DEVELOPMENT AND ANALYTICAL VALIDATION OF A GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR THE ASSESSMENT OF GASTROINTESTINAL PERMEABILITY AND INTESTINAL ABSORPTIVE CAPACITY IN DOGS

A Dissertation

by

HERIBERTO RODRIGUEZ FRAUSTO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Veterinary Microbiology

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ABSTRACT

Development and Analytical Validation of a Gas Chromatography-Mass Spectrometry Method for the Assessment of Gastrointestinal Permeability and Intestinal Absorptive Capacity in Dogs. (December 2008)

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Assessment of gastrointestinal permeability in vivo is considered a suitable method for the evaluation of gastrointestinal mucosal integrity. Probes commonly used include lactulose (L) and rhamnose (R) for the assessment of intestinal permeability, xylose (X) and 3-*O*-methylglucose (M) for the evaluation of intestinal absorptive capacity, and sucrose (S) for the assessment of gastric permeability. Traditionally, various methods have been used to quantify these markers in the urine after orogastric administration. However, urine collection is difficult and uncomfortable. A protocol based on the analysis of blood samples would be easier to perform. Thus, the aim of the first part of this project was to develop and validate a new gas chromatography-mass spectrometry (GC-MS) method for the quantification of five sugar probes in canine serum. The method was sensitive, accurate, precise, and reproducible for the simultaneous quantification of 5 sugar probes in serum. The aim of the second part of this project was to assess the kinetic profiles of these 5 sugar probes in serum after orogastric administration in dogs and to determine the optimal time point for sample collection. Dogs received a solution containing L (10 g/L), R (10 g/L), X (10 g/L), M (5 g/L), and S (40 g/L) by orogastric intubation. Baseline blood samples were collected. Subsequent timed blood samples were taken for a 24 hours period. Significant changes in serum concentrations of all 5 sugars were detected after administration of the test dose ($p<0.0001$ for all 5 probes). Serum concentrations of L and R were significantly different

from baseline concentrations from 90 to 240 and from 60 to 300 min post dosing respectively, and those of X, M, and S were significantly different from 30 to 240 min after dosing ($p<0.05$ for all 5 probes). Variations of the mean sugar concentrations of all dogs at 90, 120, and 180 minutes were analyzed using a Kruskal-Wallis test. Based on the results, only two blood samples, one taken at baseline and a second sample obtained between 90 and 180 after dosing, appear to be sufficient for assessment of intestinal permeability and mucosal absorptive capacity using these sugar probes.

DEDICATION

For Angeles, Heriberto Jr., Misael, and Sofia

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CHAPTER I

INTRODUCTION

The gastrointestinal (GI) mucosa is the largest interface between the body and the external environment.[1] It is exposed to a variety of environmental factors, which may initiate and/or perpetuate a disease. One of the major challenges for clinicians and researchers in gastroenterology is inflammatory bowel disease (IBD). In humans, the etiology of this disease remains unknown and is often idiopathic.[2] However, several diagnostic techniques are available for the characterization and localization of the mucosal lesions.[3] Additionally, an important set of biological markers has been validated for the monitoring of IBD activity.[4,5] However, these diagnostic techniques do not provide functional information about the intestinal epithelium. For several decades, gastrointestinal permeability and absorptive capacity tests have been considered to be a suitable tool for the assessment of mucosal barrier function in various gastrointestinal disease states.

GASTROINTESTINAL TRACT PHYSIOLOGY

The major functions of the GI tract include the digestion and absorption of nutrients, but it also serves as a protective barrier for digestive enzymes, pathogens, toxins, and other noxious luminal macromolecules.[6] The GI mucosa is composed of a single layer of columnar epithelium.[7] It is organized into villi and microvillus, which dramatically increases the absorptive surface area. The epithelium acts as a barrier lining the mucosal surface of the GI tract. Barrier function is achieved by several mechanisms, one of which is by the presence of well-organized intercellular junctions between the cells, known as tight junctions.[7]

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The intercellular tight junctions separate the apical from the basolateral cell surface to maintain a cellular polarity, which is essential for the regulation of passive diffusion of solutes and macromolecules through the cellular space.[8] These intercellular connections are made up by a variety of proteins that regulate permeation through the paracellular pathway.[9]

The structure of the intestinal epithelium involves a set of aqueous pores distributed along the crypt-villus axis, which exhibit a gradient in tight junction permeability.[10] Small channels (radius <6 Å) are relatively abundant at the tips of the villi, allowing the permeation of small molecules, such as mannitol, but excluding the passage of larger molecules, such as lactulose.[11,12] Intermediate sized channels (10-15 Å) are located at the base of the villi and the largest channels (50-60 Å), which occur in low abundance, are located in the crypts.[13] Therefore, the paracellular pathway through the tight junctions at the level of the crypts is thought to be the route of permeation for disaccharides. The tight junctions at the level of the villi contain more strand structures and are, therefore, more selectively permeable to monosaccharides than those at the level of the crypts.[14] However, the transcellular pathway in the area of the villi is thought to be the main route of permeation for monosaccharides, mainly because the cellular surface area is much larger than the surface area of the tight junctions.[12] .

INTESTINAL EPITHELIAL FUNCTION

Transcellular absorption. The intestinal epithelium prevents the entry of pathogens, toxins, and undigested macromolecules across the mucosa, while simultaneously digesting and selectively absorbing nutrients.[6] Such selective absorption is considered a complex mechanism, which depends on the physicochemical properties of the permeating substances (i.e., molecular size and molecular weight), as well as physiological factors, and intestinal epithelial characteristics, such as the polarized epithelial cellular structure, which ensures a selective distribution of protein transporters at the apical membrane.[15]

Macromolecules can cross the intestinal epithelium by transport through the cells (transcellular pathway) or between the cells (paracellular pathway). The transcellular transport can by permeation or by a carrier-mediated process. Carrier-mediated absorption can be active (ATP-dependent), or passive, driven by a electrochemical gradient.[16] A molecule crossing from the apical to the basolateral membrane by passive diffusion must be lipophilic, which will permit rapid absorption due to its solubility in the lipid bilayer.[17] In contrast, the transcellular absorption of hydrophilic compounds is carried out by substrate specific carriers or through active transport proteins.[18] The transcellular transport of nutrients and other substances from the apical to basolateral membrane is ATP dependent and is complemented by a transepithelial electrochemical gradient generated by the basolaterally positioned Na^+/K^+-ATPase.[16] The electrochemical gradient is essential for the passive permeation of some water-soluble substances through the paracellular pathway.[19]

Paracellular permeation. Paracellular permeability of small molecules through the tight junctions is passive, driven by an electro-osmotic gradient or in response to the presence of luminal glucose.[20] The paracellular mechanism dissipates any possibility of transepithelial gradient saturation that would stop the transcellular transport or cause back flux toward the intestinal lumen, thus maintaining an equilibrated internal and external environment.[16] A component of this equilibrium is due to the small intestine becoming progressively less permeable towards the colon, which gradually affects the permeability of polar compounds along the intestinal tract.[21] Thus, the low resistance of the small intestinal epithelial tissues at this level permits the passage of a significant proportion of solutes and water between epithelial cells (paracellular route).[22] In general, up to 95% of the permeability of the small intestine is due to the paracellular route. Conversely, the paracellular transport of a molecule has a tendency to decrease from the small intestine to the colon due to decreases in the absorptive surface area, cell, and intercellular tight junctions.[23] Thus, only approximately 5% of the mucosal permeability of the large intestine is attributable to the paracellular pathway.

The paracellular pathway is regulated by intercellular junctions, which contain specific transcellular proteins which make up a complex molecular net for the opening and closing of this pathway.[20] Claudins and adhesion molecules are the major proteins that interact with intracellular proteins, such as zonula occludens (ZO)-1, which in turn interact with the actin cytoskeleton for regulation of the paracellular pathway.[24]

FACTORS AFFECTING INTESTINAL EPITHELIAL PERMEABILITY

Enteropathogens, allergens, toxins, and viruses affect the tight junctions, leading to an increased permeability, which is a common mechanism involved in the pathogenesis of inflammatory bowel disease.[25] For example, in cell monolayers infected with *Salmonella typhimurium* (*S. typhimurium*), Zonulin-1 (ZO-1) expression was significantly decreased. Also, ZO-2, a zonula occludens protein located in the nucleus of epithelial cells and E-cadherin were downregulated in response to *Salmonella* infection.[26]

Recent studies in colonic epithelial cells infected with *Campylobacter jejuni*, showed a decrease in transepithelial electrical resistance (TER) and a redistribution and de-phosphorylation of occludin that led to the opening of tight junctions.[27] The same phenomenon was observed using an *in-vivo* permeability assay in rabbits perfused with zonula occludens toxin (Zot), a protein produced by *Vibrio cholera*, which induced modifications of cytoskeletal organization (monomer polymerization), leading to the opening of tight junctions due to the contraction of the perijunctional actin ring.[27,28] The loss of TER as well as an altered distribution of several tight junction proteins have been observed in cells infected with rotavirus.[29] Transmigration of polymorphonuclear cells by the epithelium are some other effects of cellular protein disruption caused by pathogens.[30]

Stress factors have been associated with an increased intestinal permeability.[31] Studies in rats under provoked stress showed an increase in jejunal permeability for probes such as Cr-EDTA and horseradish peroxidase (HRP). The increased intestinal permeability of Cr-EDTA in stress situations has been associated with an augmented

concentration of colonic interferon gamma (IFN-γ).[32] In another study, serum cortisol concentrations (as an indicator of stress) showed a positive correlation with an increase in the incidence of gastric ulcers and an increased intestinal permeability in response to sustained strenuous exercise.[33]

METHODS FOR ASSESSING INTESTINAL PERMEABILITY

***In vitro* methods.** *In vitro* and *in vivo* methods have been developed to assess the integrity of the intestinal epithelium. The properties of the intestinal epithelium can be assessed *in vitro* by the measurement of transepithelial electrical resistance (TER), which reflects tight junction resistance,[34] and by the measurement of permeability towards paracellular markers.[35] The success of this model depends on how closely it mimics all the *in vivo* conditions. *In vitro* models such as Ussing chambers use excised epithelial tissue, membrane preparations, and cultured cells.[36] Sections of the small intestine are prepared between two chambers containing buffer, and the measurement of transepithelial resistance and the passive or active transport of molecules across the epithelium is evaluated as indicators of intestinal barrier integrity.[12]

***In vivo* methods.** Anatomical and physiological characteristics of the mucosal epithelium play a significant role for *in vivo* methods. *In vivo* studies can be used to identify and localize lesions along the entire length of the digestive tract.[37] Such methods have been developed in humans and several animal species. , these tests consist of the oral administration of a solution containing the test markers, which will be excreted in the urine and measured as a percentage recovery of the administered amount.

MARKERS FOR THE EVALUATION OF PERMEABILITY *IN VIVO*

Markers for gastrointestinal permeability testing have to meet several properties. The ideal marker would be 1) non-metabolizable, 2) not endogenously produced, 3) non-toxic, 4) water soluble, 5) completely absorbed, 6) limited to the extracellular space, 7) excreted intact in the urine, and 8) easily measured.[38,39]

Polyethylene glycol. Polyethylene glycol (PEG) exists in many different polymer sizes. Studies evaluating the effect of PEG size on paracellular permeability have demonstrated that the bioavailability for PEG remained 100% for oligomers with a size around 600 Da.[40] The most commonly used polymer is PEG 400, which has a molecular weight of 400 g/mol.[38] PEG 400 is resistant to bacterial degradation, permeates the intestinal mucosa, and is excreted in urine in proportions ranging from 26 to 72% of the administered dose.[41,38] Smaller polymers have a molecular weight that is similar to some sugars (i.e., rhamnose with a molecular weight of 164 g/mol and 242 g/mol for PEG). Some other polymers have a similar size to ^{51}Cr-EDTA (359 g/mol). However, smaller polymers permeate the mucosa faster than rhamnose, while the permeation rate of the larger polymers (550 g/mol) is higher than those for ^{51}Cr-EDTA or lactulose.[41] Therefore, these differences in permeation rates and their tendency to be retained by tissues makes PEG less ideal for the evaluation of intestinal permeability.[42]

^{51}Cr-EDTA. Tests using radioactively labeled substances such as ^{51}Cr-EDTA, were common when permeability testing was first evaluated some decades ago. Even though the radioactivity incorporated in the substance is lower than the radiation emitted during routine abdominal radiography, the application of radioactive tests has been decreasing.[43,44] The radioactive marker ^{51}Cr-EDTA is usually given at doses that are chemically stable. ^{51}Cr-EDTA is not hydrolyzed by bacteria and the analysis of this radioisotope is considered simple. Under physiologic conditions ^{51}Cr-EDTA does not permeate any part of the intestinal mucosa. However, disease of any portion of the intestinal tract can cause an increased permeability of this marker. If combined with other markers and small intestinal lesions can thus be excluded an increase permeability towards ^{51}Cr-EDTA would suggest a disorder of the large intestine.[45]

Saccharides. Monosaccharides and disaccharides as well as synthetically modified sugars are the most common GI permeability markers used today. Rhamnose, mannitol, xylose, and 3-O-methyl-D-glucose are monosaccharides with a molecular size of 0.5-0.6 nm.[46] Lactulose, sucrose, and sucralose are the most commonly used disaccharides and have a molecular size of 342.2, 342.3 and 397.4 Da respectively. [47] In

addition, sugar markers are biologically safe for use in humans and animals.[47] However, some of these sugars may be degraded to some degree by digestive enzymes and/or luminal bacteria.[37]

Sucrose has been used as a marker of gastric barrier function in human beings and dogs. Sucrose is rapidly hydrolyzed within the small intestine and, therefore, permeability of intact sucrose implies pre-duodenal permeation due to gastric mucosal damage.[48] The enzyme sucrase isomaltase, located in the brush border of enterocytes of the small intestine, hydrolyzes sucrose into glucose and fructose.[49] Because sucrose is only present intact in the stomach and at the very proximal portion of the duodenum any increased of sucrose permeability would suggest gastric ulcers or another gastric disease.[37]

Lactulose is metabolized exclusively in the colon.[37] Therefore, lactulose permeability should be specific for the small intestine. Lactulose permeates the small intestine through paracellular pores, which are located at the crypt in a low density, while rhamnose absorption occurs mainly through transcellular aqueous pores, which are present at a higher frequency at the tip of the villi. None of these sugar probes has an affinity for carrier-mediated transport. Therefore, the ratio between these two markers is an indicator of intestinal mucosal integrity.[50]

Intestinal absorptive capacity can be estimated by carbohydrates that undergo carrier-mediated absorption, namely D-xylose and 3-O-methyl-D-glucose.[14,51] D-xylose undergoes passive carrier-mediated transport predominantly in the jejunum, whereas 3-O-methyl-D-glucose is absorbed by active carrier-mediated (Na-dependent) transport in the small intestine.[52] The ratio of D-xylose to 3-O-methyl-D-glucose absorption can be used to correct for pre- and post-mucosal factors affecting the absorption of these two monosaccharides.[53] Under physiologic conditions, sucralose, a sucrose molecule in which three hydroxyl groups have been replaced with chlorine atoms, passes the entire digestive tract without being absorbed. Moreover, sucralose is not metabolized by intestinal bacteria.[37] Thus, a sucralose permeability test may be used as an indicator of permeability of the entire gastrointestinal tract. However, the signal to noise ratio can be

high and it may be difficult to assess permeability of the colonic mucosa with these markers.

