passage from the SI to the colon; III, 4½ - 6 hr, corresponding to the passage from the proximal to distal colon) was based on the early peak in mean % recovery of mannitol and on the later peak in mean % recovery of lactulose viewed in conjunction with the data from SmartPill® (see text). Arrows on X axis indicate the time of passage of the SmartPill® into subsequent segments of the gut. Dots indicate the Mean ± SEM of the % half-hourly recoveries of the sugars. Horizontal bars indicate the temporal range of the peak in % recovery of each of the sugars between subjects.
7.4.3. Patterns of variation of each sugar within each period

The temporal profiles of the first period (period I; 0 - 2 hr) did not exhibit consistent patterns between treatments. The temporal profiles of the second (period II; 2½ - 4 hr) and third (period III; 4½ - 6 hr) periods of the temporal plots of excretion of mannitol were of broadly linear configuration, the percentage excretion tending to decrease with (Figure 7-1B) and without aspirin (Figure 7-1A).

The excretion rates of mannitol during the period III declined at a significantly lower rate than during period II both with (Figure 7-2, Table 7-1) and without aspirin (Figure 7-2, Table 7-1). The plots of the excretion rates of lactulose during period II and III differed in configuration from those of mannitol. During period II, lactulose excretion rates showed a tendency to increase, with a greater increase after dosage with aspirin (Figure 7-2, Table 7-1). It is noteworthy that the R² values are low (Figure 7-2) as there is not much variation in the data, the linear regressions used to indicate the general direction of the line (i.e. an increase or decrease in the amount of each sugar excreted during each period) and to highlight the trends in the data. The mean temporal slope of lactulose excretion after the consumption of placebo tended to increase during period II and to decrease during period III (Figure 7-2). Hence each temporal slope differed significantly from those obtained with mannitol (Period II: d.f 1,18; t = 7.07; P < 0.001, period III: d.f 1,18; t = 2.19; P < 0.05 respectively). However, after dosage with aspirin, the mean slopes of lactulose excretion differed significantly from those of mannitol only during period II (d.f 1,18; t = 4.44; P < 0.001). It is however noteworthy that the slope of the SLR for the decline in lactulose excretion during period III following consumption of aspirin was not significant (Figure 7-2). The between subjects standard error was minimized and the correlation coefficient optimized on SLR of values for excretion of both sugars during the 2½ - 4 hr of collection. This, in conjunction with the finding that the slope of excretion
for lactulose differed significantly from that for mannitol during this period, indicates this
time period is optimal for urine collection to assess differences in permeability between the
two sugars.

Table 7-1: Comparison of results of simple linear regressions from period II and III in the
temporal pattern of excretion of lactulose and mannitol in 40 healthy female participants

<table>
<thead>
<tr>
<th>Placebo v/s aspirin for each segment</th>
<th>Between periods (II vs. III) for each treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar</td>
<td>Period (hr)</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>Mannitol</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>III</td>
</tr>
<tr>
<td>Lactulose</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>III</td>
</tr>
</tbody>
</table>

df = degrees of freedom
Figure 7-2: Simple linear regressions of pooled half-hourly percentage urinary recoveries of mannitol and lactulose against time, during period II and period III after dosage with placebo or aspirin, in forty healthy female volunteers. Vertical bars are standard errors.
Chapter 7

7.4.3.1. Variation in cumulative recoveries of the probe sugars with duration of collection

Variations between subjects in the half-hourly percentage recoveries of mannitol, as indicated by the variance and the 95% confidence intervals, were lowest during period II, i.e. between the 2½ and 4 hr after both aspirin and the placebo. Variation between subjects in half-hourly percentage recoveries of lactulose was similarly lowest during this period.

The cumulative percentage recoveries of mannitol and of lactulose varied with the duration of collection both after placebo and after aspirin. Similarly, the calculated mean hourly percentage recoveries that were derived from these cumulative recoveries varied significantly between subjects at 2 hrs and 4 hrs after dosage (Table 7-2). Hence the mean hourly percentage excretion of mannitol declined with time from ingestion both with and without dosage with aspirin (Table 7-2). Conversely the mean hourly percentage excretion of lactulose increased with time from ingestion after dosage with aspirin but again did not differ after dosage with aspirin from that after dosage with placebo. Correspondingly, these differences caused the overall ratio, i.e. LMR, to vary with the duration of the period over which urine samples were bulked (Table 7-2).
Table 7-2: Variation in calculated hourly percentage excretions of urinary lactulose and mannitol with duration of collection period and consequent change in the lactulose:mannitol ratio (LMR) based on data from 40 healthy female participants

<table>
<thead>
<tr>
<th>Duration#(hr)</th>
<th>Mean hourly Mannitol</th>
<th>Mean hourly Lactulose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Aspirin</td>
</tr>
<tr>
<td>2</td>
<td>1.81 ± 0.10</td>
<td>1.97 ± 0.14*</td>
</tr>
<tr>
<td>LMR Range</td>
<td>0.053-0.058</td>
<td>0.071-0.081</td>
</tr>
<tr>
<td>4</td>
<td>1.73 ± 0.08</td>
<td>1.68 ± 0.10*</td>
</tr>
<tr>
<td>LMR Range</td>
<td>0.049-0.054</td>
<td>0.091-0.099*</td>
</tr>
</tbody>
</table>

Data are the Mean ± SEM; Duration: 2 hr = (¼ hr - 2hr)/2; 4 hr = (¼ hr - 4hr)/4
*P < 0.001: 2 hr versus 4 hr; †P < 0.001: placebo versus aspirin

7.4.3.2. Comparisons with published values

The augmented cumulative 5 hr lactulose excretions that were obtained in our studies after dosage with aspirin, were similar to those reported in patients with Crohn’s disease (Figure 7-3) but significantly higher (d.f 2,52; t = 4.17; P < 0.001) than those in the study of subjects with coeliac disease. However the cumulative 5 hr mannitol excretions in the study of the subjects with Crohn’s disease were significantly (d.f 2,85; t = 3.17; P < 0.001) higher than those of our healthy subjects without aspirin. The reported mean LMR from the study of subjects with Crohn’s disease (0.067) was lower than that from subjects in our study who had received aspirin (0.108). Again the cumulative 5 hr mannitol excretion in the study of subjects with coeliac disease was significantly higher than those of the subjects in our study both in the control (d.f 2,52; t = 2.84; P = 0.006) and aspirin (d.f 2,52; t = 2.85; P = 0.006) treatments in our study.
Figure 7-3: Comparison of calculated 5 hr cumulative recoveries of mannitol (A) and lactulose (B) in forty healthy female volunteers following dosage with placebo (a) or 600 mg aspirin (b) with those reported for subjects with pro-inflammatory conditions, i.e. coeliac disease (c 40, d 42) and Crohn’s disease(e 41)

Graphic comparison of integrated half-hourly glucose levels from fifteen subjects with non-insulin dependent diabetes mellitus\textsuperscript{44} with those of half-hourly mannitol excretion obtained from our study, showed that both peaked at approximately two hrs post dosage as would be expected if active absorption of glucose was inhibited and glucose absorbed passively (Figure 7-4). Conversely graphic comparison of the integrated half-hourly glucose levels of sixty seven healthy women (n = 67)\textsuperscript{44} with those of half-hourly mannitol excretion from this study showed that glucose levels peaked earlier than did those of mannitol and
were subsequently attenuated, this presumably resulting from active glucose uptake and disposal (Figure 7-4).

Figure 7-4: Comparison of temporal patterns of half-hourly urinary mannitol (mg) excretion from 40 healthy female volunteers with calculated half-hourly glucose absorbed (mg/½ hr) based on published blood sugar values of women with non-insulin dependent diabetes (NIDDM, n = 12) and those with normal glucose tolerance (NGT; n = 67).

7.5. Discussion

The results of this study, notably the minimization of the between subject standard error, the optimization of the between subject correlation between quantities of each sugar excreted with time and the significant between subject difference in the temporal patterns of excretion of the two sugars during this time period, indicate that the optimal period for
collection of urine used in clinical tests of small intestinal permeability is between 2½ - 4 hr post dosage. The variation in the LMR would be similarly minimized. Moreover, in the light of the concurrent SmartPill studies, the characteristics of the temporal profiles of the excretion of the two sugars suggest that this period is optimal as it avoids incorporation of gastric residence time which varies greatly between individuals. Hence, the commencement of collection at 2½ hr will ensure that in the majority of subjects the bulk of the dose of each probe will have exited the stomach and the column of digesta containing them will be advancing steadily through the small and large intestine, the low inter-subject variation reflecting the relative lack of variability between subjects in the rates of passive absorption from these structures.

Given that the permeability of the small intestine (SI) to mannitol is higher than that of the large intestine (LI)²² and that the permeability of the LI to lactulose is higher than that of the SI²², the temporal profiles of the half-hourly percentage excretion of the two saccharidic probes, fit with hypotheses that a column of digesta occupies an increasing length of small intestine as it leaves the stomach¹³,⁴⁵ and that the leading edge of this column is transiting from the small to the larger bowel at the time when the trailing edge has left the stomach.

This conclusion, that a column of digesta containing the probe occupies and is absorbed from, a length of intestine, is supported by a body of data describing the quantitative dependence of hormonal feedback loops that modulate gastric emptying on the length of intestine that is occupied by nutrient containing chyme. Hence the rate of gastric emptying is dependent upon the rate of absorption of nutrients⁴⁶ such as glucose⁴⁷ or fat⁴⁸ from the volume of digestate⁴⁹ that is in contact with small intestinal mucosa and thus to the length of small intestinal lumen that contains it.
The fact that the mean retention time of the SmartPill (1.79 hr) was close to the mean time when the absorption of mannitol was maximal (2 hrs after dosage) also fits with this hypothesis given that the SmartPill is likely have been expelled from the stomach after the bulk of the fluid phase was expelled\textsuperscript{37, 38}. Hence, as stated hitherto, the collection period that minimizes variance between subjects will occur when, in most subjects, the column of digesta containing the probes is transiting from the small to the large intestine. Again the rate at which the excretion of mannitol decreases and that of lactulose increases during this period will depend on the rate at which digesta transits from the small to the large intestine which appears to be relatively invariant between subjects and not to be influenced by aspirin.

We could find no published data for gastric emptying time after the consumption of 100 ml of clear fluid containing simple sugars. However mean gastric emptying time evaluated by the $[^{13}\text{C}]$ acetate breath test following a 250 ml liquid meal (Fresubin diabetes, Fresenius, Oberursel, Germany) containing protein, carbohydrate and fat (225 kcal) is reported to be 1.28 ± 0.31 hr\textsuperscript{50}. Given that the volume of the gastric load in relation to the volume of the stomach\textsuperscript{51, 52} and the nutrient content of the meal would influence gastric emptying\textsuperscript{47,49,53}, this value is broadly compatible to that obtained in our study.

The calculated five hr cumulative excretions of the two sugars based on our data are broadly similar to those reported in subjects with pathological disturbances in permeability\textsuperscript{41}. However the importance of examining lactulose and mannitol excretion separately\textsuperscript{22} as well as in simple ratio is manifest in these comparisons. Hence the cumulative 5 hr lactulose excretion reported in subjects with Crohn’s disease\textsuperscript{41} was similar to that in our study following dosage with aspirin whilst the cumulative 5 hr excretion of mannitol was higher than that in our healthy subjects. This caused the mean LMR of 0.067\textsuperscript{41} of subjects with Crohn’s disease to be lower than that in our study after aspirin.
(0.108) and runs contrary to the hypotheses that the absorptive surface area is compromised by inflammatory disease\textsuperscript{16-18}. However, it is noteworthy that this study included patients with differing Harvey Bradshaw grades of Crohn’s disease and included those in remission as well as those with mildly active disease. Again the sites of the lesions varied, some participants having both small and large bowel lesions\textsuperscript{41}. Further, the reported values are from studies that included both male and female patients whereas our study included only females.

The fact that the 5 hr cumulative lactulose excretion reported in one study of subjects with frank coeliac disease was lower than those in our studies and their mannitol excretions higher giving LMRs of 0.146\textsuperscript{42} and 0.018\textsuperscript{40} is further testament to the variability of the LMR and the variability of the excretion of component sugars with differing inflammatory stimuli. These examples likely indicate that inflammation from a disease and from a pro-inflammatory stimulus such as aspirin can have differing outcomes that may be better identified by comparisons based on the temporal profiles of the two probes rather than the ratios of cumulative excretion. Hence for example, inflammatory disorders of the intestine may have greater influence on the rate of transit of the column of digesta containing the probes through particular segments of the gut. In regard to the latter and in regard to the variability in gastric emptying there is a need for further research, for example to determine whether there is a place for simultaneous use of the SmartPill in determinations of permeability.

The absorption of mannitol is passive\textsuperscript{9, 54} and dependent largely on the concentration of the sugar in the periphery of the lumen. As such it can provide an index of the extent of mixing the column of digesta within the lumen that is useful in assessing effects from the physical form of the food or pharmaceutical formulation on absorption. Unlike that of glucose, the temporal profile of mannitol absorption is not augmented by
hormonal effects such as those from insulin/incretin\textsuperscript{55, 56} on active transporters. This is readily illustrated by comparisons of the half-hourly percentage cumulative excretion profile of mannitol with published blood sugar values from oral glucose tolerance tests\textsuperscript{44} that have been integrated\textsuperscript{43} to convert them from instantaneous rate of absorption/storage to the cumulative quantity of glucose required to maintain the half-hourly profile above the baseline value. Graphic comparison of integrated half-hourly glucose levels from twelve subjects with non-insulin dependent diabetes mellitus\textsuperscript{44} with those of half-hourly mannitol excretion obtained from our study shows that both peaked at approximately two hrs post dosage as would be expected if the active absorption or disposal of glucose was inhibited. Conversely graphic comparison of the integrated half-hourly glucose levels of sixty seven healthy women\textsuperscript{44} with those of half-hourly mannitol excretion from this study showed the glucose levels of healthy subjects peaked earlier and were subsequently attenuated, this presumably resulting from normal incretin and insulin mediated augmentation of glucose uptake and disposal.

In summary the results of this study indicate that in healthy subjects the quantities of lactulose and mannitol absorbed from the intestine and excreted in the urine, and the ratios between them, may be confounded by differences in the time periods over which they are collected. Hence collection periods should be standardized for use in clinical tests. In this regard the period of highest levels of consistency of absorption between subjects in assays of the absorption and urinary excretion of lactulose and mannitol was between 2½ and 4 hr judged on the basis of minimal standard error and maximal $R^2$ values of SLR. This period, was shown by concurrent dosage with the SmartPill to correspond to the period when the column of digesta containing the probes was passing from the small to the large intestine. Further, that comparison of the two sugars should include consideration of the absolute amounts of each sugar that are absorbed rather than be restricted to the ratio of
the two, as the amounts of each may be influenced differently by disease. Finally, that comparisons of the temporal patterns of sugars that are passively absorbed such as mannitol with those that are actively absorbed such as glucose may be useful in understanding the effects of diseases such as diabetes mellitus on the mucosal dynamics of absorption.

7.6. Afterword

The identification of a period over which samples can be collected to assess small intestinal permeability is critical to not only the ease at which the test can be conducted but also the accuracy of the test to sample region specific permeability.

7.7. References

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CHAPTER 8

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate’s Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate’s contribution as indicated below in the Statement of Originality.

Name of Candidate:  Ivana Roosevelt Sequeira

Name/Title of Principal Supervisor:  Prof. Roger Graham Lentle

Name of Published Research Output and full reference:  Sequeira IR, Lentle RG, Kruger MC, Hurst RD. Ascorbic acid may exacerbate aspirin induced increase in intestinal permeability.

In which Chapter is the Published Work:  Chapter 8

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:  80%
  and / or

- Describe the contribution that the candidate has made to the Published Work:

Ivana Sequeira  15.12.2014
Candidate’s Signature  Date

Principal Supervisor’s signature  22/1/2015  Date
8a. Can the increase in intestinal permeability associated with a pharmacological dose of ascorbic acid be extrapolated to that from dietary sources/intake?

This addendum to Chapter 8 addresses a key issue given the finding that a 500 mg dose of ascorbic acid increased small intestinal permeability. The chapter aims to understand if a similar effect on intestinal permeability would be achieved from consuming dietary sources of ascorbic acid.

Ascorbic acid is an essential micronutrient in the human diet\(^1\) having both enzymatic and antioxidant properties and is widely used for daily supplementation either alone or as part of a multivitamin. As discussed in Chapter 8, ascorbic acid has been co-formulated with non-steroidal anti-inflammatory drugs (NSAIDs)\(^2-4\) such as aspirin e.g. AspirinPlusC® (Bayer Pharmaceuticals, Germany). Aspirin monotherapy has been shown decrease the levels of ascorbic acid within enterocytes\(^5\) and gastric juice\(^6\) reducing the potential of intestinal mucosal cells to cope with oxidative\(^5\). Hence combined dosage therefore is presumed to ameliorate any NSAID mediated reactive oxygen species (ROS) induced gastrointestinal damage\(^7\).

However, the work in the previous chapter indicates that rather than ascorbic acid mitigating aspirin induced damage of the intestinal mucosa, it increases the absorption of lactulose either when given alone or in combination with aspirin. Hence whilst concurrent dosage with the two agents may be beneficial in the longer term from the antioxidant effects of ascorbic acid, it does not appear to mitigate the short term deleterious effect of aspirin on permeability. Importantly, the fact that dosage with ascorbic acid increases small intestinal mucosal permeability raises questions as to whether similar changes could occur when foods that are rich in ascorbic acid are consumed. It is noteworthy that the dose of 500 mg ascorbic acid used in the study is higher than the recommended daily allowance
which some researchers have proposed be increased to 200 mg in order to maximize the health benefits of ascorbic acid. Given the effect of ascorbic acid in increasing permeability it is important to consider whether a high intake of the vitamin is necessarily beneficial.

Several factors require consideration given that the quantities of ascorbic acid in a given food may vary widely and in fruits and vegetables are governed by variations in growing conditions, stage of maturity, regional and seasonal differences. In the same way that ascorbic acid in solution is susceptible to oxidation, it is likely the relative proportions of the reduced and the oxidized form also vary between different foods according to the levels of hydration. Supplementation of products with the reduced form of synthetic ascorbic acid is known to facilitate oxidation of the product. Furthermore, the addition of glucose to a food product could engender competition with dehydroascorbic acid for GLUT transporters. It is noteworthy that the active absorption of glucose via SGLT1 could further influence the tight junctions indirectly and mucosal permeability.

Of course, the absorption of ascorbic acid would also depend upon the level of interaction of the reduced or oxidized form with other dietary components such as fibre and phytochemicals (i.e. bioflavanoids). Hence, for example, the absorption of ascorbic acid from a juice containing 50 mg ascorbic acid was significantly lower than that from a simple solution of ascorbic acid (doses between 50 - 500 mg), the authors concluding that some micronutrient component within the juice had inhibited ascorbic acid absorption. Flavanoids such as quercetin and myricetin may also decrease the absorption of ascorbate and DHA, in vitro, by binding competitively with transporters. This conclusion is supported by work demonstrating that co-administration of quercetin with ascorbic acid inhibited absorption and decreased plasma levels of the latter.
Again Vinson et al. showed that ascorbate levels following co-dosage with a citrus fruit extract, containing 18% bioflavonoids, 15% proteins and 30% carbohydrates, rose more slowly than those following a simple solution of ascorbate\textsuperscript{19}. A similar effect was found in humans after dosage with citrus fruit extract\textsuperscript{20}. However, this effect appears inconsistent as the administration of ascorbic acid with whole kiwifruit\textsuperscript{21}, or with blackcurrant juice\textsuperscript{22} or orange juice\textsuperscript{23} containing flavanoids did not impede absorption. It seems likely that these differences resulted from the varying effects of the citrus fruit extracts on gastric transit times via duodenal receptors\textsuperscript{24}. Alternatively, the discrepancies in the results in these various studies could have arisen from the differences in the forms in which ascorbic acid was administered, i.e. as solutions or gels, influencing GI residence times and hence the ratios of absorption and appearance of ascorbic acid in the biological fluids tested (i.e. plasma, urine etc.).

In summary, it appears from the inconsistent evidence regarding the effects of other co-administered substances in food that the increased intestinal permeability associated with a 500 mg dose of ascorbic acid cannot be generalized and extended to ascorbic acid in foods. Intervention studies using whole foods or extracts at comparable doses using the dual sugar absorption test to assess permeability would provide a better understanding of their physiological effects on the barrier function of the mucosa. The ability of ascorbic acid to augment absorption of lactulose raises the possibility that the agent could be co-administered with therapeutic substances of similar or larger molecular weight to facilitate their absorption. Indeed, it is conceivable the co-administration of ascorbic acid with aspirin may potentiate the serum levels and analgesic effects of the latter.
8.8. References

17. Park JB, Levine M. Intracellular accumulation of ascorbic acid is inhibited by flavonoids via blocking of dehydroascorbic acid and ascorbic acid uptakes in HL-60, U937 and jurkat cells. The Journal of Nutrition 2000;130:1297-1302.
8b. Does a urine collection period 2½ - 4 hr post dosage optimally assess changes in small intestinal permeability

This section of the addendum to Chapter 8 addressed if an assessment of intestinal permeability made between the 2½ - 4 hr period, when the column of digesta is thought to reside in the small intestine, is suitable to identify mucosal permeability changes.

8b.1 Introduction

Traditionally assessments of small intestinal permeability using the lactulose mannitol test have been conducted over a six hr period\textsuperscript{1-3}. The ratio of the cumulative excretion of the sugars, i.e. the lactulose mannitol ratio (LMR), has been relied upon to determine increased intestinal permeability. However based on the duration of urine sample collection, there is a large variation in the reported values in different studies\textsuperscript{4}. As discussed in the previous chapters, this is primarily because the mucosal absorption of the larger and smaller sugars varies along the length of the intestine\textsuperscript{5}.

Previous work has confirmed that greater amounts of mannitol are absorbed in the small intestine whilst greater amounts of lactulose are absorbed in the colon\textsuperscript{5,6}. The trace of the temporal patterns of excretion of each of the sugars indicated that within 2 hr the column of digesta containing the sugars exited the stomach to move into the small intestine (2½ - 4 hr) and resides in the proximal colon over 4½ - 6 hr period of the total six hr period of urine collection\textsuperscript{6}. This was ascertained using a wireless motility capsule (WMC), the SmartPill. On the basis of both the WMC recordings and the temporal patterns of excretion of the sugars it was concluded that the column of digesta, following the consumption of the sugar solution, is contained in the small intestine over 2½ - 4 hr period\textsuperscript{7}.
In Chapter 8, the effect of a 500 mg dose of ascorbic acid on intestinal permeability was assessed over the first (0 - 3 hr) and second (3 - 6 hr) three hr period of the total 6 hr urine collection period. These consecutive three hourly time intervals were used for analysis as it allowed differences in the excretion of mannitol over the first three hrs (0 - 3 hr) and lactulose over the second three hours (3 - 6 hr) to be determined. The current chapter aims to assess if the recommendation of the 2½ - 4 hr duration is sufficiently sensitive to pick up minor changes small intestinal permeability following the administration of a single 600 mg dose of aspirin, a 500 mg dose of ascorbic acid and the combined dosage of both agents. It is reasoned that over this period any variance in the recoveries of lactulose and mannitol between subjects that could result from differences in gastric emptying would be reduced. Further that the shorter period of urine collection would reduce the burden of sample collection for researchers and participants.

8b.2 Data analysis

The data collection was as described in Chapter 8. The quantities of each sugar excreted over each half-hour period were determined by multiplication with the sample volume and the percentage recovery of the ingested dose of each excreted probe calculated. Hence values for cumulative samples were calculated by summing the component half-hourly recoveries (concentration times volume) of lactulose and of mannitol that were excreted over the 2½ - 4 hr period.

In a similar manner to the analysis conducted in Chapter 8, due to absence of the use of a placebo control (water), aspirin was used as a positive control in the study. The effect of aspirin on lactulose and mannitol excretion was with a similar dose of 600 mg aspirin used in a previous published experiment7 over the 2½ - 4 hr period.
Statistical analyses were conducted using the SYSTAT statistical software package version 13 (Systat Software Inc., Chicago, IL)\(^8\). The percentage recoveries of lactulose and mannitol were transformed using the *Johnson algorithm* in the Minitab 16 statistical package\(^9\) to render them amenable to parametric analysis. The calculated percentage excretion of each probe sugar during the 2½ - 4 hr period were each assessed by repeated measures ANOVA, for the effect of treatment (after administration of aspirin alone, ascorbic acid alone and after simultaneous dosage with both agents) with the probabilities of *post hoc* comparisons corrected by the *Bonferroni algorithm*.

The differences in the percentage excretions of lactulose and mannitol following dosage with aspirin from the study were each compared with those following the consumption of aspirin and placebo in the prior experiment\(^7\).

### 8b.3 Results

The half-hourly percentage recoveries of lactulose and mannitol were transformed using the *Johnson algorithm* \([\text{lactulose}: (y = 1.04+1.13*\ln((X+0.014)/(1.37-X))\text{ and mannitol } (y = -0.77+1.48*\text{asinh}((X-2.15)/1.67))\text{ respectively}]\) for sixty eight participants (includes the results from the 40 subjects from the previous experiment\(^7\)) and were found to be normally distributed on the *Kolmogorov Smirnov-Lilliefors test*. 
8b.3.1 Effects of the administration of ascorbic acid and aspirin

Excretion of mannitol

There were no significant differences in the amount of mannitol excreted following dosage with aspirin alone, ascorbic acid alone and the combined dosage with both agents (Figure 8b-1).

Figure 8b-1: Variation in recovery of mannitol (% ingested dose) with treatments over the 2½ - 4 hr of urine collection in 28 healthy female participants. *A= single dose of 600 mg aspirin, C = 500 mg ascorbic acid, CA= combined dosage of 500 mg ascorbic acid and 600 mg aspirin. Differences in letters indicate significant differences (p < 0.05) between treatments within the experiment
Excretion of lactulose

There were no significant differences in the amount of lactulose excreted following dosage with either ascorbic acid alone, aspirin alone and the combined dosage of both agents (Figure 8b-2).

![Bar graph showing lactulose excretion](image)

Figure 8b-2: Variation in recovery of lactulose (% ingested dose) with treatments over the 2 ½ - 4 hr of urine collection in 28 healthy female participants. *A= single dose of 600 mg aspirin, C = 500 mg ascorbic acid, CA= combined dosage of 500 mg ascorbic acid and 600 mg aspirin. Differences in letters indicate significant differences (p < 0.05) between treatments within the experiment

8b.3.2 Comparisons with results from the prior published experiment

Excretion of mannitol

The amounts of mannitol excreted were significantly greater (d.f 1,27; F = 4.92; P < 0.05) following dosage with aspirin from the study than those after intake of aspirin
from the previous experiment\textsuperscript{7} (Figure 8b-3). Conversely, there was no difference in the amount of mannitol excreted after dosage with aspirin from the current study when compared with the corresponding value obtained after the consumption of the placebo in the previous experiment\textsuperscript{7} (Figure 8b-3). It is noteworthy that in the previous experiment during the 2½ - 4 hr period significantly lower (d.f 1,39; F = 8.56; P = 0.006) quantities of mannitol were excreted following dosage with aspirin than those after the placebo in the same experiment (Figure 8b-3).