DEVELOPMENT OF GASTROINTESTINAL PERMEABILITY AND MUCOSAL FUNCTION TESTS

In dogs, a combination of various sugars has been used to assess gastrointestinal permeability. However; this combination will depend on which part of the gastrointestinal tract needs to be evaluated. For example, a 2-sugar protocol, simultaneously evaluating the permeability towards lactulose and L-rhamnose can be used for evaluation of intestinal permeability.[54] A 4-sugar protocol containing lactulose, L-rhamnose, methylglucose, and xylose can be used for evaluation of intestinal permeability and intestinal absorptive capacity.[55] A 5-sugar protocol that simultaneously measures lactulose, L-rhamnose, methylglucose, xylose, and sucrose can be used for evaluation of gastrointestinal permeability and intestinal absorptive capacity.[56] Regardless of which protocol is chosen, food is withheld the night before the procedure, and the bladder is completely emptied via catheterization before sugar administration. The sugar probes are dissolved in water and then administered by orogastric intubation.[57] Urine is subsequently collected over a period of six hours. Complete urine collection is important for accurate quantification of sugar permeation and absorption. However, passage of markers through the gastrointestinal tract and their permeation or absorption by the mucosa, as well as the distribution into the vascular space requires a lot of time. Therefore, 6 hours has been established as the routine time period to assess gastrointestinal permeability and absorptive capacity after oral administration of the five sugar markers described above in healthy dogs.[58]

Serum or urine concentrations of these markers do not only depend on the permeability of the intestinal mucosa. Non-mucosal factors, such as the rate of gastric emptying, intestinal transit time, dilution in the intestinal lumen, systemic distribution, metabolism, renal elimination, and urine removal all significantly influence the serum and urine concentrations for these markers.[59] In order to eliminate pre- and post-mucosal

factors, the calculation of a ratio of two measured values minimizes the influence of these factors to a large extent.[60] This is based on the assumption that both markers are affected in the same way by these non-mucosal factors. Thus, intestinal permeability is expressed as the ratio of the fractional excretion of a large molecule compared to a smaller one (for example lactulose to rhamnose).[60] That ratio can be interpreted as the number of intermediate sized pores as a proportion of the total number of aqueous channels.[13] As the smaller channels are concentrated at the villus tip, permeation rates of compounds across this pathway serve as an estimate of mature small-intestinal surface area. Thus, any epithelial damage at that level provides alternate routes for the permeation of larger molecules such as lactulose. As a result, an increase in the disaccharide/monosaccharide ratio can be observed [13]

APPLICATIONS OF GASTROINTESTINAL PERMEABILITY TESTING

Diseases that have been described as being associated with altered gastrointestinal permeability include acute viral gastroenteritis, *Clostridium perfringens*- and *Clostridium difficile*-associated diarrhea, gluten sensitive enteropathy, IBD, intestinal parasitism (e.g. ascarides, Giardia), protein losing enteropathy, small intestinal bacterial overgrowth (SIBO), diabetes mellitus, hepatitis, pancreatitis, and uremia.[61,62,51,63] Also, in several studies, non-steroidal anti-inflammatory drugs (NSAIDs) and cytotoxic drugs have been shown to increase gastrointestinal permeability.[64,65] Also, GI permeability tests may also be used to monitor response to therapy. Several studies in humans and dogs have reported the normalization of intestinal permeability after appropriate therapy.[66,42,67] The evaluation of intestinal permeability has been used as an early indicator for the relapse of certain diseases in humans, such as Crohn's disease[68] and also in dogs with gluten sensitive enteropathy.[69]

As mentioned above, traditionally GI permeability tests for the evaluation of mucosal damage in GI disease were set up by collecting urine for several hours and measuring urinary recoveries for sugar markers.[56] This method is considered time consuming and relatively cumbersome for patients and clinicians. In addition, renal

dysfunction, hydration status of the patients, and incomplete urine collection can potentially introduce methodical errors.[70] Moreover, methods using urine are considered invasive since some animals require bladder catheterization. All these aspects could be a source of error for this type of investigation.[71,72] Measurement of permeability markers in serum would reduce the problems associated with urinary recovery and may allow for the development of faster permeability testing based on the measurement of a single serum sample after oral administration of the test markers.

METHODS FOR SEPARATION AND DETECTION OF SUGAR MARKERS

Gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS), high-performance liquid chromatography coupled with pulsed amperometric detection (HPLC-PAD)[56] or with mass spectrometry (HPLC-MS) have all been reported for the simultaneous measurement of sugar probes in urine.[73] However, due to the physicochemical characteristics of sugars, they have to be converted into a more stable metabolite before GC analysis. This is one reason why HPLC-PAD or HPLC-MS are the most commonly used methods used for sugar analysis today. However, most detection systems coupled to HPLC have some technical drawbacks. For instance, refractive index (RI) based methods and evaporative light scattering detectors (ELSD) have poor selectivity due to the universal detection of compounds. Fluorescence based detection methods require fluorescent compounds, which are not available for sugar probes. Electrochemical detection, in particular PAD, is more suitable due to a higher sensitivity, though it also has low detection selectivity.[74]

More recently, liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (MS-ESI) has emerged as an alternative to overcome earlier problems with HPLC methods; however, this method has a low selectivity to cover a full range of metabolites. you are not trying to measure anything in a cell.[75,76] However, the literature demonstrates many examples where sugar analysis by GC-MS can be employed to enhance the sensitivity of sugar analysis because of its sensitivity and selectivity for biological constituents.[77]

Determination of metabolites containing hydroxyl and carbonyl functional groups requires derivatization before they can be analyzed by GC-based methods. Changes of the chemical structure are required to convert sugars into more volatile derivatives that are able to resist the high vapor pressure and temperatures of GC.[78] Such characteristics are achieved by reducing the polarity of functional groups. This results in a more thermally stable and volatile metabolite.[79]

The most common way to derivatize sugars that have functional groups, such as –OH, is to add a trimethylsilyl (TMS) group to form TMS-ether derivatives. TMS-ethers of mono- and disaccharides are easily prepared and separated chromatographically.[78] However, TMS-derivatization of monosaccharides often results in the formation of multiple peaks in the GC chromatogram due to their cyclic anomers resulting in five tautomeric forms of the reduced sugar.[80]

However, by converting the aldehyde- and keto-groups into oximes using hydroxylamine before the formation of the TMS groups eliminates the possibility of cyclic forms, resulting in the formation of only *syn* and *anti* structures. Several derivatization protocols can be used to produce both oximation and silylation reactions. Some advantages of TMS derivatization methods include the relative ease and rapid way to transform hydroxyl and carbonyl groups into the oxime and silyl derivatives.[81] BSTFA is a strong trimethylsilyl group donor and has demonstrated good silylation when combined with different catalyzing compounds such as pyridine and TMCS[79]

HYPOTHESIS AND SPECIFIC OBJECTIVES

The hypothesis of this project was that GC-MS is a sensitive and reproducible method that can be utilized to simultaneously detect lactulose, rhamnose, xylose, 3-O-methyl-D-glucose, and sucrose in canine serum.

The objectives of the present research project were: to 1) develop and validate a GC-MS-based method for the analysis of 5 orally administered sugar markers (lactulose, rhamnose, xylose, 3-O-methyl-D-glucose, and sucrose) in canine serum in order to establish the best suitable protocol for extraction and derivatization of these 5 sugar markers from serum and to establish the gas chromatographic conditions for the analysis of the 5 sugar markers in real serum samples; and 2) to analyze the kinetic profiles of lactulose, rhamnose, xylose, 3-O-methyl-D-glucose, and sucrose in serum of healthy dogs in order to establish the best time-point for collection of blood samples after an orogastric administration of a solution containing these 5 sugar probes.

CHAPTER II

DEVELOPMENT AND ANALYTICAL VALIDATION OF A GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR THE MEASUREMENT OF SUGAR PROBES IN CANINE SERUM

OVERVIEW

The study aim was to develop and analytically validate a gas chromatography-mass spectrometry (GC-MS) method for the quantification of lactulose (L), rhamnose (R), xylose (X), 3-O-methylglucose (M), and sucrose (S) in canine serum. Pooled serum samples from healthy dogs were spiked with these sugars at concentrations 3, 30, 100, and 350 mg/L. Mannitol was added as an internal standard. Serum samples were precipitated with methanol, derivatized to produce trimethylsilyl derivatives and then analyzed by GC-MS. The method was analytically validated by determination of dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability. Standard curves ranging from 0.5 to 500 mg/L for each sugar showed a mean r^2 of 0.997. The lower detection limit was 0.03 mg/L for L, R, M, and X, and 0.12 mg/L for S. The observed/expected ratios (O/E ratios) for dilutional parallelism had a mean ± SD of 105.6 ± 25.4% at dilutions of 1 in 2, 1 in 4, and 1 in 8. The analytical recoveries for the GC-MS assays of sugars had a range from 92.1% to 124.7% (mean ± SD 106.2 ± 13.0%). Intra-assay coefficients ranged from 6.8% to 12.9% for lactulose, 7.1% to 12.8% for rhamnose, 7.2% to 11.2% for xylose, 8.9% to 11.5% for methylglucose, and 8.9% to 12.0% for sucrose. Inter-assay coefficients of variation ranged from 7.0% to 11.5% for lactulose, 6.4% to 9.4% for rhamnose, 6.8% to 13.2% for xylose, 7.0% to 15.9% for methylglucose, and 5.5% to 9.4% for sucrose. The GC-MS method described here is accurate, precise and reproducible for the simultaneous measurement of sugar markers in serum.

INTRODUCTION

The assessment of gastrointestinal mucosal permeability and absorptive capacity provides information about gastrointestinal mucosal function. Non-metabolizable markers, such as polyethylene glycol, sugars, or radiolabeled substances have been widely used.[82] Altered intestinal permeability has been reported in humans with inflammatory bowel disease (IBD) and also in animals with experimental gastrointestinal disease.[83] In humans and dogs, intestinal permeability generally increases with the severity of the disease.[84,63] Intestinal permeability has even been postulated to be responsible for the introduction of antigenic or infectious agents through the intestinal mucosa, leading to excessive immunogenic stimulation.[85,86]

Today mono- and disaccharides are widely used to assess abnormalities of gastrointestinal permeability and mucosal absorptive capacity.[50] With the exception of mannitol, which is believed to be synthesized in only small quantities in humans,[43] but is not believed to be synthesized in animals,[87] both groups of sugars probes (mono- and disaccharides) are not considered to be endogenously synthesized in mammalian species.[43] Therefore, serum concentrations of these sugars are considered to exclusively originate from gastrointestinal permeability. The use of a mixture of mono- and disaccharide markers is based on the assumption that the intestinal epithelium is a heteroporous layer.[11] Aqueous pores are distributed along the crypt-villus axis of the small intestinal mucosa. Small channels (radius <6 Å) are relatively abundant at the tips of the villi, allowing the permeation of small molecules such as rhamnose, but excluding the passage of larger molecules, such as lactulose.[12] Therefore, molecules of the size of disaccharides (e.g., lactulose) are restricted from moving across the villus tip, whereas monosaccharides, such as rhamnose, can do so through passive diffusion. In contrast, while the monosaccharide mannitol is also absorbed by simple diffusion, it does not move freely across the villus tip. The largest channels (50-60 Å) are paracellular, exist in low abundance and are located in the crypts.[13] These paracellular pathways at the level of the tight junctions in the crypts are thought to be the route of permeation for

disaccharides such as lactulose, while the transcellular pathway at the level of the villi is thought to be the main route for permeation of monosaccharides.[12,88]

Rather than using a single permeability probe, often times a mixture of probes is being utilized. One advantage of using a mixture of different probes is that different probes remain intact only in specific compartments of the GI tract and this can help to localize the intestinal damage.[37] Class 1 probes (e.g., sucrose) are broken down upon entering the small intestine. Class 2 probes, such as lactulose and mannitol, pass through the stomach and the majority of the small bowel before undergoing bacterial degradation in the distal small intestine and especially the colon. Class 3 probes (e.g., Cr-EDTA and PEG) do not undergo metabolism or bacterial degradation and remain intact throughout the entire length of the gut.[37] However, because these markers permeate the gastrointestinal mucosa throughout its entire length the signal to noise ratio can be high and it may be difficult to assess permeability of the colonic mucosa with these markers.

Absorptive capacity of the intestinal mucosa can be measured effectively by use of non-metabolizable carbohydrates such as rhamnose, which is believed to permeate the gastrointestinal mucosa by the passive transcellular route, and thus permeability of this sugar is dependant on mucosal surface area.[89] Other carbohydrates that undergo passive and active carrier-mediated transport include D-xylose and 3-O-methyl-D-glucose respectively.[14,51] D-xylose is not absorbed by the intestine via a passive carrier (GLUT2 or GLUT5), but has a low affinity for the sodium dependent carrier SGLT-1 predominantly in the jejunum.[90,91] In contrast, 3-O-methyl-D-glucose is absorbed by active carrier-mediated transport (Na^+-dependent) throughout the small intestine.[92] These sugars are not metabolized and, after intravenous administration, are excreted intact in the urine.[53]

Many different protocols for assessment of intestinal permeability and mucosal function have been developed.[14,43] To date, protocols utilizing permeability markers excreted and recovered in urine are the most widely used.[58] Such protocols require complete urine collection over a period of 4 to 24 hrs, which is laborious and often impractical. Furthermore, incomplete urine collection may affect test results.[93] Thus, the

measurement of carbohydrate markers in serum would simplify assessment of intestinal permeability and absorptive function under clinical conditions, because serum samples can be obtained more easily in a practice setting.

Several methods for the quantification of carbohydrates in serum samples have been described. For example, sucrose has been measured in human serum using an enzymatic method.[94] In horses, a high performance liquid chromatography coupled to mass spectrometry (HPLC-MS) method has been used to measure sucrose in serum.[95] Measurement of 3-*O*-methyl-D-glucose in human serum has been reported using thin layer chromatography and densitometry.[96] Lactulose and mannitol have been measured in human serum using HPLC and pulsed amperometric detection (PAD).[93] Finally, in a recent study HPLC-PAD was used for the measurement of lactulose, rhamnose, 3-*O*-methyl-D-glucose, and xylose in dog serum.[97] For most detection systems coupled to HPLC some problems have been observed. For instance, refractive index (RI) based methods and evaporative light scattering detectors (ELSD) have poor selectivity due to universal detection of compounds, and fluorescent detection based methods require fluorescent compounds, which are not available for sugar probes. Electrochemical detection, in particular PAD, is more acceptable due to higher sensitivity, though it also has low detection selectivity.[74] Also, HPLC and PAD detection is not useful to directly analyze complex biological samples, such as serum, and serum proteins and lipids must be removed before analysis.

The use of gas chromatography - mass spectrometry (GC-MS) is considered to be a suitable method for the quantification of sugar probes, because of the high sensitivity of this method.[81] The resolution obtained with GC-MS results in good peak capacity, which is defined as the maximum number of non-overlapping peaks in a given interval.[81] GC-MS methods have been previously described for the quantification of carbohydrates in urine,[98,73] plasma,[99] aqueous solutions,[100] environmental samples, food products,[101] and serum.[102, 103]

The aim of this study was to analytically validate a GC-MS-based method for the simultaneous quantification of lactulose (L), rhamnose (R), xylose (X), methylglucose

(M), and sucrose (S) in order to assess gastrointestinal permeability and intestinal absorptive function in an accurate, fast, and practical fashion that could facilitate its use in research and clinical practice.

MATERIALS AND METHODS

Standard solutions. Stock solutions of individual sugar probes (L, R, M, X, and S)[a] were prepared by dissolving a mixture of all sugars in pooled canine serum from approximately 200 healthy dogs. Eleven standard solutions (i.e., 500.0, 250.0, 125.0, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0, 1.0, and 0.5 mg/L) were prepared for each of the 5 sugars by serial dilution of 1 in 2. Mannitol was used as an internal standard and was added to each standard solution in a final concentration of 100 mg/L.

Calibration curves were established by plotting the ratios of the area under the curve of the peaks of the sugars of interest to that of the internal standard for the different standard solutions using a polynomial curvilinear regression ($y = ax^2 + bx + c$). The sugar peak area ratios of unknown serum samples were then extrapolated from the calibration curves.

Sugars extraction and derivatization. Serum aliquots of 200 µL were mixed with 22.2 µL of an aqueous internal standard solution (mannitol in a final concentration of 100 mg/L) in 2-ml plastic vials. Then, 600 µL of methanol[b] was added to each serum sample and mixed for 20 seconds using a vortex mixer. Samples were then centrifuged[c] at 2,655 g for 7 minutes and the protein-free supernatants were transferred into 4-ml molded screw cap glass vials.[d] Samples were evaporated to dryness under a stream of nitrogen in a heating module[e] at 64°C for 30 min. Once the tubes were cooled, the dried residue was derivatized in a two-step procedure. First, 50 µL of Mox reagent (2% of Methoxyamine-HCl in pyridine)[f] and 70 µL of pure pyridine[f] were added to each tube. Tubes were capped and vortexed for 20 seconds and subsequently heated in a microwave for 2 min to promote the oximation reaction.[104] Then, samples were allowed to cool for 5 min and 100 µL of a *N,O-bis*[Trimethylsilyl]trifluoroacetamide (BSTFA)[f] containing 1% TMCS was added to each tube. The tubes were capped, vortexed for 20 seconds and

placed in a microwave for an additional 5 min to develop the silylation reaction according to the procedure described by Silva et al.[104] Once the samples were cooled at room temperature, derivatized extracts were evaporated to dryness under a nitrogen stream at 64°C for 8 minutes and the residues were dissolved in 250 µL of hexane.[g] GC-MS analysis was performed with 1 µl of this solution.

Gas chromatography. Derivatized samples were analyzed using gas chromatography[h] coupled with a mass spectrometer.[h] The 1 µL aliquots of the extracts were injected into a split/splitless inlet, operated in splitless mode, at an inlet temperature of 250°C. Separation of sugars was achieved using a DB-1MS capillary column[h] (30 m length, 250 µm inner diameter, and 0.25 µm film thickness). The following GC column temperature program was used: the initial oven temperature was set at 100°C and held for 5 minutes. Temperature was then increased from 100 to 325°C by a constant gradient of 15°C/min and held at 325°C for 5 min, resulting in a total run time of 23.33 min per sample. Helium was used as a gas carrier at a constant flow rate of 1.5 mL/min at a velocity of 33 cm/sec. The qualitative analysis was performed under full-scan acquisition mode within an *m/z* 50 to *m/z* 1050 range. Quantification of sugar concentrations was performed by using the sum of peak areas from specific retention times of each sugar using selected ion monitoring (SIM) mode. The selected ions for the sugars were: *m/z* 204, for lactulose, *m/z* 117, for rhamnose, *m/z* 147 for methylglucose, *m/z* 217 for sucrose, *m/z* 217 for xylose, and *m/z* 217 for mannitol.

Chromatograms. The derivatization procedure was evaluated by comparing the area under the curve for peaks generated from spiked serum samples with those generated from the same amount of the pure compound previously analyzed under the same derivatization and gas chromatography conditions. Sugars were identified by matching their chromatographic retention times and their characteristic mass spectrum (Table 1). In order to verify interference by other carbohydrates, an aqueous solution containing glucose, fructose, fucose, and sucralose as well as mannitol (used as an internal standard) lactulose, rhamnose, methylglucose, xylose, and sucrose, was analyzed. Figure 1 illustrates the chromatogram obtained from the analysis of a pooled

serum sample. In this chromatogram, no peak with a retention time of 14.06 (retention time of mannitol) was observed. This would indicate that mannitol is not present at detectable concentrations in canine serum. In contrast, figures 2 and 3 illustrate the chromatograms of a five sugar standard solution and an unknown canine serum sample, respectively, to which mannitol (m) has been added.

Validation. The method was validated by evaluating spiking recovery, dilutional parallelism, intra-assay variability, and inter-assay variability. For spiking recovery (accuracy of the assay), four different pooled serum samples obtained from healthy dogs were spiked with each sugar to reach a final serum concentration of 3 mg/L (sample A), 30 mg/L (sample B), 100 mg/L (sample C), and 350 mg/L (sample D), respectively. For dilutional parallelism (linearity of the assay), the spiked serum sample containing 350 mg/L (sample D) of each of the sugars and an unknown sample were diluted 1 in 2, 1 in 4, and 1 in 8 with canine serum. The results for spiking recovery and dilutional parallelism were expressed as observed to expected (O/E) ratios.

The 4 spiked serum samples (samples A through D) mentioned above were used to evaluate the precision and the reproducibility of the assay. To determine precision, intra-assay variability was assessed by running these 4 serum samples spiked with different concentrations of the five sugars probes 9 consecutives times within a single GC-MS run. Coefficient of variation was calculated for all five sugars**.** To determine reproducibility, inter-assay variability was evaluated by running the 4 serum samples spiked with different concentrations of the five sugar probes 9 times on different days.

RESULTS

Table 1 shows the GC-MS characteristics for all sugar probes. The retention times and the underlined *m/z* values belong to the peaks and the major ions, respectively, which were used for quantification. The rest of the ions are those observed according to the fragmentation pattern detected in full-scan MS mode used for the identification of the carbohydrates (chromatogram not shown). The mass spectra of the analyzed carbohydrates were dominated by ions at *m/z* 73, 117, 147, 204, 217, 361, 437, and 451,

and the retention times (RT) were constant at the GC conditions and derivatization procedures performed in this study. Figures 4 to 8 show the chromatograms for the individual sugars analyzed in SIM mode. The insets in the graphs show the SIM mass spectra with the respective quantification ion for each sugar. Fig. 4 shows the chromatogram for lactulose. A minor peak eluted at 18.01 min, whereas the two main peaks eluted at a RT of 18.42 and 18.44 min, respectively.

The inset graph shows the ion with *m/z* 204, which was used for analysis and quantification of this disaccharide. The sucrose chromatogram in Fig. 5 shows a minor peak at a RT of 17.84 min, while the prominent peak is found at a RT of 18.12 minutes. While sucrose and lactulose had peaks with RT and *m/z* close to each other, the derivatization protocol used in the present study achieved sufficient and consistent separation of these two compounds for the differentiation of these two carbohydrates. Fig. 6 displays the chromatogram for rhamnose showing peak RTs of 12.43 and 12.50 min. The chromatogram in Fig. 7 shows the chromatogram and RTs of xylose. Fig. 8 shows the chromatogram for methylglucose containing the selected ion at *m/z* 147. These m/z are in agreement with previous reports using the same derivatization reagents.

Table 1: Characteristics of sugars analyzed by GC-MS as TMS derivatives. Retention time indicates where chromatogram peaks of each sugar are located. The *m/z* depicts the GC-MS fragmentation ion pattern of each sugar. The m/z of the peaks selected for quantification of the sugars is underlined.

Sugar	Molecular mass	Retention time (min)	*m/z*
Lactulose	342	18.01, 18.42,	73, 147, <u>204</u>, 361
Sucrose	342	17.75, 18.12	73, <u>217</u>, 361, 437
Xylose	150	11.84, 11.90	73, 147, <u>217</u>, 307
Rhamnose	164	12.45, 12.50	73, <u>117</u>, 219, 277
Methylglucose	194	12.70, 13.16, 13.45	73, <u>147</u>, 205, 262
Mannitol	182	14.06	73, 205, <u>217</u>, 319

The m/z of the peaks selected for quantification of the sugars is underlined.

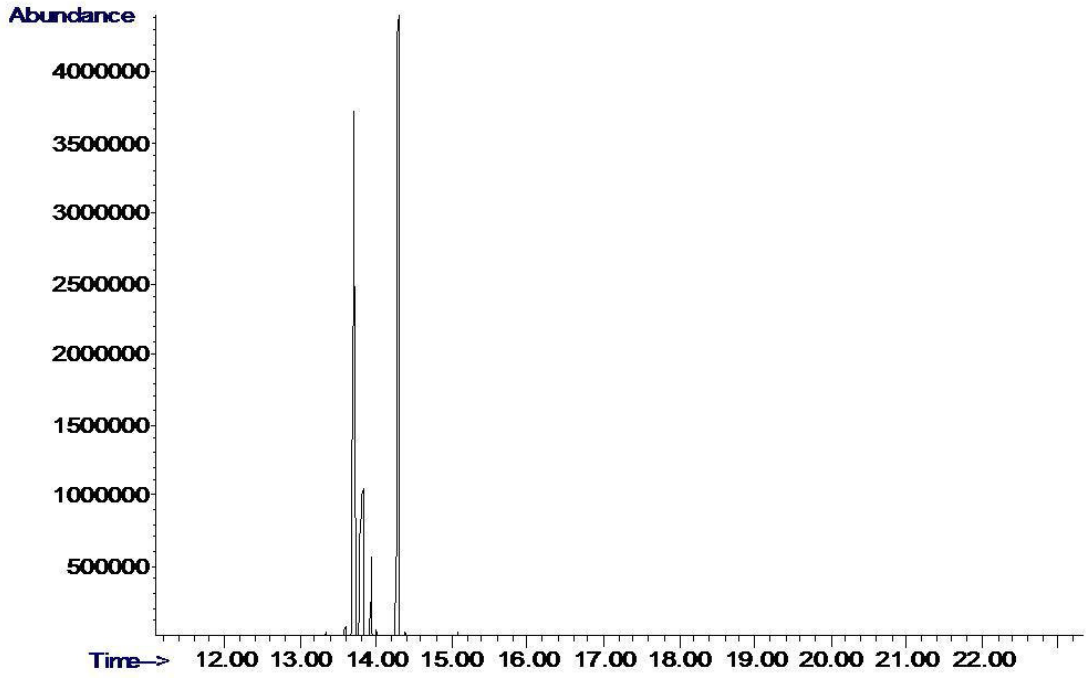

Figure 1: Total ion chromatogram for a blank serum sample. The chromatogram shown here is from a blank sample from pooled canine serum

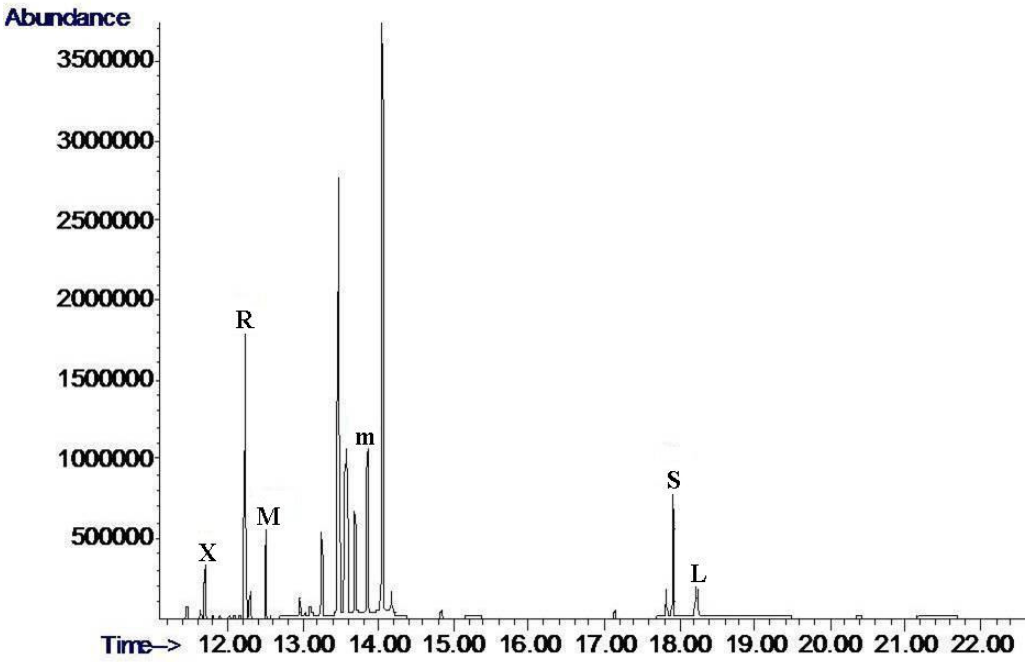

Figure 2: Total ion chromatogram for a five sugars standard solution. The chromatogram shown here is from analysis of a serum standard containing 125 mg/L of xylose (X), rhamnose (R), 3-*O*-methyl-D-glucose (M), sucrose (S), lactulose (L), and the internal standard mannitol (m).

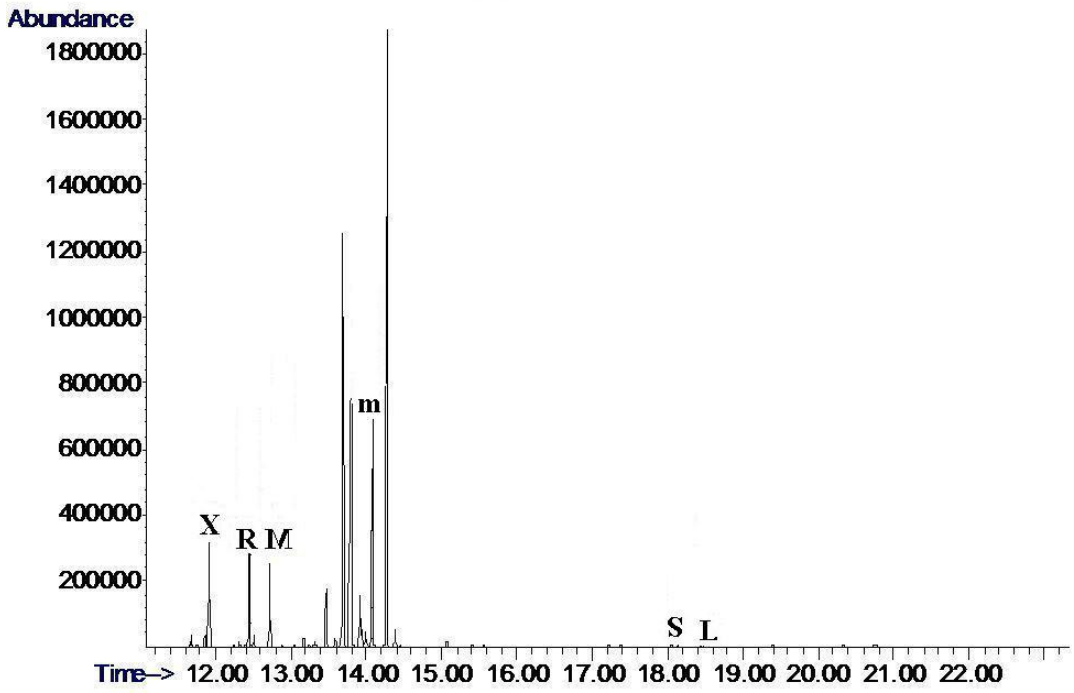

Figure 3: Total ion chromatogram of an unknown sample. The chromatogram shown here is from analysis of an unknown serum sample containing mannitol as internal standard (m).

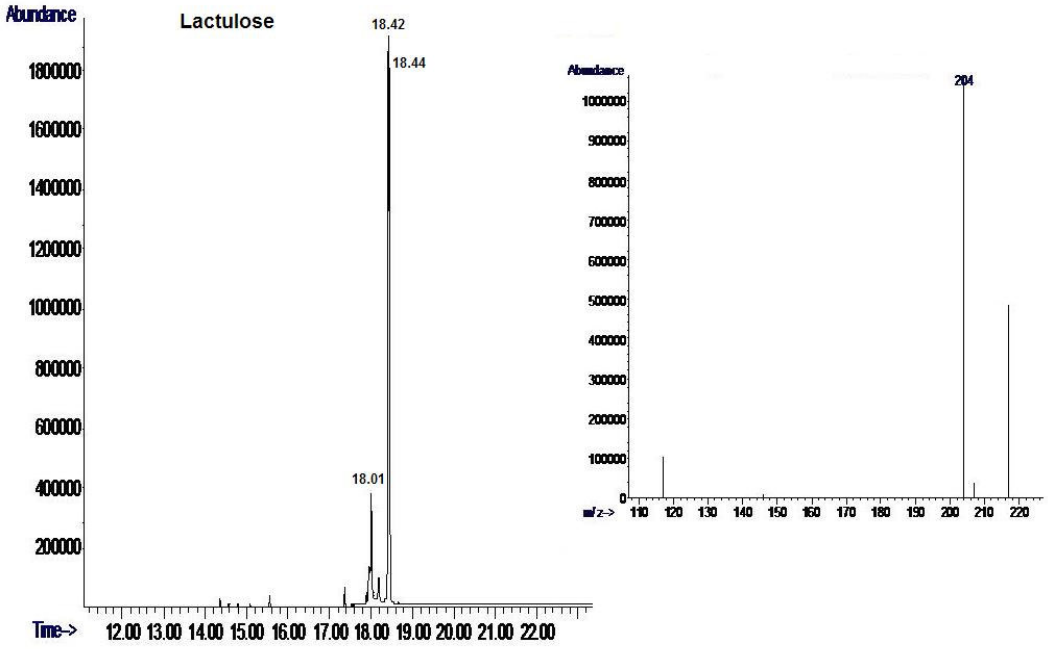

Figure 4: GC/MS chromatogram for lactulose. In the selected ion chromatogram on the left the numbered peaks indicate the retention times for the derivatized ions of lactulose. The smaller chromatogram on the right depicts the mass spectrum of the TMS-derivative of lactulose at *m/z* 204.