Figure 8b-3: Variation in recovery of mannitol (% ingested dose) with treatments over the 2½ - 4 hr of urine collection in 28 healthy females compared with results of a previous experiment in 40 healthy female participants\textsuperscript{7}. *P = 100 ml placebo (water), A = single dose of 600 mg aspirin, C = 500 mg ascorbic acid, CA= combined dosage of 500 mg ascorbic acid and 600 mg aspirin. Differences in letters indicate significant differences (p < 0.05) between treatments within each experiment.
**Excretion of lactulose**

There were no significant differences in the quantities of lactulose excreted following dosage with aspirin from the current study when compared with those obtained in the prior published experiment after dosage with aspirin (Figure 8b-4). The comparison of lactulose excretion over the period following the consumption of aspirin in the current work was significantly greater (d.f 1,27; F = 24.56; P < 0.001) than that obtained following consumption of the placebo in the prior experiment (Figure 8b-4).

Figure 8b-4: Variation in recovery of lactulose (% ingested dose) with treatments over the 2½ - 4 hr of urine collection in 28 healthy females compared with results of a previous experiment in 40 healthy female participants. *P = 100 ml placebo (water), A = single dose of 600 mg aspirin, C = 500 mg ascorbic acid, CA= combined dosage of 500 mg ascorbic acid and 600 mg aspirin. Differences in letters indicate significant differences (p<0.05) between treatments within each experiment.
8b.4 Discussion

The findings from the study exhibit that the effect of a single 600 mg dose of aspirin, a 500 mg pharmacological dose of ascorbic acid and the combined dosage of both agents on intestinal permeability could be determined over the 2½ - 4 hr period. The fact that these results are broadly similar to that when the analysis was conducted over the two three-hourly periods of collection (of the total six hr period) seems to suggest that the shorter time interval is sufficiently sensitive to pick up changes in small intestinal permeability in response to the treatments. This offers researchers and clinicians an opportunity to reduce the burden of longer periods of urine sample collection while conducting the test.

The finding that none of the three treatments had an effect on the amount of mannitol excreted over the 2½ - 4 hr period fits in with the result that mannitol excretion did not differ between treatments either in the first or the second three hrs of urine collection as reported in Chapter 8. It is noted that the amount of mannitol excreted after dosage with 600 mg aspirin in the present study was greater than that obtained from a similar dose of aspirin used in the previous published experiment. Whilst aspirin increased paracellular permeability due to its topical effects on the mucosa, its effect on decreasing mannitol absorption was not observed in this study group.

In contrast, the finding that similar amounts of lactulose was excreted following the consumption of aspirin alone, ascorbic acid alone and co-administration of both agents is different to that reported in Chapter 8. It seems likely that the analysis of small intestinal permeability over the 2½ - 4 hr period circumvents the observed differences in the excretion of lactulose following consumption of each of the treatments when the analysis was conducted over the two three hourly periods of collection. Whilst the latter analysis gave useful information, the variation in lactulose excretion following consumption of the
treatments in the first three hour period could have been due to variation in gastric emptying between subjects while the variation in the second three hour period could have been due to differences in colonic absorption of the sugar. It is noteworthy that the amount of lactulose excreted following the consumption of aspirin in the current study during the 2½ - 4 hr period is similar to that obtained from a similar dose of aspirin in previous published experiment. Again, within the previous study the effect of the single dose of aspirin on lactulose and mannitol excretion was apparent.

Hence, the results from this study support the recommendation for assessing small intestinal permeability over the 2½ - 4 hr period in healthy individuals. It must be duly noted that this time period may not be appropriate for use in clinical conditions such as inflammatory bowel disease due to variations in gastrointestinal motility patterns. Hence the test requires further standardization in such conditions. Also gender differences could influence the timing and magnitude of the temporal GI absorption profiles of these sugars and hence may limit the use of this time interval to assess permeability in males.
8b.5 References


CHAPTER 9

Summarizing discussion and conclusions

with recommendations for future research
9.1. Summarizing discussion

This chapter summarizes and discusses the findings of the thesis in light of the methodologies that were used and the significance of the results. The discussion will address three main outcomes that are relevant to clinicians, researchers and those in the food and pharmacological industry.

9.1.1. Standardization of the ‘classical’ lactulose mannitol test

A variety of different test protocols for the dual sugar absorption test, using lactulose and mannitol, that are found in the literature indicate there is a need for the protocol to be standardized. Previous workers have used a range of different periods of urine collection\(^1\),\(^2\), a number of different test probes\(^3\) at different doses\(^4\),\(^5\) that were consumed with different volumes of fluid\(^6\) conducted during fasting or when food was consumed\(^7\),\(^9\). It is due to such variations that meaningful comparisons between different studies are largely precluded.

A potential problem with the consumption of food is that it is likely to initiate a post prandial physiological status, rather than that of the inter meal interval when the pattern of antro duodenal motility is dominated by the migrating motor complex. Hence the ingestion of food\(^10\) and nutrient components\(^11\),\(^12\) would alter gastric, small intestinal and large intestinal transit times within and between subjects. As a result of the literature surveyed, it was concluded that the bulk of reported research had conducted the test in the inter meal interval and thus the research in this thesis endeavoured to develop a protocol during which this was maintained.
It was ensured that the osmolarity (720 osmol l\(^{-1}\)) of the test solution was consistent between tests using 10 g of lactulose with 5 g of mannitol in 100 ml of water. The solution did not contribute to an osmolar stimulus\(^9\). Fluid intake was restricted to 700 ml during the entire six hour period to limit any effect of diuresis on the excretion of the probe sugars\(^6,13\). The distribution of this volume of fluid over the experimental period served to further control any osmolar effects of the drink and enable the collection of half-hourly urine samples.

A further complication, in the test protocols, was that urine samples were collected over a range of different time periods and that permeability was assessed principally on the basis of ratios of the probe sugars excreted rather than the absolute amounts of the two sugars excreted. This rationale was based on the premise that the rates of excretion of the two sugars are relatively invariant over time. However, the current study showed that the rates of excretion and the timings of the peak in excretion of the smaller and larger sugars varied in healthy subjects according to the duration of the sampling period. The ratio of the rate of excretion of the two probes will thus vary depending on the sampling period.

The SmartPill studies showed that the rates, at which the smaller sugars, mannitol and rhamnose were excreted, peaked during the first two hrs, a period when they were transiting the small intestine, whilst the rate of excretion of lactulose peaked during the fourth hr at a time when it was transiting the proximal colon. Hence the ratio of the sugars excreted during earlier periods, i.e. between 0 – 3 hr were lower than those during the latter period, i.e. between 3 – 6 hr which were higher. An assessment of permeability based solely on the ratio would therefore be confounded by changes in the timing and duration of urinary sampling. Such variation is particularly important in conditions such as coeliac disease where villous atrophy causes a relative decrease in the excretion of mannitol\(^{14}\) and mucosal inflammation causes an increase in the excretion of lactulose\(^{15}\). Hence the
comparisons of the absolute quantities of each of the probes that are excreted with due regard to the sampling period will allow greater insight into the factors that are driving the changes in the ratio.

Similarly, it is recommended that the standardized tests do not employ cellobiose and rhamnose as they are reported to undergo enzymatic degradation\textsuperscript{16} and transport across erythrocytes\textsuperscript{17}. A further complication when these sugars are used interchangeably with lactulose and mannitol is that there could be discrepancies in absolute recoveries of each sugar, e.g. between mannitol and rhamnose, and consequently the ratio, that arise due to the physicochemical properties of the molecules\textsuperscript{18}. Lactulose and mannitol are commonly used to assess small intestinal permeability\textsuperscript{19}. However, trace amounts of mannitol in dietary items that are consumed in the preceding 24 hrs and some quantities endogenously produced by commensal microflora\textsuperscript{20} necessitate correction of all urine samples by subtraction of the quantity found in a baseline sample collected prior to administration of the sugar solution\textsuperscript{3}. It is noted that such a procedure assumes constancy of baseline excretion.

A further problem with the tests is that collection periods in which the permeability of a particular GI segment can be assessed differ. Urine collections over 0 - 3, 3 - 5 and 5 - 24 hr broadly correspond with permeability in the proximal small intestine, distal small intestine and colon respectively\textsuperscript{21}. There is a practical limit to the number of urine samples that can be collected over a particular time period. Hence even if the time of collection is properly delineated, the variance obtained with a particular time interval may vary with the segment.

The work with the wireless motility capsule confirmed that during the 2½ - 4 hr period the bulk of the sugars were in the small intestine and during the 4½ - 6 hr period
the bulk of the sugars were in the proximal colon (the capsule being emptied from the stomach and from the small intestine within 1.79 ± 0.31 hr and 4.64 ± 0.68 hr respectively, following the ingestion of the solution). It is noted that such retention times would likely be at or above the maximum residence time of the solution as the capsule could have been expelled from the different segments following the emptying of the solution. In healthy subjects the 2½ - 4 hr period could therefore be used to assess permeability of the small intestinal mucosa. Whilst shortened collection over this period would reduce the burden of sample collection for subjects and reduce any between or within subject variation that might occur due to differences in gastric emptying, the lower volume of urine collected with respect to the total duration of absorption in the small intestine may engender variance.

Importantly, the SmartPill work also determined that the consumption of 250 ml of the lactulose mannitol solution did not accelerate or delay transit through the stomach, small intestine and large intestine with respect to water. Hence the solution did not induce a post prandial pattern of absorption of the probe sugars supporting the hypothesis that the new protocol reflects the patterns of absorption during the inter meal interval.

It is also important to note that intestinal hurry or delay associated with inflammatory conditions such as coeliac, Crohn’s disease and ulcerative colitis may influence transit time and absorption. Hence in conditions associated with motility disorders, the recommended 2½ - 4 hr period may not be suitable for assessing permeability of the small intestine. In such cases it may be preferable to conduct half-hourly urine collections pending further investigation, i.e. quantification of the shifts patterns of absorption and in the residence time.
It is noteworthy that the work on standardization has been subsequently endorsed in an editorial of a peer review journal\textsuperscript{24}.

\textbf{9.1.2. Augmentation of the ‘classical’ test with a single dose of 600 mg aspirin}

The development of an aspirin augmented test stemmed from the concept that gut ‘health’ constituted the resistance of the mucosa to perturbation by a noxious agent or an organism. Hence gut ‘health’ could be quantified either as the resistance to a noxious stimulus (i.e. the magnitude of disturbance produced by the standardized stimulus or the time taken for recovery from the standardized stimulus). Such tests of resilience would enable the ability of health promoting agents to ameliorate the perturbation by such a noxious stimulus. The augmented test described in this study used a single dose of 600 mg aspirin taken shortly before dosage with the probe sugars. This agent was shown to augment permeability in the small intestine as assessed by the excretion of lactulose and mannitol. Further, augmentation of the test by aspirin appeared to amplify the effect of a pre-existing adverse stimulus such as that from smoking\textsuperscript{25}. Hence the test could conceivably be used to detect sub-clinical inflammation. However, further work is required to delineate the time before normal permeability to both probe sugars is re-established.

The time taken to recover from the dose of aspirin could be assessed by ongoing administration of lactulose and mannitol. This would establish a steady state in which both sugars are absorbed from all sites along the GI tract and the duration of the period when the absorption of the probes is augmented could be determined.

It is noteworthy that the use of aspirin as a perturbing stimulus may be confounded by variation between subjects. Sensitivity to aspirin appears to be to some extent genetically
predetermined\textsuperscript{26}. It is also possible that, when subjects with unidentified pre-existing inflammatory conditions are dosed with aspirin, this could act to reduce inflammation and augment mucosal integrity, i.e. have a restorative effect on permeability. Alternatively other noxious agents such as chenodeoxycholic acid (CDCA), a bile acid synthesized in the liver and generally regarded as safe, could be used\textsuperscript{27}. A 750 mg oral dose of CDCA is known to increase small intestinal permeability to lactulose\textsuperscript{28}. Alternatively, as was shown in this study, a pharmacological dose of ascorbic acid (500 mg) appears to have a similar effect on intestinal permeability to that of a single dose of 600 mg aspirin for reasons that are not fully understood.

9.1.3. Additive effects on intestinal permeability

The study found that ascorbic acid augmented intestinal permeability when it was co-administered with 600 mg of aspirin. This finding has important connotations for the clinical formulations such as ‘Aspirin® Plus C’ (\textit{Bayer Healthcare, Leverkusen, Germany}), that are formulated on the basis that ascorbic acid may reduce any oxidative damage by aspirin. The results of the study indicate that such a formulation may have more immediate effects on the apical membrane before any anti-oxidative effects are exerted. However, it is noteworthy that the tablet contains lower dose of both agents than was used in our study.

It is unknown whether this lower dose would have a similar effect. It is however possible that a number of dietary agents may have similar effects. At all events the fact that ascorbic acid augments paracellular permeability could be useful for augmenting uptake of pharmaceutical agents.
9.2. Use of the augmented lactulose mannitol test for future work

The aspirin augmented standardized lactulose mannitol test developed in the thesis has already been used in published work correlating mucosal integrity with circulating levels of bacterial products, i.e. lipopolysaccharide (LPS) or endotoxin\(^9\).

The augmented lactulose mannitol test could also be used to quantify the effects of therapeutic agents such as rebamipide\(^9\) on intestinal permeability. The latter agent is used in the treatment of peptic ulcers and has been shown to mitigate the adverse effects of NSAIDs on the small intestinal mucosa\(^31,32\).

The aspirin augmented standardized lactulose mannitol test could also be used to explore the effects of various nutraceutical agents that are reputed to be beneficial to gut health. For example, the effects of dietary antioxidants, such as anthocyanin containing berries or extracts, could be assessed using the augmented test. However it is important to note that as a result of the test design that all these assessments would be conducted in the inter meal interval. Hence the patterns of absorption of both the nutraceutical agent and the test sugars may differ from those after the consumption of a meal, i.e. the post prandial period.

With regard to the use of this test in investigating the pathogenesis of intestinal diseases, as stated hitherto, further work is necessary regarding the influence of such conditions on intestinal transit time. The simultaneous use of the SmartPill could allow any effects on transit time to be directly determined.

The lactulose mannitol test could be useful in assessing effects of encapsulation on the efficiency of absorption from the gut lumen using formulations that have the probe sugars incorporated into them. Again, it is important to note that testing of foods would be conducted in the inter meal interval.
Lastly, comparisons of the temporal patterns of absorption of passively absorbed probe sugars with actively absorbed sugars, such as glucose, may be useful in quantifying the effects of hormones such as insulin/incretin\textsuperscript{33, 34} on the kinetics of active absorption.