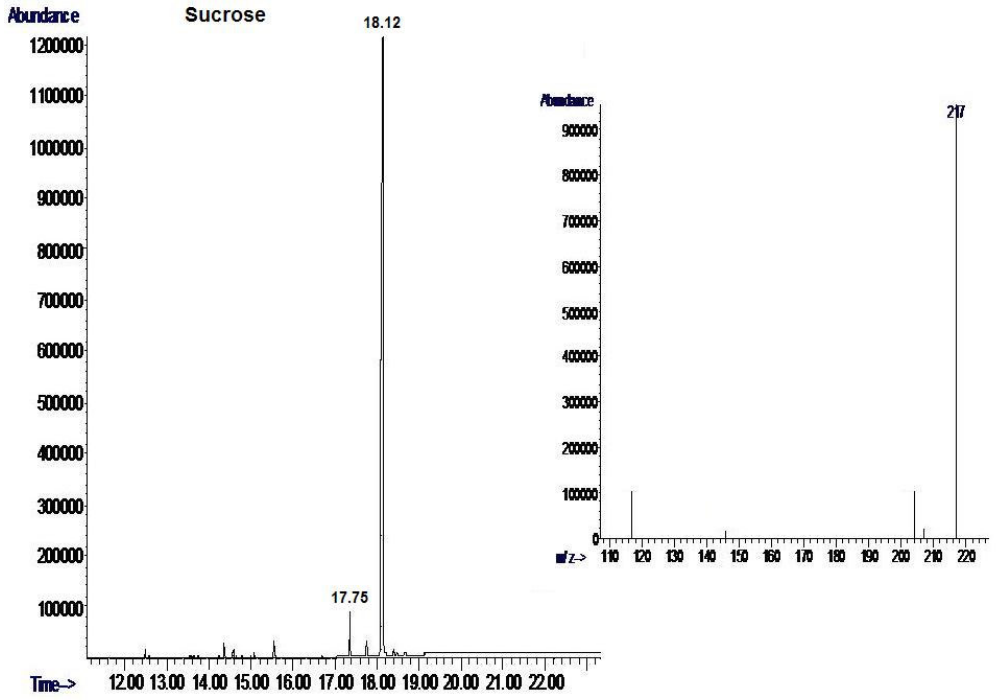

Figure 5: GC/MS chromatogram for sucrose. In the selected ion chromatogram on the left the numbered peaks indicate the retention times for the derivatized ions of sucrose. The smaller chromatogram on the right depicts the mass spectrum of the TMS-derivative of sucrose at *m/z* 217.

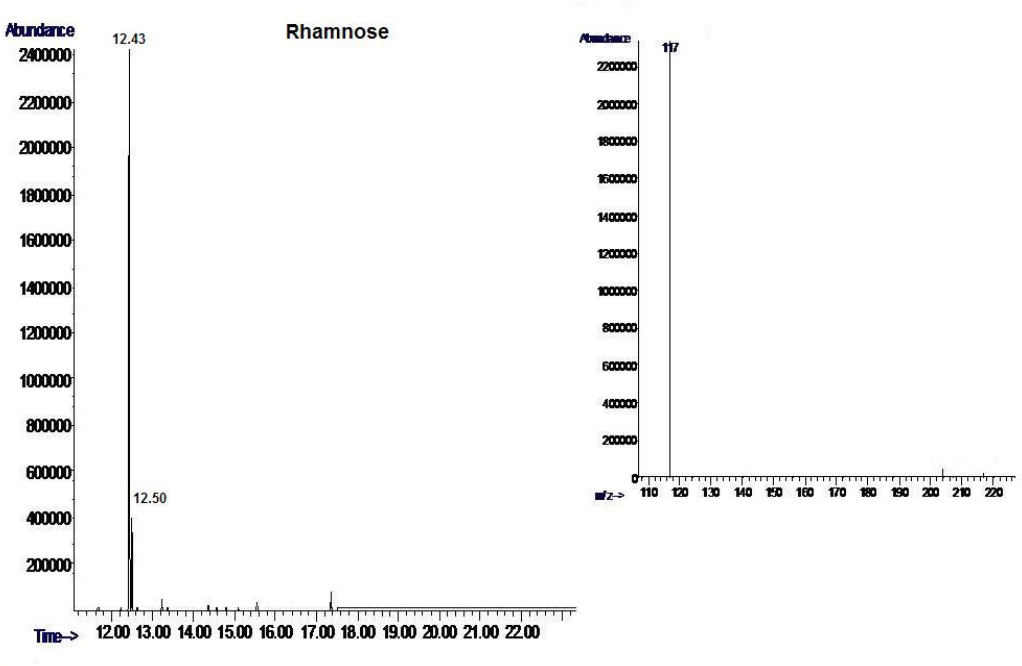

Figure 6: GC/MS chromatogram for rhamnose. In the selected ion chromatogram on the left the numbered peaks indicate the retention times for the derivatized ions of rhamnose. The smaller chromatogram on the right depicts the mass spectrum of the TMS-derivative of rhamnose at *m/z* 117.

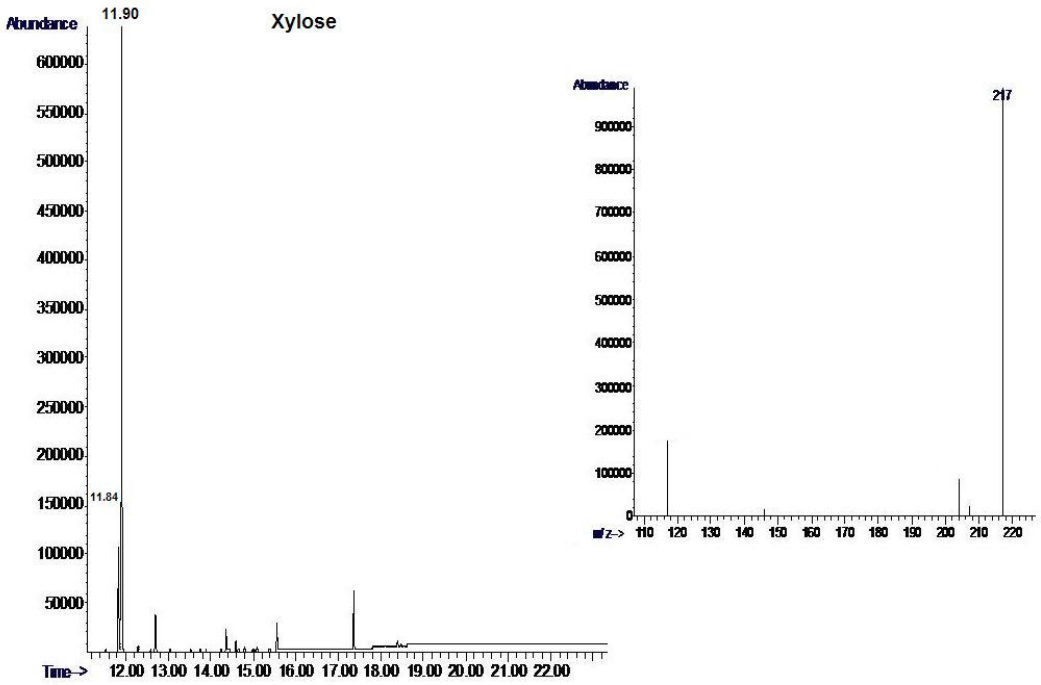

Figure 7: GC/MS chromatogram for xylose. In the selected ion chromatogram on the left the numbered peaks indicate the retention times for the derivatized ions of xylose. The *m/z* smaller chromatogram on the right depicts the mass spectrum of the TMS-derivative of xylose at *m/z* 217.

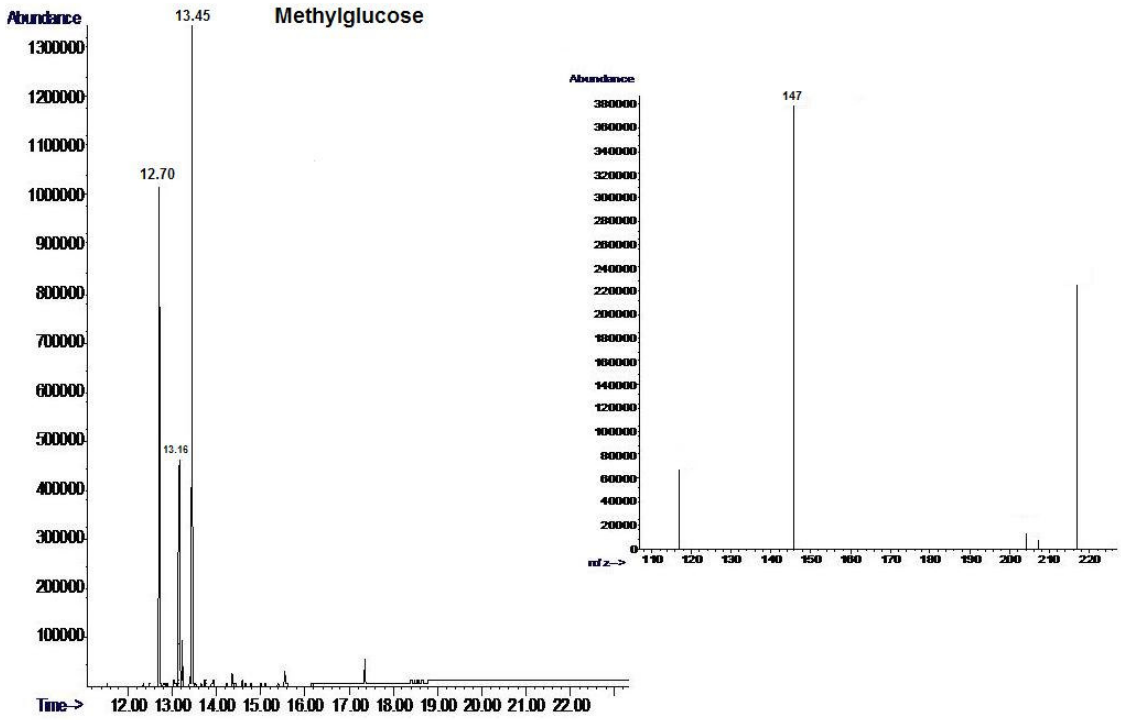

Figure 8: GC/MS chromatogram for methylglucose. In the selected ion chromatogram on the left the numbered peaks indicate the retention times for the derivatized ions of methylglucose. The smaller chromatogram on the right contains the mass spectrum of the TMS-derivative of methylglucose at *m/z* 147.

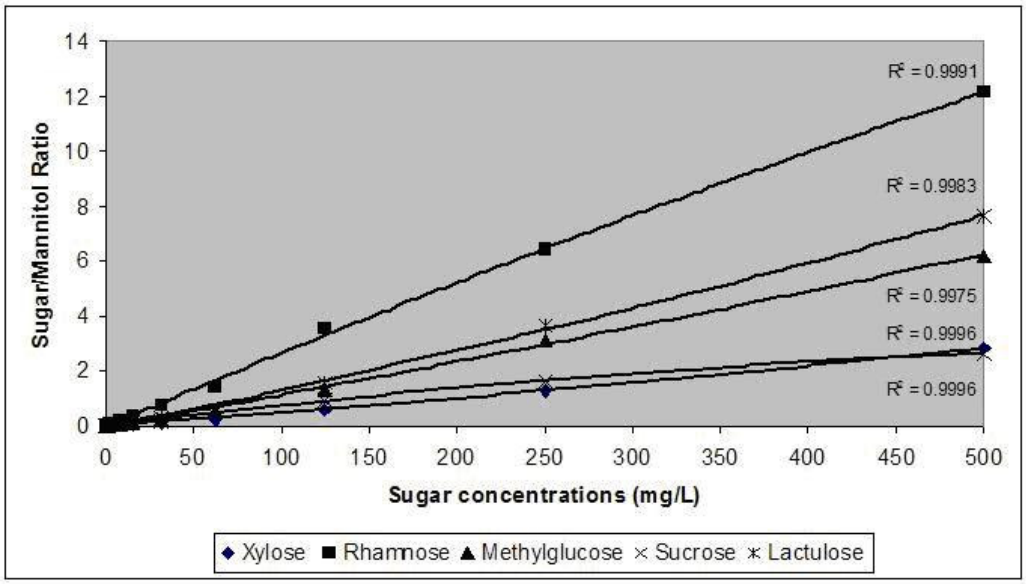

Figure 9: Standard curves. Representative standard curves of the GC-MS assays for the simultaneous determination of five sugar probes in canine serum.

Table 2: Calibration curves, linearity detection limits for sugar analysis by GC-MS. This table shows the *m/z* of the peak used for quantification of each sugar, the range of calibrators used for calculating the standard or calibration curve, linearity, and the detection limits when assayed by this GC-MS method.

Compound	Ion (m/z)	Calibration curve (mg/L)	Linearity (r^2)	Lower limit of detection (mg/L)
Lactulose	204	0.5 -500	0.9983	0.03
Sucrose	217	0.5 -500	0.9996	0.12
Xylose	217	0.5 -500	0.9996	0.03
Rhamnose	117	0.5 -500	0.9991	0.03
Methylglucose	147	0.5 -500	0.9975	0.03

Table 3: Spiking recovery. This table shows the recovery of the 5 sugars spiked into canine serum at concentrations of 3, 30, 100, and 350 mg/L. The means are expressed as observed (O)/expected (E) ratios (O/E %).

	Expected (mg/L)	Observed (mg/L)	O/E (%)
Lactulose	3	3.7	123.3
	30	34.9	116.3
	100	97.2	97.2
	350	338.4	96.7
		mean	108.4
		SD	13.5
Rhamnose	3	4.4	147.7
	30	43.5	145.1
	100	112.6	112.6
	350	326.3	93.2
		mean	124.7
		SD	26.4
Methylglucose	3	3.6	120.1
	30	25.4	84.7
	100	88.9	88.9
	350	313.5	88.3
		mean	95.5
		SD	16.5
Xylose	3	4.3	143.3
	30	31.7	105.7
	100	107.5	107.5
	350	309.2	84.9
		mean	110.4
		SD	24.2
Sucrose	3	1.7	56.7
	30	34.5	115.1
	100	111.7	111.7
	350	297.1	84.9
		mean	92.1
		SD	27.2

Table 4: Dilutional parallelism. This table shows the results of dilutional parallelism of a blank serum sample spiked with 350 mg/L of all 5 sugar probes (L, R, M, X, and S) and of an unknown serum sample. (O/E = (observed/expected)*100)

Serum sample (350 mg/L)				**Unknown serum sample**			
Dilution	O mg/L	E mg/L	O/E (%)	Dilution	O mg/L	E mg/L	O/E (%)
	Lactulose				**Lactulose**		
Neat	318.4			Neat	10.2		
1:2	142.7	159.2	89.7	1:2	6.2	5.1	121.2
1:4	69.4	79.6	87.2	1:4	3.6	2.6	149.0
1:8	30.7	39.8	77.7	1:8	2.1	1.3	164.2
	Rhamnose				**Rhamnose**		
Neat	299.4			Neat	63.9		
1:2	166.1	149.7	110.9	1:2	32.0	31.8	100.8
1:4	81.1	74.9	108.3	1:4	13.7	15.9	86.4
1:8	38.6	37.4	103.1	1:8	5.4	7.9	67.6
	Methylglucose				**Methylglucose**		
Neat	330.7			Neat	99.9		
1:2	185.3	165.3	112.1	1:2	49.7	50.0	99.6
1:4	88.7	82.7	107.3	1:4	23.3	25.0	93.2
1:8	40.3	41.3	97.6	1:8	10.5	12.5	84.4
	Xylose				**Xylose**		
Neat	304.1			Neat	106.8		
1:2	160.9	152.1	105.8	1:2	55.5	53.4	104.0
1:4	76.5	76.0	100.6	1:4	24.1	26.7	90.2
1:8	36.5	38.0	96.0	1:8	10.9	13.4	81.6
	Sucrose				**Sucrose**		
Neat	290.2			Neat	2.3		
1:2	154.5	145.1	106.5	1:2	1.3	1.1	110.7
1:4	84.9	72.5	117.0	1:4	ND		
1:8	41.5	36.3	114.3	1:8	ND		

Table 5: Intra-assay variability. Four serum samples containing 5 sugars at different concentrations were analyzed 9 times within the same assay run. Samples A, B, C, and D contained 3, 30, 100, and 350 mg/L of the 5 sugars, respectively. (%CV = (standard deviation/mean)*100)

	Sample 1 (3 mg/L)		**Sample 2 (30 mg/L)**		**Sample 3 (100 mg/L)**		**Sample 4 (350 mg/L)**	
Sugar	mean (mg/L)	CV (%)	mean (mg/L)	CV (%)	mean (mg/L)	CV (%)	mean (mg/L)	CV (%)
Lactulose	3.3	9.1	46.5	7.9	131.9	6.8	281.3	12.9
Rhamnose	3.4	11.8	43.0	8.9	123.3	7.1	364.9	12.8
Methylglucose	2.7	11.1	33.7	8.9	125.3	10.8	377.6	11.5
Xylose	2.9	10.3	38.4	11.0	136.9	7.2	384.0	11.0
Sucrose	3.1	9.7	26.4	9.4	96.7	12.0	352.5	8.4

Table 6: Inter-assay variability. Four serum samples containing 5 sugars at different concentrations were analyzed in 9 consecutive assay runs. Samples A, B, C, and D contained 3, 30, 100, and 350 mg/L of the 5 sugars, respectively. (CV% = (standard deviation/mean)*100

	Sample A (3 mg/L)		**Sample B (30 mg/L)**		**Sample C (100 mg/L)**		**Sample D (350 mg/L)**	
Sugar	mean (mg/L)	CV (%)	mean (mg/L)	CV (%)	mean (mg/L)	CV (%)	mean (mg/L)	CV (%)
Lactulose	3.7	7.0	34.9	9.6	97.2	11.5	338.4	8.0
Rhamnose	4.4	6.9	43.5	7.8	112.6	9.4	326.3	6.4
Methylglucose	3.6	11.8	25.4	12.4	88.9	15.9	313.5	7.0
Xylose	4.3	7.3	31.7	13.2	107.5	8.7	309.2	6.8
Sucrose	1.7	8.1	34.5	9.4	111.7	5.5	297.1	8.1

Assay validation. Fig. 9 shows the calibration curves for the 5 sugar probes evaluated. Each sugar standard was prepared with eleven calibration concentrations covering the range of expected values in the samples. Within the range of 0.5-500 mg/L sugar concentrations were proportional to their integrated peak areas. Similarly, the concentration of the internal standard was proportional to its integrated peak area. Due to a possible instrument variability and analyte sensitivity (i.e., changes in temperature, pressure, and electronic detector), taking a ratio of areas is more reproducible than using absolute values for individual compounds. Thus, if we assume that unknown samples/sugar standards and internal standards were processed analogously, they were exposed to similar factors. Therefore, standard curves established with an internal standard minimized the effect of variability from run-to run. Calibration curves used the ratio rather than just the peak area of the analytes to be quantified. In this case, the ratio of the concentrations of a given unknown and the internal standard is proportional to the ration of their peak areas. All curves showed good coefficients of correlation (Table 2). The best-fit line for the ratio of integrated peaks against the ratio of concentrations of each sugar to internal standard was fitted by polynomial regression. The curves showed a mean $r^2 = 0.997$ for all sugars. The polynomial second order model used here has the advantage over the straight line model that the former integrates an intercept line with the experimental background noise of the blank sample. Therefore, with a polynomial model, the intercept line runs through zero, which is useful to define detection limits and to properly measure unknown samples.[105]

The lower detection limit of the assay was 0.03 mg/L for L, R, M, and X, and 0.12 mg/L for S. These limits compare favorably with those reported for HPLC-PAD analysis (i.e., xylose 0.13 mg/L, rhamnose 0.36 mg/L sucrose 0.02 mg/L, mannitol 0.12 mg/L,[74] and lactulose between 0.4-0.8 mg/L)[72,106] or for capillary electrophoresis (i.e., 10 mg/L for lactulose).[107]

The accuracy of the assay was determined by evaluation of spiking recovery of the samples A through D (3, 30, 100, and 350 mg/L) for each sugar in serum (Table 3). Observed to expected ratios for spiking recovery of the 5 sugars ranged from 96.7% to

123.3% (mean ± SD: 108.4 ± 13.5%) for lactulose, from 93.2% to 147.7% (mean ± SD: 124.7 ± 26.4%) for rhamnose, from 88.3% to 120.1% (mean ± SD: 95.5 ± 16.5%) for methylglucose, from 84.9% to 143.3% (mean ± SD: 110.4 ± 24.2%), and from 56.7% to 115.1% (mean ± SD: 92.1 ± 27.2%) for sucrose with the mean ranging from.92.1% to 124.7% (mean ± SD 106.2 ± 13.0%). The linearity of the assay was determined by evaluating dilutional parallelism. Table 4 shows the O/E ratios for dilutions of 1 in 2, 1 in 4, and 1 in 8, which ranged from 67.6% to 164.2% (mean ± SD of 105.6 ± 25.4%). Table 5 shows the intra-assay precision with the %CV ranging from 6.8% to 12.9% between the individual sugars at 4 different concentrations represented as samples A through D. The reproducibility of the assay was determined by evaluating inter-assay precision. Results in Table 6 show the % CV which ranged from 6.4% to 15.9% for the 5 individual sugars at different concentrations.

DISCUSSION

Several methods for the measurement of sugar markers in serum and urine for gastrointestinal permeability and absorptive capacity assessment have been reported. However, some of them are associated with technical drawbacks.[108,109,110] Thin-layer chromatography is time-consuming,[108] while colorimetric/enzymatic methods do not provide information on the composition of monosaccharides,[109] and HPLC methods are considered to have a relatively low sensitivity.[110] We developed a GC-MS method that determines carbohydrate markers in serum samples from dogs. Mass spectrometry was used because of its capability of molecular identification at high sensitivity, based on retention time and fragmentation pattern[73,76]

Because saccharides are highly polar and have a low volatility, chemical derivatization is required before GC-MS analysis.[77,78,111,112] Thus, we converted L, R, M, X, and S into more volatile and thermostable sugar-derivatives before analysis. A two step derivatization procedure was employed. First, carbonyl groups (-C=O) were transformed to more stable and non-polar groups (-C=N-O-CH3) by an oximation reaction, which has been previously described.[79] This was followed by the formation of

trimethylsilyl (TMS) esters using silylating reagents (BSTFA) to replace exchangeable protons (-OH) with TMS. The oxime formation is required to eliminate undesirable slow and reversible silylation reactions with carbonyl groups, whose products can be thermally labile, while silyl derivative groups [-Si(CH3)3] allow measurement of the analyte by GC-MS.[98] Carbohydrates used in this study produced several GC peaks, but only two or four peaks were detectable. Sugars in solution constantly cycle between the ring and the straight chain forms, which leads to a dynamic equilibrium between the two forms. Therefore, two anomers (α and β) can be formed. If the anomeric center is not destroyed by the derivatization procedure, acylation of the aldose ring freezes the structure of the α and β anomeric form, producing multiple peaks from one compound during GC analysis.[113]

When multiple peak profiles are generated on the GS-MS chromatogram, analysis is performed using the standard references previously generated, which contain both the retention time and mass spectra. Formation of anomers can potentially be avoided by preparing the alditol acetate derivative through treatment with sodium borohydride**.**[73] The alditol acetate derivate can then be separated after derivatization with dry pyridine and acetic anhydride, which will lead to a single peak for some sugars.[73] However, the alditol acetate derivative method is more suitable for reducing end sugars such as glucose, fructose, and lactose,[114] which are normally present in serum. Moreover, glucose and fructose generate other alditols, such as mannitol and glucitol hexa-acetate.[114] Therefore, the alditol derivative method was not suitable for the measurement of our sugar probes due to the generation of more sugar derivatives from glucose, fructose and lactose, which are normally present in serum. Finally, many steps are required for a complete and effective reduction of sugars to their alditol acetate derivatives.[115] Thus, the number of peaks and their retention times are characteristic features of each carbohydrate, and are also dependent on the derivatization conditions. Peaks can vary in intensity. Small peaks are part of the sugar fragmentation pattern. They are a constant fraction of the major peak due to production of multiple silyl derivative.[98] Therefore, they can be used for the sugar quantitation.[116,117] As described

above, the quantification of sugar probes was performed by using the sum of the peak areas from specific retention times of each sugar using select ion monitoring (SIM) mode for specific ions of each sugar as is showed in table 1.

The saccharides used in this study showed ions at *m/z* 73, 147, 191, 204, 205, 217, 219, 307, and 319, which are in agreement with those previously reported for TMS derivatives of rhamnose, xylose, lactulose, and sucrose in specimens other than serum or urine (e.g., environmental samples and model solutions).[76,118] Similarly, disaccharides show a peak at *m/z* 361, which is typically found as part of the fragmentation pattern of these compounds. Also, ions at *m/z* 437 and *m/z* 451 are typical for sucrose and lactulose, respectively.[76]

Observed to expected ratios for all 5 sugars spiked into serum at the 4 concentrations ranged from 92.1% to 124.7% (mean ± SD 106.2 ± 13.0%) for all sugars. The results of these spiking recovery experiments showed an increased variability for rhamnose and xylose at the lowest concentrations. These variations were observed as an effect of a wide range of the standard curves used for the method validation. However, when a sugar test is performed, xylose and rhamnose are found in significantly higher concentrations in healthy and disease states, with concentrations well within the linear mid-range of the standard curve. Thus, the effect of high variation in the lower range is not important for the assay performance for analysis of clinical samples. Suboptimal recovery rate was observed at the lowest spiking concentration of 3 mg/L. This was especially the case for the recovery of sucrose. Low accuracy for detection and quantification of sucrose at small concentrations compared with higher concentrations has been reported using gas chromatography.[119] Recovery can be influenced by loss of the analyte during extraction and oximation derivatization. Sucrose degradation may occur as result of the extraction method and oxime derivatization.[120] The %CV for intra-assay variability for the 5 sugars ranged from 6.8% to 12.9% in the 4 samples evaluated. Finally, inter-assay precision was 6.4% to 15.9% for all sugars in the 4 different serum samples.

In conclusion, a GC-MS method was successfully developed for the simultaneous quantification of lactulose, rhamnose, methylglucose, xylose, and sucrose in dog serum. Gas chromatographic conditions and sugar derivatization by converting the relative oximes before the silylating reaction showed acceptable linearity, precision, and reproducibility to be used for further studies of intestinal permeability and mucosal function testing. However, the accuracy of the assay was limited for low concentrations, especially for sucrose.

Notes

[a.] D-(+)-Xylose ($C_5H_{10}O_5$), D-Sucrose ($C_{12}H_{22}O_{11}$), D-Lactulose ($C_{12}H_{22}O_{11}$), L-Rhamnose monohydrated ($C_6H_{12}O_5 \cdot H_2O$), 3-***O***-Methyl-D-glucopyranose ($C_7H_{14}O_6$), D-Mannitol ($C_6H_{14}O_6$), Sigma-Aldrich, St Louis, Mo.

[b.] Methanol, EDM Chemicals Inc. Gibbstown, N.J.

[c.] Centrifuge 5417C, Eppendorf, Brinkmann Instruments Inc, Westbury, N.Y.

[d.] Glass vials molded screw cap, VWR International, West Chester, Pa.

[e.] Reactive therm III, Heating module. Pierce, Rockford, Ill.

[f.] *N,O-bis*[Trimethylsilyl]trifluoroacetamide (BSTFA) 1% TMCS, MOX Reagent (2% Methoxyamine•HCL in pyridine), Pyridine, Pierce, Rockford, Ill.

[g.] Hexane, EDM Chemicals Inc. Gibbstown, N.J.

[h.] Gas chromatograph (6890N GC) coupled with Mass Spectrometer (5975 MSD) and DB-1MS capillary column (122-0132), Agilent Technologies, Palo Alto, Calif.

CHAPTER III

KINETIC ANALYSIS OF FIVE SUGAR PROBES IN DOG SERUM AFTER OROGASTRIC ADMINISTRATION*

OVERVIEW

The study aim was to describe the kinetic of sugar probes in serum for the assessment of gastrointestinal permeability and intestinal absorptive capacity in eight healthy dogs. Based on their body weight, dogs received specific amounts of a mixed solution containing lactulose (L), rhamnose (R), methylglucose (M), xylose (X), and sucrose (S) by orogastric intubation. Baseline blood samples were taken before dosing. Subsequently, timed blood samples were collected during 24 hours. Sugars in serum were assayed by the analytically validated gas chromatography-mass spectrometry (GC-MS) method described in chapter II. Sugar concentrations in serum were quantified using an internal standard. Statistical analysis was performed using a Friedman test with Dunn's multiple comparison post test and a Kruskal-Wallis test. Statistical significance was set at a p-value <0.05. Sugars in serum were detected in all dogs after administration of the test dose ($p<0.0001$). Concentrations of L and R were significantly different from the baseline between from 90 to 240 and 60 to 300 min respectively, and those of X, M, and S were different between 30 and 240 min post dosing ($p<0.05$ for all 5 probes). Maximum concentrations of L and R were obtained at 180 min, while X, M, and S reached their maximum concentrations at 90 min post dosing. For all sugars, no statistically significant differences were found between concentrations at 90, 120, and 180 min or between the coefficients of variation (%CV) of the mean concentrations for these 3 time points.

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Based on these data, the collection of two blood samples, one taken at baseline and a second sample obtained between 90 and 180 after dosing, might be sufficient for the determination of all five sugar probes in canine serum.