### 9.3. Limitations of the thesis

There are a number of limitations in respect to the tests of permeability outlined in this thesis:

Firstly, the work is based on the assumption that the administered dose of lactulose and mannitol are excreted at a steady rate by the kidneys in direct proportion to the amount absorbed over the entire 6 hr period. Some reports indicate that there is ongoing urinary excretion of mannitol and lactulose beyond the 6 hr period\textsuperscript{21}. Further work is needed to investigate the dynamics of absorption and excretion throughout the entire 6 hr period, preferably correlating excretion with serum levels.

Secondly, it is important to note that the studies involving the effect of ascorbic acid on mucosal permeability did not include a placebo treatment within the same cohort. Rather, the effect was assessed by comparison with placebo results obtained from a previous study carried out by us under identical test conditions\textsuperscript{35}. Further work with direct comparisons with a placebo (water) is required.

Thirdly, it is noteworthy that the decrease that was noted in the excretion of mannitol after dosage with aspirin was not statistically significant in all of the studies. Hence further work with larger number of subjects is required to validate this effect.

Finally, only female subjects were used in our studies. A number of reports have suggested that gastric emptying varies with gender, women having a slower rate of
empting than men\textsuperscript{36}. However it is not clear whether permeability varies with gender\textsuperscript{37-39}. Further work is needed in this regard. Until such findings are completed, our findings in female subjects cannot necessarily be extrapolated to males.

9.4. References

29. Gnauck A, Lentle RG, Kruger MC. Aspirin-induced increase in intestinal paracellular permeability does not affect the levels of LPS in venous blood of healthy women. Innate Immunity 2014;1753425914557101.


This appendix describes the series of questions that were used to screen participants during the recruitment process for inclusion into the study.
HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

Screening Questionnaire

Name: ____________________________
DOB: ____________________________
Telephone #: _____________________
Cell phone#: _____________________
Address: __________________________
Email address: ____________________

Please answer the following questions with a tick or cross i.e. ✓ if yes or ✗ if no.

<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do you suffer from heartburn, indigestion or have a history of gastrointestinal disease other than appendicitis?</td>
<td></td>
</tr>
<tr>
<td>Diabetes or persistent sugar in the urine?</td>
<td></td>
</tr>
<tr>
<td>Endocrine disease (hormone trouble)?</td>
<td></td>
</tr>
<tr>
<td>Thyroid disease (eg goitre)?</td>
<td></td>
</tr>
<tr>
<td>Kidney problems?</td>
<td></td>
</tr>
<tr>
<td>Disorders of the liver?</td>
<td></td>
</tr>
<tr>
<td>Recent major abdominal surgery?</td>
<td></td>
</tr>
<tr>
<td>Impaired immunity or recent immunisation?</td>
<td></td>
</tr>
<tr>
<td>A recent gut infection</td>
<td></td>
</tr>
<tr>
<td>Any bleeding from the bowel?</td>
<td></td>
</tr>
<tr>
<td>Any chronic or recent constipation?</td>
<td></td>
</tr>
<tr>
<td>Diarrhoea in the last 1 month?</td>
<td></td>
</tr>
</tbody>
</table>

Do you smoke? If so how many a day? ...........................................

How many drinks containing alcohol do you have a day?

Have you ever been told you have a high alcohol consumption rate?
HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

Are you allergic to or unable to take aspirin?

Do you take any of the following:

<table>
<thead>
<tr>
<th>Vitamin supplements</th>
<th>Mineral supplements</th>
<th>Iron supplement</th>
<th>Other health foods</th>
<th>Extra calcium</th>
<th>Pills for anaemia</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Pills or medicine for gastric ulcer or stomach disorder</th>
<th>Blood thinning pills eg warfarin or aspirin</th>
<th>Probiotic or prebiotic supplements eg lactulose</th>
<th>Immuno-suppressants or steroids (oral, spray or cream)</th>
<th>Antibiotics (within the last month)</th>
</tr>
</thead>
</table>

Please list any medications and supplements you are currently taking below
APPENDIX 2

SYSTAT data output

The following SYSTAT output shows the models used in the analysis of all the reported data described in the results section of Chapter 4. The vast data and large body of statistical analyses conducted in the thesis precludes all the output data to be attached in the Appendix. This model therefore is an example of the manner in which the data was handled, i.e. the factors/variables that were used on the doubly repeat measure ANOVA and repeat measure ANOVA. Similar models have been used to analyze data in subsequent chapters to determine the effect of treatment and time on the excretion of lactulose and mannitol during the three periods of urine collection, i.e. the first three hours, the second three hours and the entire six hour period.
1. Doubly repeated measure analysis of variance of the lactulose mannitol ratio in urine samples collected over the first three hours and the entire six hour period in subjects following the consumption of the aspirin and the placebo drink.

**Nonparametric: Kolmogorov-Smirnov Test**

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPIRINTHREE</td>
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<td>0.300</td>
<td>0.000</td>
</tr>
<tr>
<td>PLACEBOTHREE</td>
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<td>0.134</td>
<td>0.467</td>
</tr>
<tr>
<td>ASPIRINSIX</td>
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<td>0.243</td>
<td>0.003</td>
</tr>
<tr>
<td>PLACEBOSIX</td>
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<td>0.152</td>
<td>0.259</td>
</tr>
</tbody>
</table>

**General Linear Model**

Effects coding used for categorical variables in model. The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
<th>NS = non-smokers; S = smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>NS  S</td>
<td></td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

**Dependent Variable Means**

<table>
<thead>
<tr>
<th>LOGASPIRINTHREE</th>
<th>LOGPLACEBOTHREE</th>
<th>LOGASPIRINSIX</th>
<th>LOGPLACEBOSIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.728</td>
<td>-3.561</td>
<td>-2.199</td>
<td>-3.067</td>
</tr>
</tbody>
</table>

**Repeated Measures Factors and Levels of Dependent Variables**

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>1.000</td>
<td>2.000</td>
<td>2.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.000</td>
<td>2.000</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

**Univariate and Multivariate Repeated Measures Analysis**

### Between Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
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<td>1</td>
<td>0.809</td>
<td>0.311</td>
<td>0.584</td>
</tr>
<tr>
<td>Error</td>
<td>46.795</td>
<td>182</td>
<td>0.073</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>G-G-F</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5.194</td>
<td>70.903</td>
<td>0.000</td>
<td></td>
</tr>
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<td>0.000</td>
<td>0.001</td>
<td>0.973</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>1.319</td>
<td>180</td>
<td>0.073</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>15.103</td>
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<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment *SMOKINGSTATUS$</td>
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<td>1.042</td>
<td>1.635</td>
<td>0.217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>11.466</td>
<td>18</td>
<td>0.637</td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period*Treatment</td>
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<td>0.004</td>
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<td>0.854</td>
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<td></td>
</tr>
<tr>
<td>Period<em>Treatment</em>SMOKINGSTATUS$</td>
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<td>0.033</td>
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<td>0.574</td>
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<td></td>
</tr>
<tr>
<td>Error</td>
<td>1.825</td>
<td>18</td>
<td>0.101</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Repeat measure analysis of variance of the lactulose mannitol ratio in urine samples collected over the first three hours and the entire six hour period in subjects who had received the placebo drink.

▼ Nonparametric: Kolmogorov-Smirnov Test

Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUMLMR</td>
<td>80</td>
<td>0.281</td>
<td>0.000</td>
</tr>
<tr>
<td>LOGCUMLMR</td>
<td>80</td>
<td>0.069</td>
<td>0.419</td>
</tr>
</tbody>
</table>
\section*{General Linear Model}

Data for the following results were selected according to SELECT (TREATMENT$ = 'Placebo')

Effects coding used for categorical variables in model. The categorical values encountered during processing are

\begin{tabular}{|c|c|}
\hline
Variables & Levels \\
\hline SUBJECT$ (2 levels) & NS $|$ S \\
\hline
\end{tabular}

\[ NS = \text{non-smokers}; \ S = \text{smokers} \]

N of Cases Processed: 20

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Dependent Variable} & \textbf{Means} \\
\hline
LOGTHREEHR & -3.561 \\
LOGSIXHR & -3.067 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Repeated Measures Factors and Levels of Dependent Variables} & \\
\hline Within Factor & 1 & 2 \\
Period & 1.000 & 2.000 \\
\hline
\end{tabular}
\end{table}

\section*{Univariate Repeated Measures Analysis}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Between Subjects} & \textbf{Source} & \textbf{SS} & \textbf{df} & \textbf{Mean Squares} & \textbf{F-Ratio} & \textbf{p-Value} \\
\hline
SUBJECT$ & 1.843 & 1 & 1.843 & 1.493 & 0.237 & \\
Error & 22.213 & 18 & 1.234 & & & \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Within Subjects} & \textbf{Source} & \textbf{SS} & \textbf{df} & \textbf{Mean Squares} & \textbf{F-Ratio} & \textbf{p-Value} \textbf{G-G} & \textbf{H-F} \\
\hline
Period & 2.463 & 1 & 2.463 & 47.426 & 0.000 & . & . \\
Period*SUBJECT$ & 0.018 & 1 & 0.018 & 0.354 & 0.559 & . & . \\
Error & 0.935 & 18 & 0.052 & & & & \\
\hline
\end{tabular}
\end{table}

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.
Appendix

General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Placebo')

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
<th>LOGTHREEHR</th>
<th>LOGSIXHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3.561</td>
<td>-3.067</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Within Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>Greenhouse-Geisser Epsilon</th>
<th>Huynh-Feldt Epsilon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period</td>
<td>2.445</td>
<td>1</td>
<td>2.445</td>
<td>48.739</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>0.953</td>
<td>19</td>
<td>0.050</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Plot of Residuals vs. Predicted Values
3. Repeat measure analysis of variance of the lactulose mannitol ratio in urine samples collected over the first three hours and the entire six hour period in subjects who had received the aspirin drink.

▼General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Aspirin')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUBJECT$</td>
<td>2 levels</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHR</td>
</tr>
<tr>
<td>-2.728</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables
Within Factor

<table>
<thead>
<tr>
<th>Period</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUBJECT$</td>
<td>0.007</td>
<td>1</td>
<td>0.007</td>
<td>0.004</td>
<td>0.952</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>36.048</td>
<td>18</td>
<td>2.003</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Within Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-GH-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period*SUBJECT$</td>
<td>0.015</td>
<td>1</td>
<td>0.015</td>
<td>0.122</td>
<td>0.731</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>2.208</td>
<td>18</td>
<td>0.123</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.
Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Aspirin')

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHR LOGSIXHR</td>
</tr>
<tr>
<td>-2.728 -2.199</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeated Measures Factors and Levels of Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within Factor 1 2</td>
</tr>
<tr>
<td>Period          1.000 2.000</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Within Subjects Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>2.803</td>
<td>1</td>
<td>2.803</td>
<td>23.952</td>
<td>0.000</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Error</td>
<td>2.223</td>
<td>19</td>
<td>0.117</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.
4. Doubly repeated measure analysis of variance of the lactulose mannitol ratio in urine samples collected over the first three hours and the second three hour period in subjects following the consumption of the aspirin and the placebo drink.

▼ Nonparametric: Kolmogorov-Smirnov Test

Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGASPIRINTHREE</td>
<td>20</td>
<td>0.117</td>
<td>0.710</td>
</tr>
<tr>
<td>LOGPLACEBOTHREE</td>
<td>20</td>
<td>0.148</td>
<td>0.301</td>
</tr>
<tr>
<td>LOGASPIRINSECONDTHREE</td>
<td>20</td>
<td>0.123</td>
<td>0.620</td>
</tr>
<tr>
<td>LOGPLACEBOSECONDTHREE</td>
<td>20</td>
<td>0.080</td>
<td>1.000</td>
</tr>
</tbody>
</table>

▼ General Linear Model

Effects coding used for categorical variables in model. 
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>NS S</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

Dependent Variable Means

<table>
<thead>
<tr>
<th></th>
<th>LOGASPIRINTHREE</th>
<th>LOGPLACEBOTHREE</th>
<th>LOGASPIRINSECONDTHREE</th>
<th>LOGPLACEBOSECONDTHREE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2.728</td>
<td>-3.561</td>
<td>-1.663</td>
<td>-2.491</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>1.000</td>
<td>2.000</td>
<td>2.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.000</td>
<td>2.000</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate and Multivariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SMOOKINGSTATUS$</td>
<td>1.332</td>
<td>1</td>
<td>1.332</td>
<td>0.695</td>
<td>0.415</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>34.483</td>
<td>18</td>
<td>1.916</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Within Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>G-GH-F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period</td>
<td>22.329</td>
<td>1</td>
<td>22.329</td>
<td>25.171</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Period *SMOKINGSTATUS$</td>
<td>0.060</td>
<td>1</td>
<td>0.060</td>
<td>0.068</td>
<td>0.797</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>15.968</td>
<td>18</td>
<td>0.887</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5. Repeat measure analysis of variance of the lactulose mannitol ratio in urine samples collected over the first three hours and the second three hour period in subjects who had received the placebo drink.

\(\text{\textbf{Nonparametric: Kolmogorov-Smirnov Test}}\)

\(\text{\textbf{Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution}}\)

\(\text{\textbf{General Linear Model}}\)

Data for the following results were selected according to SELECT ( TREATMENT$ = 'Placebo')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

\(\text{\textbf{Variables Levels}}\)

\(\text{\textbf{N of Cases Processed: 20}}\)
### Dependent Variable Means

<table>
<thead>
<tr>
<th></th>
<th>LOGTHREEHR</th>
<th>LOGSECONDTHREEHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3.561</td>
<td>-2.491</td>
</tr>
</tbody>
</table>

### Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

### Univariate Repeated Measures Analysis

#### Between Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUBJECT$</td>
<td>1.579</td>
<td>1</td>
<td>1.579</td>
<td>1.492</td>
<td>0.238</td>
</tr>
<tr>
<td>Error</td>
<td>19.060</td>
<td>18</td>
<td>1.059</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>11.495</td>
<td>1</td>
<td>11.495</td>
<td>32.940</td>
<td>0.000</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Period *SUBJECT$</td>
<td>0.056</td>
<td>1</td>
<td>0.056</td>
<td>0.160</td>
<td>0.694</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Error</td>
<td>6.282</td>
<td>18</td>
<td>0.349</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon: .
Huynh-Feldt Epsilon: .