INTRODUCTION

The gastrointestinal tract (GIT) provides barrier and transport functions preventing the passage of pathogens, toxins, and other luminal contents to extra-intestinal tissues while selectively absorbing essential nutrients. Such functions can be assessed via intestinal permeability testing for macromolecules which are passively diffused or absorbed via carrier-mediated transport.[43] Permeation and absorption of solutes through the gastrointestinal epithelium are determined by the structure of the membrane, the physicochemical properties of the solute, and its interaction with the media or solvent.[82]

Approximately 90% of the absorption in the GIT occurs in the small intestine while the rest is carried out by the colon and the cecum. The enterocyte is the most abundant cell type with a surface area of approximately 2 X 10^6 cm^2, due to surface-amplification through villi and microvilli.[6,1]

The route by which a compound crosses the intestinal epithelium is transcellular (small pores) or paracellular (large pores). Transcellular absorption from the lumen to the blood requires carrier mediated transport. The paracellular permeability depends on the regulation of intercellular tight junctions which consist of channels formed by adjacent enterocytes. Only a small number of compounds can cross the paracellular space due to the fact that the surface area available for these channels is estimated to only be about 0.01% of the total surface area of the small intestine.[121,122]

Three distinct pores are present along the crypt-villus axis, which affect GIT permeability due to molecular size restrictions.[123] At the tips of the villi, there is an abundance of small pores (radius <6 Å), while in the crypts much larger pores (50-60 Å) are found in low density. Intermediate sized (10-15 Å) channels, which are not exposed

to luminal content, can be found at the base of the villi. Thus, tight junctions of the villus epithelium are more restrictive than those in the epithelium of the crypts.[121]

This prevents molecules of the size of disaccharides (e.g. lactulose) from moving across the villus tip; whereas, monosaccharides like mannitol can cross with relative freedom.[13] The epithelial junctions become progressively tighter from the small intestine to the colon, decreasing the permeability to polar compounds along the intestinal tract.[21]

Disorders of the intestinal barrier tend to decrease the transcellular permeability reflecting a diminished number of mucosal cells; whereas, paracellular permeability tends to increase, reflecting damage to the tight junctions.[123,124] According to a review by Cave et.al., an increased intestinal permeability has been reported in several intestinal diseases in dogs and cats, including gluten sensitivity enteropathy, small intestinal bacterial overgrowth, intestinal ischemia-reperfusion, and non-steroidal anti-inflammatory drug-induced injury.[125]

Intestinal permeability is assessed by measuring urinary excretion of orally administered water-soluble, non-degradable test molecules. The test compares the intestinal permeation of larger molecules which occurs only between cells at or near the crypts with smaller molecules which are normally absorbed along the entire crypt-villus axis.[43] Calculation of a ratio of the large to the small marker molecule reduces the potential influences of pre-mucosal, mucosal, and post-mucosal factors.[43,126] An increased ratio could reflect an increase in the paracellular permeability due to factors such as loss of villus height, which would allow an amplified permeation through the larger pores formed by adjacent crypt cells.[88]

The most commonly used markers for permeability tests are mono- and disaccharides, ^{51}Cr-EDTA, polyethylene glycols (PEGs), and dextran. Inert sugars, such as lactulose and L-rhamnose, are commonly used as markers of small intestinal permeability. While lactulose permeates through paracellular pores of low frequency and L-rhamnose crosses the intestinal epithelium mainly by transcellular passive diffusion through aqueous pores at a much higher rate, but neither lactulose nor rhamnose undergo carrier-mediated transport.[43,14] D-xylose and 3-*O*-methyl-D-glucose are non-metabolized

monosaccharides that are absorbed by the intestinal epithelial cells via carrier-mediated transport and they are commonly used as markers of intestinal absorptive capacity. D-xylose undergoes passive carrier mediated transport, principally in the jejunum; whereas; whereas 3-*O*-methyl-D-glucose is absorbed by active carrier transport throughout the small intestine.[127] Finally, sucrose has been introduced as a probe to measure gastric mucosal permeability. Sucrose is hydrolyzed in the very proximal small intestine, and thus the presence of the intact sucrose in serum or urine implies pre-duodenal permeation due to gastric mucosal damage.[128]

Several methods for the assessment of intestinal permeability and mucosal absorption have been developed.[14,43] To date, protocols utilizing permeability markers excreted and recovered in urine are the most widely used.[58] However, these protocols require complete urine collection over a period of at least 4 hrs and sometimes up to 24 hrs. These methods are laborious and often impractical. Also, errors may be introduced through incomplete urine collection, which may affect the test results.[93]

The measurement of carbohydrates in serum would simplify the assessment of intestinal permeability and absorptive function under clinical conditions. Some GI permeability studies using different sugar markers in serum have been reported. For instance, sucrose has been measured in serum by an enzymatic method for the detection of gastric damage in humans,[94] and was also used for the assessment of gastric permeability in horses with gastric ulceration using a high performance liquid chromatography-mass spectrometry (HPLC-MS) method.[95] Determination of 3-*O*-methyl-D-glucose in serum has been performed as a measure of gastric emptying time using thin layer chromatography and densitometry.[96] Lactulose and mannitol have been measured in serum for the assessment of intestinal permeability in humans.[93] Finally, a recent study has described assessment intestinal permeability and absorptive capacity in dogs using an HPLC-based method for the measurement of lactulose, rhamnose, 3-O-methyl-D-glucose, and xylose in serum.[97] However, there is limited experience using these assays in serum with gas chromatography-mass spectrometry (GC-MS) for the assessment of intestinal permeability and absorptive function. An essential advantage of

using GC-MS would be an improved sensitivity when compared to HPLC-MS methods. We have recently analytically validated a GC-MS method for sugar analysis in serum .[129] Due to improved sensitivity and specificity obtained from the GC-MS method relative to previously mentioned assays, this method appears to be superior to previous method for carbohydrate analysis for gastrointestinal permeability and mucosal function testing.[116] However, analysis of any polar, nonvolatile substance, such as sugars, by GC-MS requires derivatization prior to analysis.

The aim of this study was to simultaneously analyze the kinetics of D-(+)-Xylose D-Sucrose, D-Lactulose, L-Rhamnose monohydrate, and 3-***O***-Methyl-D-glucopyranose in serum from healthy dogs.

MATERIALS AND METHODS

This study was approved by the University Animal Care and Use Committee (ULAC) at the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University. Gastrointestinal permeability and absorption tests were performed in 8 healthy research dogs. All animals were adults between 2 and 7 years of age. All dogs were monitored on a daily basis, and none showed any history related to gastrointestinal tract disease. The overall health status of the dogs was assessed by a baseline analysis of complete blood count and serum biochemistry analysis. The permeability and absorption test protocol required a 48 h period before the test in which the dogs did not receive any agent that could alter small bowel permeability. Dogs were kenneled in individual cages 5 days before the experiment. To ensure that no dietary sugars would be present in the serum, food was withheld for at least 18 hours prior to the gastrointestinal permeability and absorption tests.

Hyperosmolar solutions may cause gastrointestinal side effects, such as diarrhea.[130] In addition, gastrointestinal permeability has been shown to be altered by either hypo- or hyperosmolar silutions.[131,132] Therefore, all dogs received an approximately isoosmolar sugar solution (318.3 mosm/L). In order for the dogs to receive similar amounts of sugar per body weight the volume of the sugar solutions used

was roughly modified based on body weight. Thus, dogs received either 100 ml (dogs less than 10 kg body weight), 200 ml (dogs 10-20 kg body weight), or 400 ml (dogs with a body weight of more than 20 kg) of a solution containing sterile water, 10 g/L of lactulose (L-7877; Sigma Chemical Company, St. Louis, Missouri, USA), 10 g/L of L-rhamnose (R-3875; Sigma Chemical Company) (rhamnose), 10 g/L of D(+)xylose (X-1500; Sigma Chemical Company) (xylose), 5 g/L of 3-*O*-methyl-D-glucosepyranose (M-4879; Sigma Chemical Company) (methylglucose), and 40 g/L sucrose (S-9378; Sigma Chemical Company) by orogastric intubation. A baseline blood sample was obtained from each dog. After the sugar solution was administered, further blood samples were taken at 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 720, and 1440 min post-administration from the jugular vein. Serum was separated immediately by centrifugation at 3000 x *g* for 10 min. Serum samples were then transferred to collection tubes and stored at -80°C.

Sample preparation. Stock solutions of carbohydrate standards (1 mg/mL) were prepared in serum and stored at -20°C. Mannitol (M-9546 Sigma Chemical Company) was used as an internal standard and was added at a concentration of 100 mg/L to serum samples, blank samples, and to serial 1:2 dilutions of standard solutions containing each sugar at concentrations ranging from 0.5-500 mg/L. Sets containing blanks, unknown samples, and standard solutions were extracted, derivatized, and analyzed in a GC-MS session as described in the following protocol.

Serum aliquots of 200 µL each were thawed and pipetted into 2-ml plastic vials. Deproteinization was achieved by adding 600 µL of methanol (EDM Chemicals, Inc. Gibbstown, New Jersey, USA) to each serum sample and mixing the sample for 20 seconds using a vortex mixer. Samples were then centrifuged (Centrifuge 5417C, Eppendorf, Brinkmann Instruments Inc. Westbury, New York, USA) at 2,654 x g at room temperature for 7 min, and the supernatant was transferred to a 4-ml molded screw cap glass vial (VWR International, West Chester, Pennsylvania, USA). Samples were evaporated to dryness under a stream of nitrogen in a heating module (Reacti-Therm III, Pierce, Rockford, Illinois, USA) at 64°C for 30 min. Once the tubes were cooled, the

dried residue was derivatized in a two-step procedure. First, 50 µL of Mox reagent (2% of Methoxyamine-HCl in pyridine) (Pierce) and 70 µL of pure pyridine (Pierce) were added to each tube. Tubes were capped, vortexed for 20 seconds, and subsequently heated in a microwave oven at 100% energy level (800 W) for 2 min to promote the oximation reaction. [104] Then, samples were allowed to cool for 5 min and 100 µL of *N,O-bis*[Trimethylsilyl]trifluoroacetamide (BSTFA) (Pierce) containing 1% TMCS were added to each tube. The tubes were capped, vortexed for 20 seconds and placed in a microwave oven at 100% energy level (800 W) for an additional 5 min to develop the silylation reaction according to the procedure described by Silva et al.[104] Once the samples were cooled at room temperature, the derivatized extracts were evaporated to dryness under a nitrogen stream at 64°C for 8 min and then, the residues were dissolved in 250 µL of hexane (EDM Chemicals, Inc). GC-MS analysis was performed with 1 µl of this solution.

Gas chromatography analysis. Derivatized samples were analyzed using gas chromatography (Gas chromatograph 6890N GC, Agilent Technologies, Inc. Headquarters, Santa Clara, California, USA) coupled with a mass spectrometer (Agilent Technologies, Inc.). Sugars were separated using a DB-1MS capillary column (30 m length, 250 µm inner diameter, and 0.25 µm film thickness) (Agilent Technologies, Inc.). The following GC column temperature program was used: the initial oven temperature was set at 100°C and held for 5 minutes. The temperature was then increased from 100 to 325°C by a constant gradient of 15°C/min and held at 325°C for 5 min, resulting in a total run time of 23.33 min per sample. Helium was used as a carrier gas at a constant flow rate of 1.5 mL/min and at a velocity of 33 cm/sec. Sugar identification and characterization was performed under full-scan acquisition mode within the *m/z* 50 to *m/z* 1050 range. Sugar analysis and quantification were performed in selected ion monitoring (SIM) mode using *m/z* 204 for lactulose (L), *m/z* 117 for rhamnose (R), *m/z* 147 for methylglucose (M), and *m/z* 217 for sucrose (S), xylose (X), and mannitol (m).

Kinetic analysis. Calibration curves were established by plotting the ratios of the area under the curve of the peaks of the sugars of interest to that of the internal standard

for each of the different standard solutions using a polynomial curvilinear regression ($y = ax^2 + bx + c$). Calibration curves ranged from 0.5 to 500 mg/L for each sugar with a mean $r^2 = 0.997$. The sugar peak area ratios of unknown serum samples were then extrapolated from the calibration curves. Statistical analysis was carried out using a commercial software program (GraphPad Prism 5). Sugar concentrations at all time points were analyzed for each sugar using a Friedman test with Dunn's multiple comparison post test. Variation of the mean sugar concentrations of all dogs at 90, 120, and 180 minutes was analyzed using a Kruskal-Wallis test. Statistical significance was set at a p-value <0.05.

RESULTS

The GC-MS analysis showed good peak resolution for all sugar probes. Fig. 3 depicts representative total ion chromatograms (TIC) of the five target sugar probes in canine-serum samples at 180 min after oral administration. No overlapping peaks were observed among the five sugar standards (L, R, M, X, and S) and the internal standard mannitol (m).

Permeation profiles. Table 7 shows the mean ± SD concentrations (mg/L) of lactulose, rhamnose, methylglucose, xylose, and sucrose in serum at individual time points after oral administration of the 5-sugar solution. The serum sugar showed significant concentrations of all five sugars at 30 min post dosing (the first sampling time). Monosaccharides (X, R, and M) reached the serum rapidly, while disaccharides (L and S) reached the serum more slowly. Quantifiable concentrations for all sugar markers were reached until the last sampling time point.

Figures 10 to 14 show the mean ± SD concentration profiles for each sugar in serum, while the profiles for all five sugars are shown in Figure 15. Significant changes in the serum concentrations of all 5 sugars were detected after administration of the test dose ($p<0.0001$ for all 5 probes). Serum concentrations of lactulose and rhamnose were significantly different from the baseline at 90, 120, 180, 240 min, and at 60, 90, 120, 180, 240, and 300 min post dosing respectively; those of xylose, methylglucose, and sucrose were significantly different from the baseline at 30, 60, 90, 120, 180, and 240 min post dosing ($p<0.05$ for all 5 probes).

Maximum concentrations of lactulose (Fig. 10) and rhamnose (Fig. 11) were obtained at 180 min (mean ± SD: 8.2 ± 4.9 and 35.6 ± 4.0 mg/L, respectively) followed by a gradual decline, while xylose (Fig. 12), methylglucose (Fig. 13), and sucrose (Fig. 6) reached their peak concentrations at 90 min post dosing (mean ± SD: 224.0 ± 72.6, 214.8 ± 80.7, and 4.1 ± 1.7 mg/L, respectively) followed by a gradual decline. For all of the 5 sugar probes, no statistically significant differences were found between concentrations measured at 90, 120, and 180 min time points or between the coefficients of variation (%CV) of the mean concentrations for those 3 time points.

Table 7: Mean ± SD concentrations (mg/L) and coefficient of variation (CV) of sugar probes in serum. This table shows the five sugar concentrations in serum for each time period from dogs after orogastric administration of a 5-sugar solution.

Time	Xylose			Methylglucose			Rhamnose			Sucrose			Lactulose		
min	mean	SD	CV%	mean	SD	CV%	mean	SD	CV%	mean	SD	CV%	mean	SD	CV%
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	121.4	51.1	42.1	140.4	74.1	52.8	9.1	2.6	28.7	3.2	1.3	40. 7	1.1	0.9	83.1
60	216.1	43.3	20.1	201.7	47.5	23.6	19.5	4.4	22.7	4.0	1.3	33.4	3.1	1.9	61.6
90	224.0	77.6	34.7	214.8	86.2	40.1	29.9	8.8	29.5	4.1	1.8	33.4	5.1	3.2	62.0
120	212.4	62.3	29.3	193.1	73.1	37.9	33.9	8.6	25.2	3.5	1.6	45.2	6.0	3.7	62.0
180	153.2	50.2	32.8	144.8	44.2	30.6	35.6	4.3	12.0	2.5	0.9	38.1	8.2	4.9	60.0
240	85. 5	35.1	41.1	91.9	39.9	43.5	26.5	10.1	38.3	1.7	0.5	31.5	6.1	3.8	61.8
300	53.7	26.4	49.2	61.8	18.1	45.5	18.0	6.8	38.0	1.3	0.5	36.2	4.6	3.7	80.8
360	28.4	12.1	42.5	44.4	16.1	36.3	11.9	4.0	33.5	1.1	0.3	29.1	2.1	3.0	138.6
420	15.1	8.6	57.1	26.0	6.6	25.5	8.5	2.5	29.3	0.9	0.2	20.0	1.5	2.7	175.1
480	11.5	5.1	44.6	22.0	8.5	38.8	6.6	2.5	37.5	0.8	0.2	22.2	0.6	1.2	220.3
720	4.3	1.2	28.0	12.4	5.7	45.7	3.7	1.2	34.0	0.2	0.1	36.7	0.0	0.0	0.0
1240	2.9	0.7	23.5	3.8	1.4	37.4	2.1	0.2	10.7	0.1	0.0	37.9	0.0	0.0	0.0

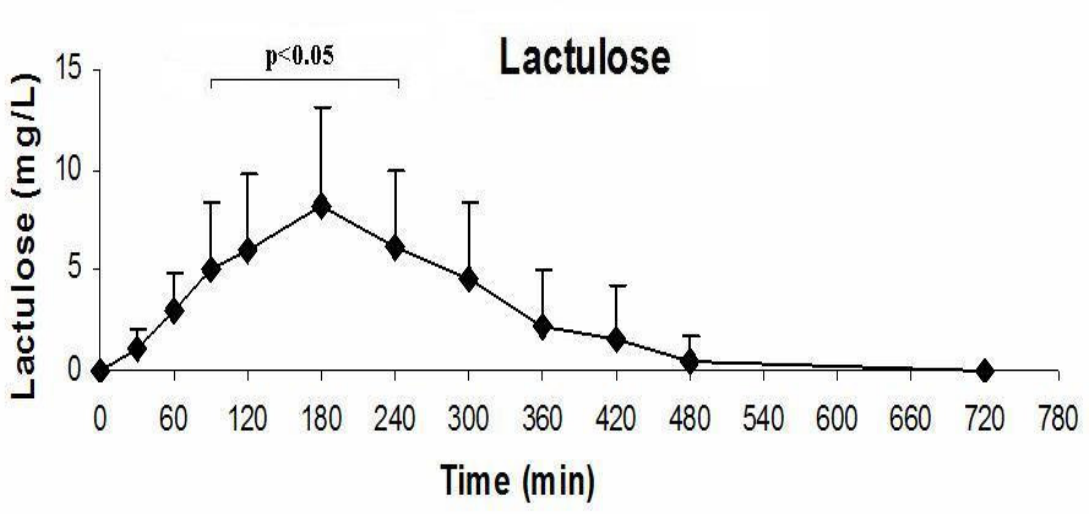

Figure 10: Mean ± SD concentration-time profile of lactulose. This figure shows the changes of lactulose concentrations in serum after orogastric administration in healthy dogs (n= 8). Serum concentrations of lactulose were significantly different from baseline at 90, 120, 180, and 240 minutes post dosing ($p<0.05$), reaching the maximum peak concentration at 180 minutes post dosing (mean ± SD: 8.2 ± 4.9 mg/L).

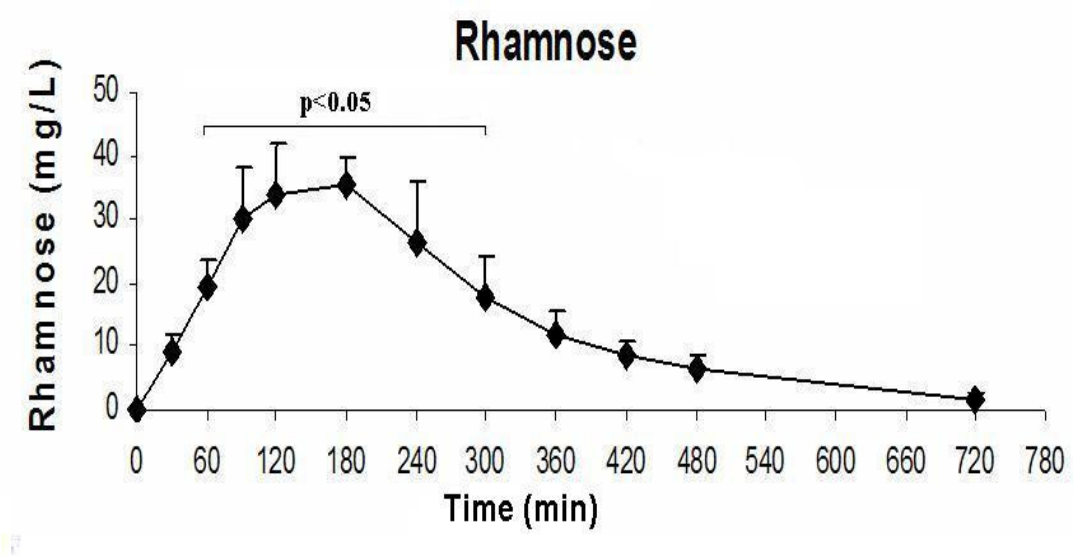

Figure 11: Mean ± SD concentration-time profile of rhamnose. This figure shows the changes of rhamnose concentrations in serum after orogastric administration in healthy dogs (n= 8). Serum concentrations of rhamnose were significantly different from baseline at 60, 90, 120, 180, 240 and 300 minutes post dosing ($p<0.05$), reaching the maximum peak concentration at 180 min post dosing (mean ± SD: 35.6 ± 4.4 mg/L).

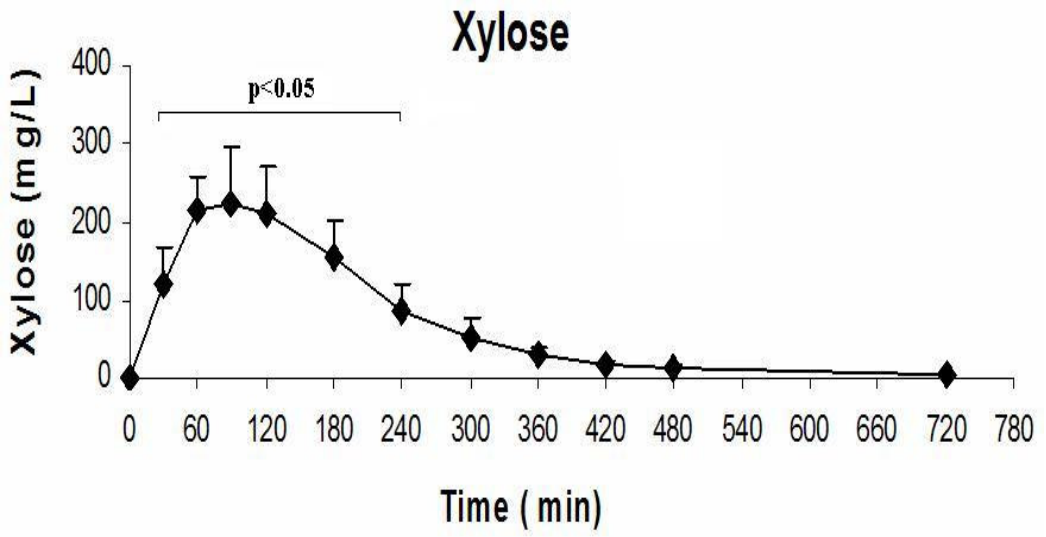

Figure 12: Mean ± SD concentration-time profile of xylose. This figure shows the changes of xylose concentrations in serum after orogastric administration in healthy dogs (n= 8). Serum concentrations of xylose were significantly different from baseline at 30, 60, 90, 180, and 240 min post dosing ($p<0.05$), reaching the maximum peak concentration at 90 min post dosing (mean ± SD: 224.0 ± 72.6 mg/L).

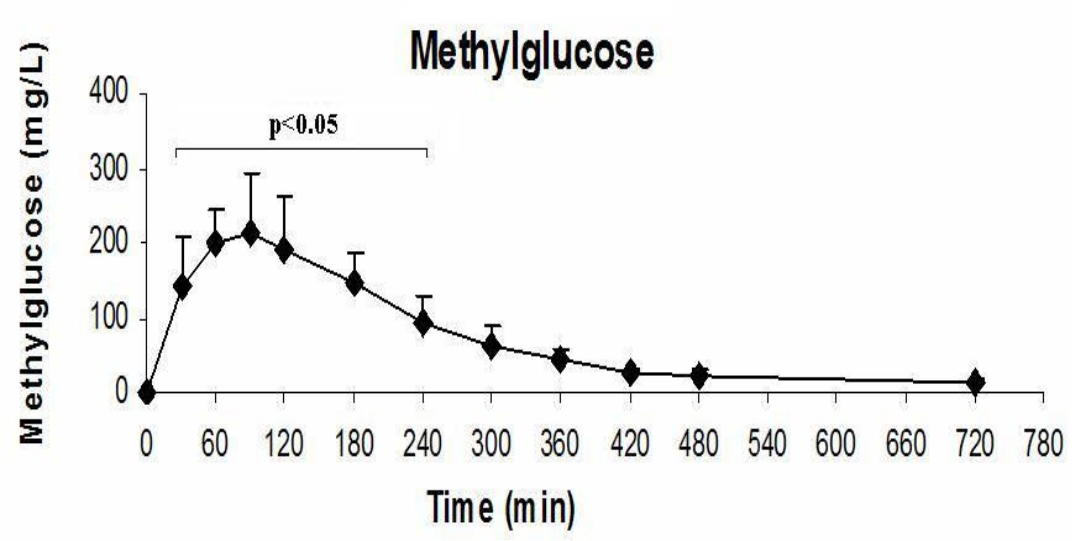

Figure 13: Mean ± SD concentration-time profile of methylglucose. This figure shows the changes of methylglucose concentrations in serum after orogastric administration in healthy dogs (n= 8). Serum concentrations of methylglucose were significantly different from baseline at 30, 60, 90, 120, 180, and 240 min post dosing ($p<0.05$), reaching the maximum peak concentration at 90 min post dosing (mean ± SD: 214.8 ± 80.7 mg/L).

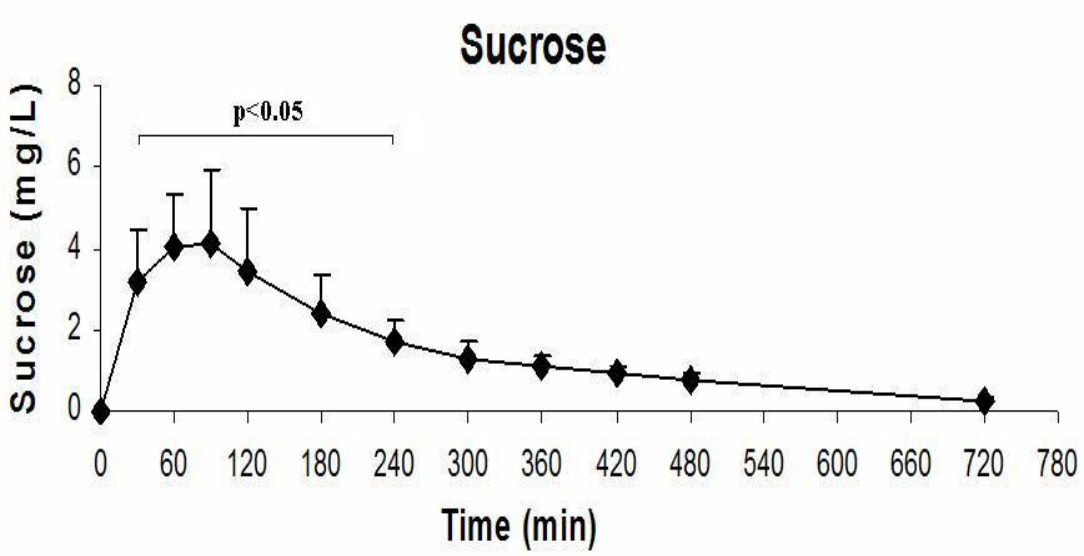

Figure 14: Mean ± SD concentration-time profile of sucrose. This figure shows the changes of sucrose concentrations in serum after orogastric administration in healthy dogs (n= 8). Serum concentrations of sucrose were significantly different from baseline at 30, 60, 90, 120, 180, and 240 min post dosing ($p<0.05$), reaching the maximum peak concentration at 90 min post dosing (mean ± SD: 4.1 ± 1.7 mg/L).

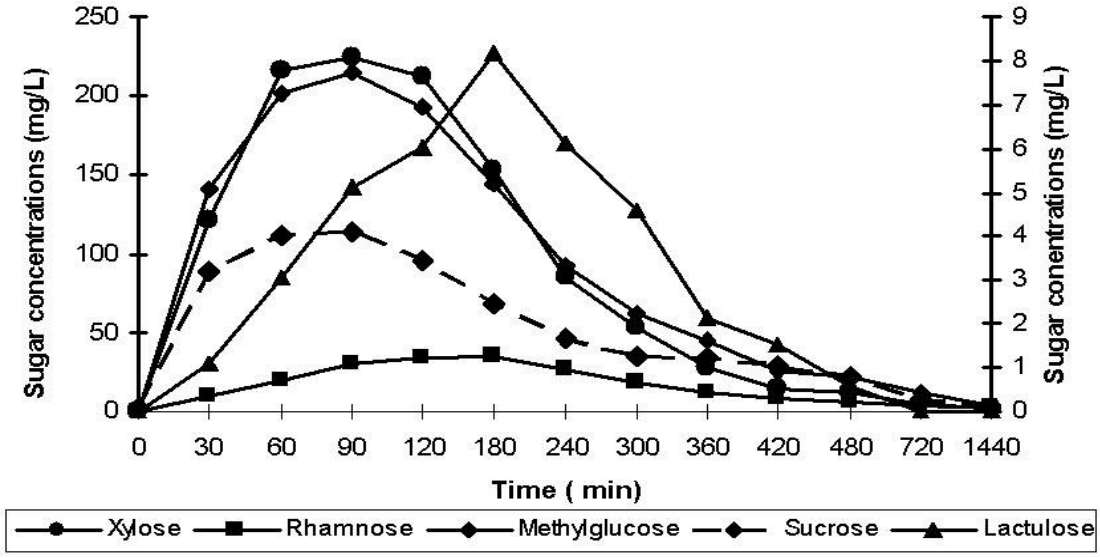

Figure 15: Serum concentration-time curves of the five sugars after orogastric administration. Serum concentrations of xylose, rhamnose, and methylglucose are displayed on the y-axis on the left. Serum concentrations of lactulose and sucrose are displayed on the y-axis on the right. For all five sugars, no significant differences were found between concentrations measured at 90, 120, and 180 min time points or between the coefficients of variation (CV %) of the mean concentrations of these three points.

DISCUSSION

The serum kinetics for 5 sugar probes commonly used for gastrointestinal permeability and intestinal absorptive capacity testing were assessed in this study**.** In general, sugar profiles in serum reflected a permeation and absorption pattern based on their physical and chemical characteristics. Taking into account the molecular dimensions, the monosaccharides xylose, methylglucose and rhamnose were absorbed faster and in higher amounts than the disaccharides. Therefore, the permeation pattern of lactulose and sucrose showed their selective size range to permeation by the gastrointestinal mucosa.

Kinetic profiles of xylose and methylglucose in serum were consistent throughout the experiment. Both sugars showed identical appearance and clearance patterns in serum after the ingestion of the sugar solution. These findings compare well with results of kinetic studies of intestinal permeability in humans using xylose and methylglucose, which showed similar kinetic characteristics in plasma.[52] In the present study, the maximum serum concentrations for these sugars were established at 90 min, and the X:M ratio with a mean ± SD of 1 ± 0.09 was consistently stable from 30 to 240 min.

The absorption of both monosaccharides occurs by mediated transport. Xylose is actively absorbed from the proximal small intestine (i.e., duodenum and jejunum), whereas methylglucose is absorbed throughout the length of the entire small intestine. Thus, a considerable reserve capacity exists for absorption of methylglucose, but not for xylose.[127] Therefore, a decrease in the X:M ratio is a more reliable indicator of decreased absorptive capacity than xylose alone,[133] because xylose is impaired to a greater extent than methylglucose in cases of mucosal damage, resulting in a subsequent decrease of the plasma X:M ratio.[52]

Lactulose and rhamnose permeation showed a similar kinetic pattern through the study. Concentrations of these sugars showed a constant increase in serum until 180 min after oral administration. Serum concentrations from both sugars had a ratio of 0.27 ±

0.06 mean ± SD at this sampling time period in where their concentrations were significantly different from the baseline.