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

▼ General Linear Model

Data for the following results were selected according to
SELECT (TREATMENT$ = 'Placebo')

N of Cases Processed: 20

#### Dependent Variable Means

<table>
<thead>
<tr>
<th></th>
<th>LOGTHREEHR</th>
<th>LOGSECONDTHREEHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3.561</td>
<td>-2.491</td>
</tr>
</tbody>
</table>

#### Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>
Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>11.450</td>
<td>1</td>
<td>11.450</td>
<td>34.328</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>6.337</td>
<td>19</td>
<td>0.334</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon: 
Huynh-Feldt Epsilon: 

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

6. Repeat measure analysis of variance of the lactulose mannitol ratio in urine samples collected over the first three hours and the second three hour period in subjects who had received the aspirin drink.

▼ General Linear Model

Data for the following results were selected according to 
SELECT ( TREATMENT$ = 'Aspirin')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUBJECT$</td>
<td>(2 levels)</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20
### Dependent Variable Means

<table>
<thead>
<tr>
<th></th>
<th>LOGTHREEHR</th>
<th>LOGSECONDTHREEHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2.728</td>
<td>-1.663</td>
</tr>
</tbody>
</table>

### Repeated Measures Factors and Levels of Dependent Variables

**Within Factor**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

### Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUBJECT$</td>
<td>0.141</td>
<td>1</td>
<td>0.141</td>
<td>0.120</td>
<td>0.733</td>
</tr>
<tr>
<td>Error</td>
<td>21.219</td>
<td>18</td>
<td>1.179</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>10.839</td>
<td>1</td>
<td>10.839</td>
<td>11.462</td>
<td>0.003</td>
</tr>
<tr>
<td>Period*SUBJECT$</td>
<td>0.341</td>
<td>1</td>
<td>0.341</td>
<td>0.360</td>
<td>0.556</td>
</tr>
<tr>
<td>Error</td>
<td>17.022</td>
<td>18</td>
<td>0.946</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon

Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

### General Linear Model

Data for the following results were selected according to

SELECT ( TREATMENT$ = 'Aspirin')

N of Cases Processed: 20

<table>
<thead>
<tr>
<th></th>
<th>LOGTHREEHR</th>
<th>LOGSECONDTHREEHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2.728</td>
<td>-1.663</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>
Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error</td>
<td>17.362</td>
<td>19</td>
<td>0.914</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

7. Doubly repeated measure analysis of variance of lactulose excreted in urine samples collected over the first three hours and the entire six hour period in subjects following the consumption of the aspirin and the placebo drink.

▼Nonparametric: Kolmogorov-Smirnov Test

Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGASPIRINTHREEHOUR</td>
<td>20</td>
<td>0.135</td>
<td>0.446</td>
</tr>
<tr>
<td>LOGPLACEBOTHREEHOUR</td>
<td>20</td>
<td>0.152</td>
<td>0.264</td>
</tr>
<tr>
<td>LOGASPIRINSIXHOUR</td>
<td>20</td>
<td>0.130</td>
<td>0.521</td>
</tr>
<tr>
<td>LOGPLACEBOSIXHOUR</td>
<td>20</td>
<td>0.127</td>
<td>0.559</td>
</tr>
</tbody>
</table>
Appendix

▼ General Linear Model

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>NS S</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGASPIRINTHREE-HOUR</th>
<th>LOGPLACEBOTHREE-HOUR</th>
<th>LOGASPIRINSIXHO-LPPLACEBOSIXHO-HOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.594</td>
<td>2.871</td>
<td>4.561</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>1.000</td>
<td>2.000</td>
<td>2.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.000</td>
<td>2.000</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate and Multivariate Repeated Measures Analysis

Between Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>5.206</td>
<td>1</td>
<td>5.206</td>
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<tr>
<td>Error</td>
<td>36.109</td>
<td>18</td>
<td>2.006</td>
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</table>

Within Subjects

<table>
<thead>
<tr>
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<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
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</thead>
<tbody>
<tr>
<td>Period</td>
<td>17.670</td>
<td>1</td>
<td>17.670</td>
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<tr>
<td>T Period *SMOKINGSTATUS$</td>
<td>0.011</td>
<td>1</td>
<td>0.011</td>
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<td>0.723</td>
</tr>
<tr>
<td>Error</td>
<td>1.514</td>
<td>18</td>
<td>0.084</td>
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Greenhouse-Geisser Epsilon: .
Huynh-Feldt Epsilon: .

Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>11.057</td>
<td>1</td>
<td>11.057</td>
<td>16.834</td>
<td>0.001</td>
</tr>
<tr>
<td>Treatment *SMOKINGSTATUS$</td>
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<td>1</td>
<td>0.000</td>
<td>0.983</td>
<td>0.354</td>
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<tr>
<td>Error</td>
<td>11.823</td>
<td>18</td>
<td>0.657</td>
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Greenhouse-Geisser Epsilon: .
Huynh-Feldt Epsilon: .

Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period* Treatment</td>
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<td>1</td>
<td>0.014</td>
<td>0.124</td>
<td>0.729</td>
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<tr>
<td>Period* Treatment *SMOKINGSTATUS$</td>
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<td>1</td>
<td>0.004</td>
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Greenhouse-Geisser Epsilon: .
Huynh-Feldt Epsilon: .
Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error</td>
<td>2.054</td>
<td>18</td>
<td>0.114</td>
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<td></td>
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</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Successfully saved file H:\Systat\results\Cumlactuloseexcretion.syz
Processed 9 Variables and 20 Cases.

8. Repeat measure analysis of variance of lactulose excreted in urine samples collected over the first three hours and the entire six hour period in subjects who had received the placebo drink.

▼ Nonparametric: Kolmogorov-Smirnov Test

Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THREEHOUR</td>
<td>40</td>
<td>0.245</td>
<td>0.000</td>
</tr>
<tr>
<td>SIXHOUR</td>
<td>40</td>
<td>0.250</td>
<td>0.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOUR</td>
<td>40</td>
<td>0.105</td>
<td>0.302</td>
</tr>
<tr>
<td>LOGSIXHOUR</td>
<td>40</td>
<td>0.097</td>
<td>0.433</td>
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</tbody>
</table>

▼ General Linear Model

Data for the following results were selected according to SELECT ( TREATMENT$ = 'Placebo')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>(2 levels) NS S</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSIXHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.871</td>
<td>3.788</td>
</tr>
</tbody>
</table>
Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

### Between Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>2.564</td>
<td>1</td>
<td>2.564</td>
<td>2.116</td>
<td>0.163</td>
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<td></td>
</tr>
<tr>
<td>Error</td>
<td>21.815</td>
<td>18</td>
<td>1.212</td>
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</table>

### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>8.341</td>
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<td>8.341</td>
<td>190.578</td>
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<td>.</td>
</tr>
<tr>
<td>Period *SMOKINGSTATUS$</td>
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<td>0.001</td>
<td>0.021</td>
<td>0.888</td>
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<tr>
<td>Error</td>
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<td>0.044</td>
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</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Placebo')

N of Cases Processed: 20

**Dependent Variable Means**

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSIXHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.871</td>
<td>3.788</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>
Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>8.408</td>
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<td>8.408</td>
<td>202.545</td>
<td>0.000</td>
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<td>.</td>
</tr>
<tr>
<td>Error</td>
<td>0.789</td>
<td>19</td>
<td>0.042</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

9. Repeat measure analysis of variance of lactulose excreted in urine samples collected over the first three hours and the entire six hour period in subjects who had received the aspirin drink.

▼ General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Aspirin')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>2 levels</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20
Appendix

### Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSIXHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.594</td>
<td>4.561</td>
</tr>
</tbody>
</table>

### Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

### Univariate Repeated Measures Analysis

#### Between Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>2.642</td>
<td>1</td>
<td>2.642</td>
<td>1.821</td>
<td>0.194</td>
</tr>
<tr>
<td>Error</td>
<td>26.118</td>
<td>18</td>
<td>1.451</td>
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</tr>
</tbody>
</table>

#### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>9.343</td>
<td>1</td>
<td>9.343</td>
<td>60.474</td>
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</tr>
<tr>
<td>Period *SMOKINGSTATUS$</td>
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<td>0.014</td>
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<td>Error</td>
<td>2.781</td>
<td>18</td>
<td>0.154</td>
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</table>

**Greenhouse-Geisser Epsilon**

**Huynh-Feldt Epsilon**

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

▶ General Linear Model

Data for the following results were selected according to

SELECT ( TREATMENT$ = 'Aspirin')

N of Cases Processed: 20

### Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSIXHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.594</td>
<td>4.561</td>
</tr>
</tbody>
</table>

### Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>
Appendix

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
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<tr>
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<td>0.147</td>
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</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

10. Doubly repeated measure analysis of variance of lactulose excreted in urine samples collected over the first three hours and the second three hour period in subjects following the consumption of the aspirin and the placebo drink.

▼ Nonparametric: Kolmogorov-Smirnov Test

Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGASPIRINTHREEHOUR</td>
<td>20</td>
<td>0.135</td>
<td>0.446</td>
</tr>
<tr>
<td>LOGPLACEBOTHREEHOUR</td>
<td>20</td>
<td>0.152</td>
<td>0.264</td>
</tr>
<tr>
<td>LOGASPIRINSECONDTHREE</td>
<td>20</td>
<td>0.122</td>
<td>0.635</td>
</tr>
<tr>
<td>LOGPLACEBOSECONDTHREE</td>
<td>20</td>
<td>0.140</td>
<td>0.389</td>
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</tbody>
</table>
Appendix

General Linear Model

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>(2 levels) NS S</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

Dependent Variable Means

<table>
<thead>
<tr>
<th></th>
<th>LOGASPIRINTHREEHOUR</th>
<th>LOGPLACEBOTHREEHOUR</th>
<th>LOGASPIRINSECONDDTHREE</th>
<th>LOGPLACEBOSECONDDTHREE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.594</td>
<td>2.871</td>
<td>3.979</td>
<td>3.155</td>
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</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>1.000</td>
<td>2.000</td>
<td>2.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.000</td>
<td>2.000</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate and Multivariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SMOKINGSTATUS$</td>
<td>3.799</td>
<td>1</td>
<td>3.799</td>
<td>1.706</td>
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<td>40.072</td>
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<td>2.226</td>
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</table>

Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
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<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>2.355</td>
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<td>2.355</td>
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</tr>
<tr>
<td>Period * SMOKINGSTATUS$</td>
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<td>1</td>
<td>0.191</td>
<td>1.511</td>
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</tr>
<tr>
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<td>18</td>
<td>0.126</td>
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<td>.</td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

<table>
<thead>
<tr>
<th>Within Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>11.795</td>
<td>1</td>
<td>30.762</td>
<td>0.000</td>
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<td>.</td>
<td></td>
</tr>
<tr>
<td>Treatment * SMOKINGSTATUS$</td>
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<td>1</td>
<td>0.004</td>
<td>0.010</td>
<td>0.923</td>
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<td>.</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>6.902</td>
<td>180.383</td>
<td>18</td>
<td>0.410</td>
<td></td>
<td></td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

<table>
<thead>
<tr>
<th>Within Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period * Treatment</td>
<td>0.052</td>
<td>0.052</td>
<td>1</td>
<td>0.127</td>
<td>0.726</td>
<td>.</td>
<td>.</td>
<td></td>
</tr>
<tr>
<td>Period * Treatment * SMOKINGSTATUS$</td>
<td>0.000</td>
<td>1</td>
<td>0.000</td>
<td>0.001</td>
<td>0.977</td>
<td>.</td>
<td>.</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>7.380</td>
<td>180.410</td>
<td>18</td>
<td>0.410</td>
<td></td>
<td></td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>
Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

11. Repeat measure analysis of variance of lactulose excreted in urine samples collected over the first three hours and the second three hour period in subjects who had received the placebo drink.

▼ Nonparametric: Kolmogorov-Smirnov Test

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SECONDTHREEHOUR</td>
<td>40</td>
<td>0.233</td>
<td>0.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGSECONDTHREEHOUR</td>
<td>40</td>
<td>0.098</td>
<td>0.416</td>
</tr>
</tbody>
</table>

▼ General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Placebo')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>NS   S</td>
</tr>
<tr>
<td>(2 levels)</td>
<td></td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOUR LOGSECONDTHREEHOUR</td>
</tr>
<tr>
<td>2.871 3.155</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeated Measures Factors and Levels of Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within Factor</td>
</tr>
<tr>
<td>Period</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects Source</th>
<th>SS</th>
<th>df Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>1.783</td>
<td>1.783</td>
<td>1.528</td>
<td>0.232</td>
</tr>
<tr>
<td>Error</td>
<td>21.005</td>
<td>18.1.167</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>0.854</td>
<td>1</td>
<td>0.854</td>
<td>4.095</td>
<td>0.058</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Period *SMOKINGSTATUS$</td>
<td>0.088</td>
<td>1</td>
<td>0.088</td>
<td>0.420</td>
<td>0.525</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Error</td>
<td>3.752</td>
<td>18</td>
<td>0.208</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

▼ General Linear Model

Data for the following results were selected according to
SELECT (TREATMENTS = 'Placebo')

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOURLOGSECONDTHREEHOUR</td>
</tr>
<tr>
<td>2.871</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeated Measures Factors and Levels of Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within Factor</td>
</tr>
<tr>
<td>Period</td>
</tr>
</tbody>
</table>

Plot of Residuals vs. Predicted Values
Univariate Repeated Measures Analysis

### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>0.808</td>
<td>1</td>
<td>0.808</td>
<td>3.997</td>
<td>0.060</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>3.840</td>
<td>19</td>
<td>0.202</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon: 
Huynh-Feldt Epsilon: 

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

12. Repeat measure analysis of variance of lactulose excreted in urine samples collected over the first three hours and the second three hour period in subjects who had received the aspirin drink.

▼ General Linear Model

Data for the following results were selected according to 
SELECT (TREATMENT$ = 'Aspirin')

Effects coding used for categorical variables in model.  
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>(2 levels) NS S</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

### Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSECONDTHREEHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.594</td>
<td>3.979</td>
</tr>
</tbody>
</table>

### Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

### Between Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>2.019</td>
<td>1</td>
<td>2.019</td>
<td>1.400</td>
<td>0.252</td>
</tr>
<tr>
<td>Error</td>
<td>25.968</td>
<td>18</td>
<td>1.443</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>1.554</td>
<td>1</td>
<td>1.554</td>
<td>4.740</td>
<td>0.053</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix

Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>Huynh-Feldt Epsilon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time*SMOKINGSTATUS$</td>
<td>0.104</td>
<td>1</td>
<td>0.104</td>
<td>0.316</td>
<td>0.581</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>5.901</td>
<td>18</td>
<td>0.328</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

▼ General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Aspirin')

N of Cases Processed: 20

### Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSECONDTHREEHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.594</td>
<td>3.979</td>
</tr>
</tbody>
</table>

### Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Plot of Residuals vs. Predicted Values
Univariate Repeated Measures Analysis

Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>1.490</td>
<td>1</td>
<td>1.490</td>
<td>4.713</td>
<td>0.053</td>
</tr>
<tr>
<td>Error</td>
<td>6.005</td>
<td>19</td>
<td>0.316</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

13. Doubly repeated measure analysis of variance of mannitol excreted in urine samples collected over the first three hours and the entire six hour period in subjects following the consumption of the aspirin and the placebo drink.

▼ Nonparametric: Kolmogorov-Smirnov Test

Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGASPIRINTHREEHOUR</td>
<td>20</td>
<td>0.123</td>
<td>0.626</td>
</tr>
<tr>
<td>LOGPLACEBOTHREEHOUR</td>
<td>20</td>
<td>0.121</td>
<td>0.644</td>
</tr>
<tr>
<td>LOGASPIRINSIXHOUR</td>
<td>20</td>
<td>0.148</td>
<td>0.298</td>
</tr>
<tr>
<td>LOGPLACEBOSIXHOUR</td>
<td>20</td>
<td>0.203</td>
<td>0.031</td>
</tr>
</tbody>
</table>

▼ General Linear Model

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
<th>NS = non-smokers; S = smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS</td>
<td>NS, S</td>
<td></td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
<th>LOGASPIRINTHREEHOUR</th>
<th>LOGPLACEBOTHREEHOUR</th>
<th>LOGASPIRINSIXHOUR</th>
<th>LOGPLACEBOSIXHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.629</td>
<td>5.738</td>
<td>6.067</td>
<td>6.156</td>
</tr>
</tbody>
</table>
### Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.00</td>
<td>1.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.00</td>
<td>2.00</td>
<td>1.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

### Univariate and Multivariate Repeated Measures Analysis

#### Between Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>0.215</td>
<td>1</td>
<td>0.215</td>
<td>0.613</td>
<td>0.444</td>
</tr>
<tr>
<td>Error</td>
<td>6.313</td>
<td>18</td>
<td>0.351</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>3.559</td>
<td>1</td>
<td>3.559</td>
<td>210.760</td>
<td>0.000</td>
</tr>
<tr>
<td>Period * SMOKINGSTATUS$</td>
<td>0.041</td>
<td>1</td>
<td>0.041</td>
<td>2.401</td>
<td>0.139</td>
</tr>
<tr>
<td>Error</td>
<td>0.304</td>
<td>18</td>
<td>0.017</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon: 

Huynh-Feldt Epsilon: 

#### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.153</td>
<td>1</td>
<td>0.153</td>
<td>1.843</td>
<td>0.191</td>
</tr>
<tr>
<td>Treatment * SMOKINGSTATUS$</td>
<td>0.244</td>
<td>1</td>
<td>0.244</td>
<td>2.930</td>
<td>0.104</td>
</tr>
<tr>
<td>Error</td>
<td>1.498</td>
<td>18</td>
<td>0.083</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon: 

Huynh-Feldt Epsilon: 

#### Within Subjects

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period * Treatment</td>
<td>0.001</td>
<td>1</td>
<td>0.001</td>
<td>0.109</td>
<td>0.745</td>
</tr>
<tr>
<td>Period * Treatment * SMOKINGSTATUS$</td>
<td>0.005</td>
<td>1</td>
<td>0.005</td>
<td>0.441</td>
<td>0.515</td>
</tr>
<tr>
<td>Error</td>
<td>0.220</td>
<td>18</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon: 

Huynh-Feldt Epsilon: 

---

334
14. Repeat measure analysis of variance of lactulose excreted in urine samples collected over the first three hours and the entire six hour period in subjects who had received the placebo drink.

▼ Nonparametric: Kolmogorov-Smirnov Test

Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THREEHOUR</td>
<td>40</td>
<td>0.108</td>
<td>0.275</td>
</tr>
<tr>
<td>SIXHOUR</td>
<td>40</td>
<td>0.135</td>
<td>0.065</td>
</tr>
</tbody>
</table>

LOGTHREEHOUR
LOGSIXHOUR

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOUR</td>
<td>40</td>
<td>0.085</td>
<td>0.659</td>
</tr>
<tr>
<td>LOGSIXHOUR</td>
<td>40</td>
<td>0.134</td>
<td>0.069</td>
</tr>
</tbody>
</table>

▼ General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Placebo')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>(2 levels) NS S</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOUR 5.738</td>
</tr>
<tr>
<td>LOGSIXHOUR 6.156</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects Source</th>
<th>SS</th>
<th>df Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>0.000</td>
<td>0.000</td>
<td>0.003</td>
<td>0.956</td>
</tr>
<tr>
<td>Error</td>
<td>2.606</td>
<td>180.145</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Within Subjects Source</th>
<th>SS</th>
<th>df Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.711</td>
<td>1.711</td>
<td>135.503</td>
<td>0.000</td>
</tr>
<tr>
<td>Period *SMOKINGSTATUS$</td>
<td>0.008</td>
<td>0.008</td>
<td>0.648</td>
<td>0.431</td>
</tr>
<tr>
<td>Error</td>
<td>0.227</td>
<td>180.013</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

General Linear Model

Data for the following results were selected according to
SELECT (TREATMENT$ = 'Placebo')

N of Cases Processed: 20

### Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSIXHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.738</td>
<td>6.156</td>
</tr>
</tbody>
</table>

### Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

### Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.752</td>
<td>1.752</td>
<td>141.3890.000</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
15. Repeat measure analysis of variance of mannitol excreted in urine samples collected over the first three hours and the entire six hour period in subjects who had received the aspirin drink.

▼ General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Aspirin')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>2 levels</td>
</tr>
<tr>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOUR</td>
</tr>
<tr>
<td>5.629</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables
Within Factor
<table>
<thead>
<tr>
<th>Period</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>0.458</td>
<td>1</td>
<td>0.458</td>
<td>1.586</td>
<td>0.224</td>
</tr>
<tr>
<td>Error</td>
<td>5.205</td>
<td>18</td>
<td>0.289</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Within Subjects Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.849</td>
<td>1</td>
<td>1.849</td>
<td>112.326</td>
<td>0.000</td>
</tr>
<tr>
<td>Period *SMOKINGSTATUS$</td>
<td>0.038</td>
<td>1</td>
<td>0.038</td>
<td>2.293</td>
<td>0.147</td>
</tr>
<tr>
<td>Error</td>
<td>0.296</td>
<td>18</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

▼ General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Aspirin')

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOUR</td>
</tr>
<tr>
<td>5.629</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Plot of Residuals vs. Predicted Values

<table>
<thead>
<tr>
<th>ESTIMATE(1)</th>
<th>ESTIMATE(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RESIDUAL(1)</td>
<td>RESIDUAL(1)</td>
</tr>
<tr>
<td>RESIDUAL(2)</td>
<td>RESIDUAL(2)</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Within Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Period</td>
</tr>
<tr>
<td>Error</td>
</tr>
</tbody>
</table>
Appendix

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

16. Doubly repeated measure analysis of variance of mannitol excreted in urine samples collected over the first three hours and the second three hour period in subjects following the consumption of the aspirin and the placebo drink.

▼ Nonparametric: Kolmogorov-Smirnov Test

Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGASPIRINTHREEHOUR</td>
<td>20</td>
<td>0.123</td>
<td>0.626</td>
</tr>
<tr>
<td>LOGPLACEBOTHREEHOUR</td>
<td>20</td>
<td>0.121</td>
<td>0.644</td>
</tr>
<tr>
<td>LOGASPIRINSECONDTREE</td>
<td>20</td>
<td>0.136</td>
<td>0.431</td>
</tr>
<tr>
<td>LOGPLACESECONDTREE</td>
<td>20</td>
<td>0.112</td>
<td>0.798</td>
</tr>
</tbody>
</table>

▼ General Linear Model

Effects coding used for categorical variables in model. The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$ (2 levels)</td>
<td>NS = non-smokers; S = smokers</td>
</tr>
</tbody>
</table>

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGASPIRINTHREEHOUR - LOGPLACEBOTHREEHOUR</td>
</tr>
<tr>
<td>5.629</td>
</tr>
<tr>
<td>4.950</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1.000</td>
<td>1.000</td>
<td>2.000</td>
<td>2.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.000</td>
<td>2.000</td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate and Multivariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>0.013</td>
<td>0.013</td>
<td>0.028</td>
<td>0.870</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>8.750</td>
<td>180.486</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
17. Repeat measure analysis of variance of mannitol excreted in urine samples collected over the first three hours and the second three hour period in subjects who had received the placebo drink.

**Nonparametric: Kolmogorov-Smirnov Test**

**Kolmogorov-Smirnov One-Sample Test using Normal(0.00, 1.00) Distribution**

<table>
<thead>
<tr>
<th>Variable</th>
<th>N of Cases</th>
<th>Maximum Difference</th>
<th>Lilliefors Probability (2-Tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SECONDTHREEHOUR</td>
<td>40</td>
<td>0.089</td>
<td>0.577</td>
</tr>
<tr>
<td>LOGSECONDTHREEHOUR</td>
<td>40</td>
<td>0.117</td>
<td>0.171</td>
</tr>
</tbody>
</table>

**General Linear Model**

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Placebo')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are
Variables

<table>
<thead>
<tr>
<th>Levels</th>
<th>SMOKINGSTATUS$ (2 levels)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = non-smokers; S = smokers

N of Cases Processed: 20

Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSECONDTHREEHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.738</td>
<td>5.033</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SMOKINGSTATUS$</td>
<td>0.041</td>
<td>1</td>
<td>0.041</td>
<td>0.217</td>
<td>0.647</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>3.384</td>
<td>18</td>
<td>0.188</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Within Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period</td>
<td>5.038</td>
<td>1</td>
<td>5.038</td>
<td>48.819</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Period *SMOKINGSTATUS$</td>
<td>0.073</td>
<td>1</td>
<td>0.073</td>
<td>0.712</td>
<td>0.410</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>1.858</td>
<td>18</td>
<td>0.103</td>
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<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon:

Huynh-Feldt Epsilon:

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

▼ General Linear Model

Data for the following results were selected according to
SELECT ( TREATMENT$ = 'Placebo')

N of Cases Processed: 20

Dependent Variable Means

<table>
<thead>
<tr>
<th>LOGTHREEHOUR</th>
<th>LOGSECONDTHREEHOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.738</td>
<td>5.033</td>
</tr>
</tbody>
</table>

Repeated Measures Factors and Levels of Dependent Variables

<table>
<thead>
<tr>
<th>Within Factor</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.000</td>
<td>2.000</td>
</tr>
</tbody>
</table>
## Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
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<td>1</td>
<td>4.967</td>
<td>48.868</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>1.931</td>
<td>19</td>
<td>0.102</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

18. Repeat measure analysis of variance of mannitol excreted in urine samples collected over the first three hours and the second three hour period in subjects who had received the aspirin drink.

▼ General Linear Model

Data for the following results were selected according to
SELECT (TREATMENT$ = 'Aspirin')

Effects coding used for categorical variables in model.
The categorical values encountered during processing are

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKINGSTATUS$</td>
<td>(2 levels) NS</td>
</tr>
</tbody>
</table>
N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOURLOGSECONDTHREEHOUR</td>
</tr>
<tr>
<td>5.629</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeated Measures Factors and Levels of Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within Factor</td>
</tr>
<tr>
<td>Period</td>
</tr>
</tbody>
</table>

Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Between Subjects</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SMOKINGSTATUS$</td>
<td>0.134</td>
<td>1</td>
<td>0.134</td>
<td>0.339</td>
<td>0.568</td>
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<td>Error</td>
<td>7.096</td>
<td>18</td>
<td>0.394</td>
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<table>
<thead>
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<th>Within Subjects</th>
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<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>4.782</td>
<td>37.916</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Period * SMOKINGSTATUS$</td>
<td>0.256</td>
<td>1</td>
<td>0.256</td>
<td>2.028</td>
<td>0.172</td>
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<tr>
<td></td>
<td>Error</td>
<td>2.270</td>
<td>18</td>
<td>0.126</td>
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<td></td>
</tr>
</tbody>
</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.

General Linear Model

Data for the following results were selected according to
SELECT (TREATMENT$ = 'Aspirin')

N of Cases Processed: 20

<table>
<thead>
<tr>
<th>Dependent Variable Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOGTHREEHOURLOGSECONDTHREEHOUR</td>
</tr>
<tr>
<td>5.629</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeated Measures Factors and Levels of Dependent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within Factor</td>
</tr>
<tr>
<td>Period</td>
</tr>
</tbody>
</table>
Univariate Repeated Measures Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Squares</th>
<th>F-Ratio</th>
<th>p-Value</th>
<th>G-G</th>
<th>H-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period</td>
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<td>1</td>
<td>4.609</td>
<td>34.672</td>
<td>0.000</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Error</td>
<td>2.526</td>
<td>19</td>
<td>0.133</td>
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<td></td>
<td></td>
<td></td>
</tr>
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</table>

Greenhouse-Geisser Epsilon
Huynh-Feldt Epsilon

Since your repeated measure has only two levels, Greenhouse-Geisser and Huynh-Feldt Epsilon corrections are not necessary.
APPENDIX 3

Letter published by

Rafati DS, Shulman RJ. Mucosal permeability testing: should interpretation change or stay the same? Neurogastroenterology & Motility 2013;25:854-854
LETTER TO THE EDITOR

Mucosal permeability testing: should interpretation change or stay the same?

To the Editors

We read with interest the paper by Sequeira et al.1 The authors reported the temporal pattern of urinary excretion of mannitol and lactulose in smokers and non-smokers in the presence and absence of aspirin, concluding that sampling methodologies may influence interpretation of results. Their data support our previous work demonstrating the temporal pattern of these and other sugars used in gastrointestinal permeability testing.2 Based on a previous study cited by Sequeira et al., and their own data, the authors contend that the recovered quantities of individual sugars (i.e., mannitol) rather than the lactose-to-mannitol ratio (LMR) should form the basis for assessing permeability changes in the GI tract. Although this is a provocative hypothesis, the available data cast significant doubt on the veracity of this contention. A large body of work has shown that mannitol absorption and subsequent urinary recovery is related to transit time, mucosal integrity, and surface area (the latter being the most important factor [see Ref. 3 and 4, among others]). Furthermore, Elia et al., among others, have shown that urinary recovery of intravenously administered mannitol and lactulose continues up to 24 hours after administration.5 Thus, urinary excretion of mannitol and lactulose is also dependent on renal clearance. Based on our data and theirs, we agree that interpretation of the LMR depends on the doses administered and the timing of the urine collection.1,2 That said, the wealth of studies demonstrating the utility of the LMR in evaluating small intestinal mucosal permeability (or damage) in inflammatory bowel disease, celiac disease, damage from non-steroidal anti-inflammatory drugs, allergic disease, environmental enteropathy, and following chemotherapy, and many other clinical conditions underscores the utility of the LMR when used properly.

D. S. Rafati*,† a) R. I. Shulman*, ‡,†,‡
*Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA, †Department of Pediatrics, Texas Children’s Hospital, Houston, TX, USA and ‡Children’s Nutrition Research Center, Houston, TX, USA

REFERENCES
Response to the letter published as

Appendix

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate’s Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate’s contribution as indicated below in the Statement of Originality.

Name of Candidate: Ivana Roosevelt Sequeira

Name/Title of Principal Supervisor: Prof. Roger Graham Lente

Name of Published Research Output and full reference: Sequeira IR, Lentle RG, Kruger MC, Hnr R.D.

In which Chapter is the Published Work: Appendix §

Please indicate either:
• The percentage of the Published Work that was contributed by the candidate: 75%
and/or

• Describe the contribution that the candidate has made to the Published Work:

Ivana Sequeira
Digitally signed by Ivana Sequeira
Date 2014.12.15T15:52:13 -1300

Candidate’s Signature

15.12.2014
Date

Principal Supervisor’s Signature

27/1/2015
Date

GRS Version 3 – 16 September 2011

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LETTER TO THE EDITOR

Mucosal permeability testing: response

To the Editors

We concurred with the view of the correspondents that the lactulose mannitol ratio (LMR) is a useful parameter for the clinical assessment of gut permeability. However, their letter appears to misquote our work in that they mention only mannitol rather than lactulose and mannitol. This statement is founded on the need to distinguish the effects of the two variables that contribute to the LMR if we are to increase its sensitivity.

The use of the LMR was originally founded on the premise that mannitol would be absorbed in proportion to the number of enterocytes in the absorbing segments and hence enable comparisons between subjects with gut components of differing surface area. However, the use of a ratio as a surface standardized index of permeability to large probes can only be as strong as the invariance of the denominator. As the correspondents point out, the levels of excretion of mannitol are variant in this respect. Moreover, the published work suggested, and subsequent work (currently in submission) further confirmed, that the rate of excretion of mannitol peaks at 2 h in contrast to that of lactulose which remains relatively constant over 6 h. Hence, mannitol excretion could differently confound the LMR when it is determined from urine collected over a single 2 h or 3 h period. Furthermore, when the test is carried out after a single dose of aspirin, an agent that is known to influence small intestinal permeability, the excretion of lactulose is increased while that of mannitol is decreased, with the timings of the peaks unaffected. Hence, mucosal disruption or inflammation is likely to increase the magnitude of the effect of mannitol in confounding the ratio. We repeated the same work using dimethoate and obtained the same effect.

Our opinion is that the fidelity of the test, and comparability with other tests taken over the same time periods, will be increased if due regard is paid to any temporal variation in excretion of both sugars. Hence, the test should quantify the excretion of the two sugars over a minimum of two time intervals so as to allow the relative temporal variation in each to be assessed. A suitably standardized test using this data could increase sensitivity and thus be more likely to detect subjects with borderline disturbances of intestinal permeability.

I. R. Sequeira, R. G. Lestie, M. C. Kruger, & R. D. Hurst* Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand and *Food & Wellness Group, The New Zealand Institute for Plant & Food Research Ltd, Palmerston North, New Zealand

REFERENCES

APPENDIX 5

Health Screening Questionnaire used in Chapter 5
HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

Health Screening Questionnaire

Name
DOB…………………………
Telephone #……………………
Cell phone#……………………
Address……………………………………………………..
Email address………………………………………

A) GENERAL HEALTH

Please answer the following questions with a tick or cross i.e. ✓ if yes or X if no

Do you suffer from heartburn, indigestion or have a history of any other digestive disease other than appendicitis?

Do you suffer from any endocrinological disease e.g. diabetes (NIDDM/IDDM), persistent sugar in the urine or thyroid disease like goitre or have hormonal trouble?

Do you have any renal (kidney) disorders e.g. renal stones, proteinuria (protein in urine) or suffer from uraemia or recurrent UTI’s?

Do you have any hepatic (liver) disorders?

Have you had any recent major abdominal surgery?

Do you suffer from impaired immunity or have had any recent immunisation?

Have you had a recent gut infection

Do you have any bleeding from the bowel or any blood in the urine?

Have you had any chronic or recent constipation?

Have you had diarrhoea in the last 1 month?

Do you have nausea, vomiting?

Have you had mucus in stools?

Are your periods regular? Please provide details e.g. the number of days between cycles (we need this information so as to schedule your visits to the lab on the days you are not menstruating)

Have you had any recent UTI’s?
HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

B) URINATION – during each experimental session you will be asked to provide a specimen of urine every half hour (you are assured privacy for the same)

Do you have any difficulty in urinating that would prevent you from providing the required specimen?

C) VAGINAL DISCHARGE – Vaginal discharge during the experiment will interfere with the test.

Do you have any current condition that causes you to have a vaginal discharge? (asked as this will interfere with the test) Provision for paper towels for use by you prior to you providing the specimen will be made.

D) ASPIRIN SENSITIVITY – as part of this study you will be asked to take a single dose of 600mg soluble aspirin.

Are you allergic to aspirin, unable to take aspirin or have a tendency to develop any problems after having taken it (if so please give details)?

Are you currently taking any medications that interfere with aspirin, notably any Anticoagulants?
HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

Do you take aspirin regularly or any other NSAIDs e.g. ibuprofen (Neurofen), indomethacin, naproxen, diclofenac (Voltaren), Ponstan?

E) LIFESTYLE HABITS

Do you smoke (cigarettes, pipe tobacco, or any other herbs)?

How many drinks containing alcohol do you have a day/ if a weekend drinker how many drinks do you have? Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

Have you ever been told you have a high alcohol consumption rate?
**HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE**

**F) Do you take any of the following supplements? Please answer the following questions with a tick or cross i.e. V if yes or X if no**

<table>
<thead>
<tr>
<th>Vitamin supplements</th>
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<tbody>
<tr>
<td>Mineral supplements</td>
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<tr>
<td>Iron supplement</td>
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<tr>
<td>Other health foods</td>
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<tr>
<td>Extra calcium</td>
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<tr>
<td>Pills for anaemia</td>
<td></td>
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</tbody>
</table>

**G) Do you take any of the following medications:**

| Pills for gastric ulcer or stomach disorder |          |
| Blood thinning pills e.g. warfarin or aspirin |          |
| Probiotic or prebiotic supplements e.g. lactulose, fibre supplements |          |
| Immuno-suppressants or steroids (oral, spray or cream) |          |
| Antibiotics (within the last month) |          |
| Pain killers |          |
| Vaginally administered prescription or preparations |          |

Please list any other medications or supplements you are currently taking below

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HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

H) DIETARY INTAKE

As part of your daily diet how much fruit and vegetable would you consume a day?

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

Do you have a particular liking for any fruit or vegetable? If so please describe along with how much you eat of them.

________________________________________________________________________
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________________________________________________________________________

Are you allergic to any foods?

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** At the end of each experimental session you will be provided with a meal.
Appendix

APPENDIX 6

Editorial published commending the work published in Chapter 5

in Clinical and Experimental Pharmacology and Physiology
EDITORIAL COMMENTARY

Clinical tests for intestinal permeability: ‘Sweet’ transition from clinical to basic physiology

The integrity of the intestinal barrier is of major importance for health and quality of life, as corresponding perturbations are integral to a number of diseases (as discussed in an excellent recent review). Clinical tests for intestinal permeability are a remarkably useful tool for the assessment of the recovery of the intestinal barrier after inflammatory conditions accompanying the associated diseases, such as inflammatory bowel disease, Crohn’s disease and rheumatoid arthritis, to name a few. As with any analytical technique, there is room for improvement, and further basic and clinical research is required to find novel methods and fine tune existing assays for examining the integrity of the mucosal barrier.

Although highly invasive or in vitro methods can be used for basic research purposes, this is not possible for clinical research. However, several sugar absorption tests have been introduced to measure gastric, small intestinal and large intestinal permeability. In principle, it is only required that participants drink a test solution containing a mixture of different sugars, and urine is subsequently collected for a period of up to 24 h. Excreted sugars are then measured in the urine samples, normally by HPLC and related techniques. The percentage of sugar that is excreted or a ratio of the amount of excreted sugars is then a measure for the permeability in different regions of the gut. These tests are non-invasive and well tolerated by patients or test participants. They have been used for a number of years and numerous studies have been published that use these methods.

Although these tests are increasingly used, they suffer from several drawbacks:

1. The mechanisms that lead to the different absorption rates of sugars with different molecular weights are incompletely understood. A deeper insight in these mechanisms would help not only to diagnose changes in permeability, but could also help to elucidate the pathological processes that lead to these changes.

2. The impact of factors, such as age, sex and race, has been studied only to a limited extent. This could lead to the large variations in experimental data that make it necessary to use high n-numbers to achieve statistically meaningful results. A better understanding of factors that influence data variation and updated test procedures could help to reduce interindividual variations in the data. A supplementary option would be the use of mathematical models that lead to transformations of the raw data to achieve results with lower standard deviations.

3. The test procedure has not been globally standardized. Different sugars/sugar combinations, different sampling schemes and calculations are used. Some authors use parametric or non-parametric statistical tests, whereas others use transformations to convert non-normally distributed data and always use parametric tests. If used correctly, both strategies are sound, but because of these differences, results might not be comparable between different groups.

The study by Sequeira et al. published in this issue addresses some of these points. The authors carefully tested the excretion profiles of three sugars (lactulose, D-mannitol and L-rhamnose) over 6 h in females receiving either placebo or aspirin. The excretion profiles over time for all sugars were found to be bimodal with an early and a late component. These two components are attributed by the authors to differences in the permeability in different segments of the gut (small intestine vs colon). The excretion rates and consequently the total excretion of mannitol were larger than the corresponding values for rhamnose. Aspirin was found to increase the cumulative excretion of lactulose, whereas it decreased the excretion of mannitol and rhamnose (Fig. 1). The determination of the temporal profiles showed interesting differences in the excretion rates of the sugars. This information could help to find sampling schemes that are adapted to specific problems. Patients would benefit considerably from sampling periods that are kept at the minimum that would be required to detect significant changes in the excretion ratios. Additionally, Sequeira et al. used mathematical and statistical manipulations, such as the ‘reduced major axis’, that are not so common in this field. Such methods might look tricky or less straightforward than our accustomed methods. However, if they are used correctly, as in the present work, they help to describe

Fig. 1 Changes in the differential trafficking of saccharide probes in healthy female volunteers under placebo or aspirin treatment in clinical tests of intestinal permeability, GI, gastrointestinal.
data better and reveal hidden features. The detailed approach used by Sequeira et al. is a good example of how solid clinical physiology studies should be planned, paving the way for valuable improvements of existing methods to benefit both patients and clinicians.

Additionally, this study also reveals some important basic physiology questions that should be addressed. Given the powerful arsenal of in vivo imaging techniques available today, such as real-time imaging of deep tissue fluorescence and transgenic mice that express fluorescent proteins, there is a golden opportunity to study the actual sites of absorption of specific sugars in vivo, and at the same time assess the fraction of sugars metabolized by gastrointestinal residing bacteria. This would provide evidence that would help us to functionally explain the excretion of sugar fractions. Furthermore, more studies like this might give us data that can be used for corresponding prediction using state-of-the-art simulation methodologies that are currently used in the gastrointestinal tract field.

Overall, the important study of Sequeira et al. provides novel, useful data that will help us to improve our understanding of the physiological counterpart of the clinical tests for intestinal permeability that use saccharide probes. Sequeira et al. do not give ready-made solutions for the aforementioned problems, but they show that many helpful techniques are already available. Importantly, this study will lead to new questions that can be answered at the level of base and theoretical physiology, and then used in clinical physiology studies.

REFERENCES


Klaus Michel
Sotiris G Zarogiannidis

*Department of Human Biology, Technische Universität München, Freising, Germany
*Department of Physiology, University of Thessaly Medical School, Biopolis, Larissa, Greece

Email: kmichel@www.tum.de

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APPENDIX 7

*Health Screening Questionnaire used in Chapter 6 & 8*
HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

Health Screening Questionnaire

An assessment of the effect of food/neutraceuticals on gut transit time

Name
DOB.
Telephone #
Cell phone#
Address
Email address
Height
Weight

A) GENERAL HEALTH

Please answer the following questions with a tick or cross i.e. √ if yes or X if no

<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do you suffer from heartburn, indigestion or have a history of any other digestive disease other than appendicitis?</td>
<td></td>
</tr>
<tr>
<td>Do you have a history of any other digestive disease other than appendicitis i.e. dysphagia (difficulty in swallowing), gastric bezoars, fistulas, bowel obstruction, diverticulitis (these conditions lead to the accumulation of ingested material in the gastrointestinal tract)?</td>
<td></td>
</tr>
<tr>
<td>Have you been diagnosed with Crohn’s disease, ulcerative colitis, irritable bowel syndrome (IBS)?</td>
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<tr>
<td>Have you had any recent major abdominal/gastrointestinal surgery?</td>
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<tr>
<td>Have you had a recent gut infection</td>
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<tr>
<td>Do you have any bleeding from the bowel or any blood in the urine?</td>
<td></td>
</tr>
<tr>
<td>Have you had any chronic or recent constipation?</td>
<td></td>
</tr>
<tr>
<td>Have you had any chronic or recent constipation?</td>
<td></td>
</tr>
<tr>
<td>Have you had diarrhoea in the last 1 month?</td>
<td></td>
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<tr>
<td>Do you have nausea, vomiting?</td>
<td></td>
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<tr>
<td>Have you had mucus in stools?</td>
<td></td>
</tr>
</tbody>
</table>
Appendix

HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Have you had an earlier diagnosis of any endocrinological disease e.g. diabetes (NIDDM/IDDM), persistent sugar in the urine or thyroid disease like goitre or have hormonal trouble?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have any renal (kidney) disorders e.g. renal stones, proteinuria (protein in urine) or suffer from uraemia or recurrent urinary tract infections?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have any hepatic (liver) disorders i.e. hepatitis?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you suffer from impaired immunity or have had any recent immunisation?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are your periods regular? Please provide details e.g. the number of days between cycles (we need this information so as to schedule your visits to the lab on the days you are not menstruating)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you had any recent urinary tract infections (UTI’s)?</td>
<td></td>
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</tr>
</tbody>
</table>

B) ASPIRIN SENSITIVITY – as part of this study you will be asked to take a single dose of 600mg soluble aspirin.

Are you allergic to aspirin, unable to take aspirin or have a tendency to develop any problems after having taken it (if so please give details)?

_____________________________________________________

Are you currently taking any medications that interfere with aspirin, notably any Anticoagulants?

_____________________________________________________

_____________________________________________________

Do you take aspirin regularly or any other NSAIDs e.g. ibuprofen (Neurofen), indomethacin, naproxen, diclofenac (Voltaren), Ponstan?

_____________________________________________________

_____________________________________________________

_____________________________________________________

2
C) LIFESTYLE HABITS

Do you smoke (cigarettes, pipe tobacco, or any other herbs)?

How many drinks containing alcohol do you have a day? If a weekend drinker how many drinks do you have? Please state the kind of drink and amount e.g. 3 cans/pints of beer (330ml), one glass of wine etc.

D) Do you take any of the following supplements? Please answer the following questions with a tick or cross i.e. √ if yes or X if no

<table>
<thead>
<tr>
<th>Vitamin supplements</th>
<th>Mineral supplements</th>
<th>Iron supplement</th>
<th>Other health foods</th>
<th>Extra calcium</th>
<th>Pills for anaemia (iron, B12, folate)</th>
</tr>
</thead>
</table>
HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

E) Do you take any of the following medications:

| Pills for gastric ulcer or stomach disorder |
| Blood thinning pills e.g. warfarin or aspirin |
| Proton pump inhibitors that alter gastric pH (omeprazole, lansoprazole) |
| Histamine blockers (cimetidine, famotidine, ranitidine hydrochloride) |
| Prokinetics (cisapride, domperidone, Metaclopramide - Reglan) |
| Probiotic or prebiotic supplements e.g. lactulose, fibre supplements |
| Immuno-suppressants or steroids (oral, spray or cream) |
| Antibiotics (within the last month) |
| Antacids |
| Painkillers |

Please list any other medications or supplements you are currently taking below


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F) DIETARY INTAKE

As part of your daily diet how much fruit and vegetable would you consume a day?

---
HUMAN NUTRITIONAL STUDIES LABORATORY PROCEDURE

Do you have a particular liking for any fruit or vegetable? If so please describe along with how much you eat of them.

________________________________________

________________________________________

Are you allergic to any foods?

________________________________________

________________________________________

** At the end of each experimental session you will be provided with a meal.
APPENDIX 8

Food avoidance List given to participants in Chapter 6
An assessment of the effect of food/neutraceuticals on transit time through various components of the human gut

AVOIDANCE OF PARTICULAR FOODS DURING THE COURSE OF THE STUDY

If you participate in this study you will be asked to avoid the following a day before you are scheduled to come for your laboratory session as we are looking to assess the effect that a berry drink and a vitamin C drink (two of the five treatments that you will be randomised to) will have on your gut. Hence you will have to avoid any fruits or vegetables (and their products) that are blue purple and yellow orange (as well as dark green leafy vegetables) in colour. Below is a guide to help you.

- Cantaloupe
- Citrus fruits and juices, such as orange and grapefruit
- Kiwi fruit
- Mango
- Papaya
- Pineapple
- Strawberries, raspberries, blueberries, cranberries
- Blackcurrants
- Any berries that are of the red/purple and blue varieties
- Any grapes that are of the black and red varieties
- Any juice that are of berry, grape, citrus flavour
- Any fruits and vegetables that have a red/purple/blue colouration (red cabbage, plums, aubergine/egg plant)
- Guava
- Watermelon
- Broccoli, Brussels sprouts, cauliflower
- Green and red peppers
- Spinach, cabbage, turnip greens, and other leafy greens
- Sweet and white potatoes
- Tomatoes and tomato juice
- Winter squash
- Black rice/ noodles
- Any juice that are of berry or grape flavours
- Blackcurrant juice or cordial, cranberry
- Any conserves that contain berries (blackcurrant, blackberry boysenberry)
- Red wine

Some cereals and other foods and beverages are fortified with vitamin C. Fortified means a vitamin or mineral has been added to the food. Check the product labels to see how much vitamin C is in the product.
NOTE: Consumption of vitamin C-rich foods in their fresh, raw form is the best way to maximize vitamin C intake. The vitamin C content of food may be reduced by prolonged storage and by cooking. Hence, as the list contains many of the vegetables that are part of our daily diet, if you do have to eat it — reduce the amount you will eat and make sure that it is not raw i.e. in a salad or a quick stir fry.

Meat (chicken, pork, beef, lamb) and meat products, rice, noodles, couscous, bread, can be eaten freely. Avocados, bananas, pears, asparagus are low in vitamin C. Also cucumbers, mushrooms, beans such as garbanzo beans, kidney beans, pinto beans, cooked corn are low in vitamin C.

| Additionally, dinner the previous night before you are scheduled to come to lab should not be a high fat meal. We would also ask you to limit the amount of salads/fruits that you will have with your meal. Kindly also take care to avoid any foods that could cause you GI irritation i.e. spicy foods. |

Any questions you might have with regard to your food choices will be discussed with you by telephone or email or in person by Ivana Sequeira.
APPENDIX 9

Standard Operating Procedures for the use of SmartPill data logger

used in Chapter 6
GENERAL GUIDELINES AND SOP FOR USE OF SMARTPILL® DATA RECEIVER

You will be given a Data Receiver to be worn by you on a lanyard at all times during the study right from the ingestion of the SmartPill® (Capsule) until the day you pass it out.

- It is important that for accurate measurements you:
  - MUST be fasted for the required time mentioned on the information sheet before ingesting the capsule
  - MUST NOT be taking any medications that influence gastrointestinal transit or alter gastric pH (we will assess your eligibility when you come in for your screening session). If however you do start to take ANY MEDICATION thereafter you MUST notify us of the same.

- We will calibrate the capsule before you swallow it and ensure that the data receiver is receiving information from the capsule.
  - It is important for that you attend your session on the day scheduled. If you are UNABLE to come to the laboratory for any unforeseen reasons kindly contact us at the earliest. This is because once the SmartPill® has been calibrated and turned on we have to use it within a 2 hour window period.

- We will give you a Card that states your name and our details on it.
  - This Card must be carried by you at all times when you are on the Test. The purpose of this is to inform any person, in healthcare or otherwise that you have ingested the SmartPill. This is a precautionary measure as you cannot undergo a Magnetic Resonance Imaging (MRI) until confirmation of the passage of the Capsule.

- The Capsule normally passes naturally within 2-5 days. You will be required to visually confirm this and record it in the Diary given to you and mark it as an ‘event’ on your Receiver (see below).

- You will be contacted everyday that you are on the Test at a time convenient for you to ensure that you are not experiencing any discomfort.

IF at any time you experience any discomfort or have queries do not hesitate to contact Ivana Sequeira on the number provided in the Information Sheet and Card.

- Restrictions after capsule ingestion and during the test:
  - If the Capsule and the Receiver are separated by more than five feet data will be lost. Therefore, ensure that you keep the Data Receiver on the body whenever possible throughout the test.
- DO NOT wear the Receiver while bathing or showering, keep the receiver dry and as close to the body as possible.

- You must wear the Receiver suspended by the lanyard and kept as close to the abdominal area as possible.

- However, do not use the lanyard while sleeping. Ensure that the receiver is kept close to you when you are in bed.

- **Recording an `Event`:**

  - To place a marker in the electronic record you must depress the EVENT button on the Receiver. We will demonstrate this when you come in for your screening session.

  - The marker maybe used to record significant `events` you have identified i.e. ingestion of the Capsule, a meal, a bowel movement.

  - Pressing the EVENT button on the Receiver inserts a marker in the test data record but it does not identify the type of `event`. Therefore, it is essential that you manually write down all `events` in the Diary that is given to you. Use the time displayed on the Receiver to record `events`. 
Emergency Card given to participants in Chapter 6
SUBJECT NAME: __________________________

This person has swallowed a monitoring device called the SmartPill®. As this Capsule contains metal this person should not be allowed to undergo MRI scanning. In case of any medical emergencies or other difficulties day or night kindly contact:

Ivana Sequeira  
Institute of Food, Nutrition and Human Health  
Massey University, Palmerston North  
Tel/Text: 06 3569099 (81469) or 022 6751145  
E mail: I.R.Sequeira@massey.ac.nz

This project has been reviewed and approved by the  
Massey University Human Ethics Committee: Southern A, Application 12/42.  
If you have any concerns about the conduct of this research, please contact  
Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A.  
Telephone 06 350 5799 x 8717, email humanethicsouthea@massey.ac.nz.
APPENDIX 11

Diary to record daily ‘events’ given to participants in Chapter 6
<table>
<thead>
<tr>
<th>Date &amp; Time</th>
<th>Event</th>
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**SmartPill**

*The Measure of GI Health*

**DIARY**

Name: ________________

Test Start Date: ________________________

Press the EVENT button when you have a bowel movement and record the DATE and TIME of the EVENT in your DIARY. (E.g., eating a meal, getting up in the morning, passing gas, going to bed at night, nausea, vigorous activity, cramping/pain)
APPENDIX 12

Standard Operating Procedures for fecal sample collection

given to participants in Chapter 6
Standard operating procedure (SOP) for Collection of Faecal Samples

We require you to collect your stool samples as it is important that we monitor the passage of the capsule from your GI tract. This procedure had to be adhered to every time you have a bowel movement.

You will be provided with the following:

- Plastic bowl
- Gloves
- Sealable plastic bags
- Large Biohazard bag

First open the pack and then place the plastic bowl in the middle of the toilet bowl (this sits well in the bowl so that all faeces will be collected comfortably).

*Label the bag with the date and the time* and then place the sealable bag on top of the plastic bowel and draw the ends of the bag over the toilet seat.

**DO NOT** worry if urine is mixed with the faecal matter.

Once you have finished let the stool remain in the bag for 3 minutes, this time allowing the data receiver to detect any temperature changes so that it can record when the SmartPill® has been passed out of your body.

You will also be asked to visually confirm the evacuation of the capsule which you must record on your diary.

Wear the gloves and seal the small plastic bag provided and place it in the biohazard bag provided for storage.

The biohazard bag should be placed in a convenient cool place e.g. in a garage, garden shed pending collection/drop off by you.

All bags that have been returned to us will then be frozen until they are collected for disposal by incineration by interwaste.

*NOTE: We will demonstrate how you can place the bowl and the plastic bags onto the toilet bowl when you come to the laboratory so that you can see what is expected of you. Ivana will be happy to answer any questions and discuss any problems etc.*
Food avoidance List used in Chapter 8
AVOIDANCE OF PARTICULAR FOODS DURING THE COURSE OF THE STUDY

If you participate in this study you will be asked to avoid during the course of the study and a week prior the following:

All fruits and vegetables contain some amount of vitamin C.

Foods that are the highest sources of vitamin C include:

- Cantaloupe
- Citrus fruits and juices, such as orange and grapefruit
- Kiwi fruit
- Mango
- Papaya
- Pineapple
- Strawberries, raspberries, blueberries, cranberries
- Blackcurrants
- Guava
- Watermelon

Vegetables that are the highest sources of vitamin C include:

- Broccoli, Brussels sprouts, cauliflower
- Green and red peppers
- Spinach, cabbage, turnip greens, and other leafy greens
- Sweet and white potatoes
- Tomatoes and tomato juice
- Winter squash

Some cereals and other foods and beverages are fortified with vitamin C. Fortified means a vitamin or mineral has been added to the food. Check the product labels to see how much vitamin C is in the product.