The kinetic profiles of lactulose and rhamnose from the present study were similar to those reported in plasma from healthy dogs as previously measured by HPLC.[97] These profiles suggest that both sugars diffuse the intestinal mucosa by two distinct pathways with lactulose passing the intestinal mucosa through relatively large pores present at low abundance that is associated with the paracellular tight junction. Rhamnose crosses mainly through the small aqueous pores present at high abundance at the epithelial cell membrane. Intestinal disease leading to injury of the small intestinal mucosa leads to an abnormal mucosal permeability that is characterized by an increased L:R ratio.[52]

Many diseases affecting the small intestinal mucosa are characterized by villous atrophy which may lead to a reduced mucosal surface area for the diffusion of rhamnose, allowing an increased diffusion of lactulose in the crypt region due to a wide availability of large pores accompanying the mucosal damage. Serum L:R ratios were used to distinguish between subjects with villous atrophy and those with normal biopsies at 60, 90, and 120 min post-ingestion in humans.[72]

In regard to sucrose, this sugar was observed in small concentrations in serum. Under normal circumstances, ingested sucrose is rapidly hydrolyzed in the proximal small intestine by the enzyme sucrase into sucrose and. The normal gastric mucosa only allows a small quantity of sucrose to be absorbed before it is exposed to sucrase activity in the small intestine. During studies in animal models to identify site specific gastrointestinal permeability, sucrose could never be found beyond the stomach after rats received a gavage containing mixed sugar solutions containing sucrose.[37] An increase in gastric mucosal permeability to sucrose in NSAID gastropathy has been shown in humans.[134]

Reports of gut permeability to inert sugars in animal models (rabbits and mice) have shown an appropriate measure of lactulose to be at 180 min post dosing, whereas rhamnose showed its best expression at 120 min post dosing.[71] These values fall into the

range obtained from dogs in this study. The sucrose kinetic pattern observed in the present study showed significant statistical difference from the baseline at 30, 60, 90, 120, 180, and 240 min post dosing. This kinetic pattern closely resembles that reported for serum sucrose in human beings. Those studies showed that, in humans, gastroduodenal injury can be detected using serum at any determined point between 30 and 300 min.[135]

In conclusion, after orogastric administration of a sugar solution containing lactulose, rhamnose, xylose, methylglucose, and sucrose serum concentrations could be detected in all dogs after 30 minutes of administration. While the peaks of serum concentrations differed for the 5 sugar probes, there was no significant difference in serum concentrations between the 90 to 180 minutes timed blood sampling periods. Thus, it may be feasible to detect gastrointestinal injury (reflected by alterations in permeability and absorptive capacity) by collecting a baseline sample and a 90-180 min post administration sample.

CHAPTER IV

DISCUSSION

The assessment of gastrointestinal permeability and intestinal absorptive capacity has been used as a clinical research tool in human and veterinary medicine, and has also been used to evaluate the gastrointestinal barrier function in patients with a variety of gastrointestinal and systemic diseases.[136] The barrier function of the gastrointestinal tract can be assessed by evaluating intestinal permeability through the assessment of marker molecules that diffuse passively through the mucosa.[43] In contrast, intestinal absorptive capacity can be assessed by evaluating molecules that are absorbed by carrier-mediated transport.[43]

Measurement of marker molecules in urine after oral administration has been established as a tool for noninvasive investigation of small intestinal permeability and mucosal absorptive capacity.[72] However, one major source of error for this type of investigation lies in the ability to collect all of the urine samples over a period of several hours.[137] In addition, the most common methods used for the measurement of these marker are characterized by a lack of sensitivity to the marker molecules in urine.[138] In this study, the development and validation of a GC-MS method for the simultaneous measurement of lactulose, rhamnose, 3-O-methyl-D-glucose, xylose, and sucrose is described. Furthermore, serum profiles for these sugars in dogs are evaluated to provide the basic elements for an alternative for urine collection.

All sugars used in the GC-MS method described here have been previously used safely in humans[14] and companion animals.[48,51,58,97,139] Thus, these sugars have been a good choice for use as probes to assess intestinal permeability and mucosal absorptive capacity.[137,140] For example, lactulose and rhamnose are the most commonly used sugar probes.[37] The disaccharide lactulose crosses the intestinal epithelium by passive diffusion through paracellular tight junctions, while the monosaccharide rhamnose crosses the intestinal epithelium by transcellular passive diffusion through aqueous

pores.[141] The lactulose to rhamnose urinary recovery ratio is a well-established method for assessing intestinal permeability.[37] In addition, xylose and methylglucose are monosaccharides that are absorbed by intestinal epithelial cells via passive and active carrier-mediated transport, respectively.[141] Finally ,sucrose has been described as a marker for assessment of gastric permeability.[140]

Traditionally, gastrointestinal permeability has been estimated by the measurement of sugars in urine after oral administration of a solution containing a mixture of sugar probes. Subsequently, measurements are then performed on urine collected during a five to six hour test period. However, the collection of urine samples during this test period can be difficult, particularly in pediatric and veterinary patients.[97,141,] In addition to mucosal factors affecting permeability of the intestinal epithelium, a variety of pre-mucosal and post-mucosal factors might influence the urinary excretion of an orally administrated probe.[142] Also, the urinary recovery of some permeability markers (e.g., mannitol) can produce confounding factors due to alterations in renal function, more specifically, a low glomerular filtration rate.[142] More recently, several studies in dogs and humans have reported about the measurement of sugar permeability markers in serum rather than urine.[97,143] However, these initial studies have utilized HPLC for separation of the sugar probes and PAD for quantification, which has proved to be difficult for these closely related mono- and disaccharides. Consequently, interest in GC-MS analysis of these mono- and disaccharides has arisen.

In the first part of this study (Chapter II), an accurate, precise, and practical GC-MS method for the simultaneous analysis of lactulose, rhamnose, methylgluccose, xylose, and sucrose in spiked serum was developed and analytically validated. Subsequently, the GC-MS method was used for the measurement of these sugars in serum from healthy dogs (Chapter III) in order to establish the kinetic profiles of GI permeability-absorption markers once administered by orogastric intubation.

The refinement of the validated method was achieved by focusing on the critical steps related to metabolite analysis by GC-MS: extraction, derivatization, and separation.[144] A proper estimation of the concentration of any metabolite in a biological

sample is difficult because the composition of these samples contains a wide variety of constituents and chemicals with varying natures.[120] Serum is a complex matrix for chromatography, as it contains many substances. These substances all have the potential to affect the chromatographic measurements of the marker molecules of interest. Therefore, one goal of this study was to develop a method with as high extraction efficiency and reproducibility as possible for the GC-MS analysis without affecting the target probes themselves. Therefore, several methods, such as the addition of organic solvents[145,75] or salts,[146] and acidification using strong acids were used to precipitate proteins from biological fluids for the extraction of metabolites to be measured by GC-MS.[147]

Some overlapping peaks between endogenous glucose and our sugar markers of interest have previously been reported for urine samples. Pretreatment of urine by enzymatic degradation of glucose was needed to overcome this problem.[110] Similarly, in a previous report using HPLC-PAD for measurement of serum sugar probes, xylose eluted in close proximity to the endogenous serum sugar glucose, making measurement of serum xylose concentration difficult. Therefore, enzymatic pretreatment with glucose catalase and plasma protein precipitation were both used in that study as part of the extraction procedure to eliminate the interference of endogenous glucose. However, that procedure was considered time consuming and, therefore, impractical.[97] In an HPLC-PAD assay for the measurement of five sugar probes in serum previously developed and validated in our laboratory, enzymatic pretreatment and deproteinization were also considered necessary to obtain a more pure sample (unpublished data). Despite such pretreatment, the method failed to produce reproducible results and was thus abandoned.

For the sugar analysis reported here, multiple pretreatments of the serum samples were not necessary. Consequently, the extraction method is relatively simple compared to the previously described methods. Thus, serum proteins were precipitated by a combination of centrifugation and by addition of the organic solvent methanol as reported previously for a microwave-assisted derivatization method.[145] Although the use of methanol as a solvent for sugar extraction has been debated due to the potential

problems it may introduce, such as transmethylation of sugar esters, methanol is a more efficient extraction solvent than either ethanol or acetonitryl.[120] For example, sugar extractions using 80% methanol have been shown to have a recovery rate of 97% as determined by CG-MS.[144] In addition, in a subsequent step, methanol evaporation allowed the aqueous phase of the reaction mixture to be removed. This step is considered crucial because moisture in the extracted samples can produce derivatization artifacts or poorly derivatized compounds due to hydrophobic characteristics of the derivatization reagents.[148]

Therefore, the effectiveness of the extraction procedure used in this study was assessed by the measurement of the studied markers in serum samples that were spiked with increasing concentrations of the 5 sugar markers. This allowed for the determination of dilutional parallelism, spiking recovery, and coefficients of variation for intra- and inter-assay variability. Furthermore, the assays for all five sugars performed to acceptable standards, suggesting that the extraction and measurement procedures were effective.

The use of GC-MS rather than HPLC allows for better resolution of sugars[119] and is, therefore, considered superior for the measurement of serum concentrations of sugar markers. However, one of the major concerns with the use of GC-MS is that chromatograms contain multiple peaks that are generated by derivatized sugars.[149] Therefore, a suitable derivatization procedure was required prior to the GC-MS sugar analysis. As a result, many derivatization procedures have been described in the literature for carbohydrate analysis.[80,111,112] One such derivatization procedure is characterized by the substitution of polar groups (e.g., hydroxyl groups).[80]

In this study, several combinations of established derivatization protocols were evaluated in both individual and mixed serum samples. The purpose of this derivatization step is to increase the volatility and thermostability of the extracted samples.[111] Volatility is desirable, as it allows the marker to enter the gas phase and consequently the GC column.[112] The increased thermostability decreases the likelihood that a marker molecule will fragment under the high temperature conditions present in

the GC-MS. Such fragmentation could lead to the formation of multiple peaks in the chromatogram.[150] Other derivatization methods have been described to be used for sugars. Alditol acetate derivatization has been considered very useful for the measurement of reduced end sugars.[73] However, the alditol acetate derivatization reaction was not considered appropriate for this study, as this reaction can be associated with the generation of other sugar derivatives from glucose and fructose, such as mannitol.[114] Glucose and fructose are endogenous sugars present in serum samples and thus could be available in unpredictable quantities in each serum sample. Therefore, the alditol acetate derivatization reaction could have led to chromatograms with overlapping peaks due to the generation of carbohydrates that are structurally closely related to the carbohydrates of interest.[151] Additionally, the generation of mannitol would have altered the concentration of the internal standard, making quantification of the sugar markers impossible.

Another derivatization procedure, per-*O*-methylation, has been developed for the measurement of carbohydrates in aqueous solution.[149] Per-*O*-methylation requires the presence of water for the interaction of carbohydrates with solvents during the derivatization procedure. Therefore, this reaction avoids the oxidation of carbohydrates generated by the interaction between dimethyl sulfoxide and methyl iodide, the major derivative components of this method.[149]

Because of the limitations of the two previously discussed procedures, the oxime-trimethylsilyl derivatization procedure was selected for the chemical conversion of the sugar probes used of this study. Polar compounds containing functional groups, such as –OH were derivatized by adding a trimethylsilyl (TMS) group, called TMS-ethers. Because TMS groups lead to more volatile and thermostable compounds, TMS-ethers of mono- and disaccharides are easily prepared and separated chromatographically.[80,81] Sugar chromatographic characteristics were evaluated before sugar analysis. TMS-derivatization of monosaccharides usually results in the formation of multiple peaks.[152] For the protocol used here, the peak issue was solved by converting the aldehyde and keto groups into oximes before forming TMS ethers to reduce the

number of tautomeric forms as suggested previously by Curtis et al.[153] This structural change has been explained as a limited rotation along the C=N bond, resulting only in the formation of *syn* and *anti* forms.[154]

The oxime-trimethylsilyl derivatization procedure was evaluated during several assay runs, and a two-step derivatization protocol was chosen. The first reaction, oximation, was performed for two minutes and then the second one, silylation, was performed for five minutes, immediately before the samples were introduced into the GC-MS instrument. Both reactions were carried out in a microwave oven. Microwave-assisted derivatization is being used with increasing frequency.[155,104,156] Also, microwave use allowed for a reduced derivatization time in this study, compared to a recently reported method for which sugar oximation step had to be carried out for 12 or more hours.[120] Also, in comparison with conventional heating derivatization methods, the chromatograms generated by the metabolites resulting from microwave-assisted derivatization showed a well-defined resolution.

For sugar identification, a full scan MS method (m/z 50 to 1050) was used to select the appropriated ions. In order to increase the sensitivity of the analysis, a SIM mode was selected, which provided a much higher response than the full scan mode for the target sugars. For a better sensitivity and sugar identification, the most abundant fragments of each sugar were selected. This resulted in being able to easily identify and quantify the sugars by selecting single ion chromatograms from their selected ions as has been suggested in previous studies.[157]

Ion chromatograms were generated for each sugar at which point the chromatography peaks of the various sugars and their retention times were identified. In fact, the peaks and specific retention times generated for each sugar (Table 1) represent a potentially useful method for their direct identification. Also, the mass spectra for each sugar were recorded and compared with those previously reported.[76] Using the protocol developed here, the chromatograms did not display overlapping peaks among the sugars analyzed. Furthermore, no overlap was observed between the peaks created by the sugar markers of interest and endogenous sugars.

The oxime-TMS derivatization method is able to reduce the number of trimethylsilyl isomers.[158] Some sugars such as rhamnose (Figure 6) and xylose (Figure 7) generated two chromatogram peaks, which represent the *syn* and *anti* configuration forms, where one form greatly predominates over the other.[159] It has been reported that by measuring the larger peak the presence of the minor peak has no effect on assay reproducibility.[160] In contrast, methylglucose and lactulose showed three peaks in their chromatograms. However, all 3 peaks could be well separated. Sucrose, which is classified as a non-reducing end sugar, usually generates a single peak with most derivatization procedures.[73,149] In Figures 2 and 3 it can be observed that the chromatographic peaks eluted according to the number of carbon atoms. The smaller the number of carbon atoms of the monosaccharide, the shorter the retention time.[151]

The stability of TMS of mixed standards in serum was examined. During the examination of the standards, derivatized compounds were analyzed immediately after derivatization and were stored at room temperature or at 4º Celsius and analyzed again after 3 days. When comparing the chromatograms from samples analyzed immediately after derivatization and the same samples that had been derivatized and stored for several days, the same chromatogram resolution and peak intensity was observed. This observation demonstrates the stability of the derivatized marker molecules. Silyl derivatized products generated with BSTFA have demonstrated good stability at room temperature for 3 days after derivatization.[101] Stability of derivatized sugar samples is crucial when the interval between analysis of the first and the last samples of a GC-MS assay run is more than 12 hours.

The relative retention times of the sugars monitored and their selected ions allowed unequivocal identification and differentiation of all sugar probes used for this study. This can be observed in the representative ion chromatograms (Figure 2) of a serum sample spiked with all sugars. The ion chromatogram indicates adequate separation of the sugar probes. This is in contrast to previous assays methods, which resulted in difficulties for disaccharides identification and separation.[138] The use of relative retention time has been advocated to enhance reproducibility.[161] Because some

sugar probes used for this experiment are chemically closer to each other, the combination of the mass spectrometry and retention times have great significance for the differentiation of substances with similar mass spectra.[161] Thus, for compound identification, GC retention times from unknown samples were continuously correlated with those of the standards. The mass spectra of our target sugars correlated with the most common ions reported for pentoses and hexoses under oxime-TMS derivative forms.[118] Therefore, these mass spectra were adjusted to a single intensity for convenience of sugar identification and quantification using a selected ion monitoring method (SIM).[98] The characteristics of the sugar chromatograms were consistent during all GC-MS runs. Also, the detection limits for the sugar probes analyzed in this study are low when compared with values reported for analysis of the same sugars by capillary electrophoresis[107] and HPLC-based methods.[72,106]

This study represents the first analytical method for simultaneous separation and identification of five sugar probes in canine serum by GC-MS. The method was carried out following trimethylsilyl derivatization using the target and referenced ions (Table 1) to help reduce the influence of extraneous peaks and to ensure that any peak appearing in the SIM mode belonged to the monosaccharides and disaccharides used in this experiment. Table 7 shows that calibration curves for each sugar probe were linear within the measured range between 0.5 to 500 mg/L with mean correlation coefficients (r^2) of 0.997.

In this study, the absolute recoveries for the sugars showed a mean ± SD of 106.2 ± 13.0%. Slightly higher recoveries were observed for L, R, X, and M (Table 3) in serum samples spiked at the lowest concentrations. It is likely that the wide range of the standard curves used for this study were responsible for this overestimation of sugar concentrations. A similar overestimation was observed in successive studies when sugar concentrations were estimated using the same range (0.5 to 500 mg/L) employed for the present experiment. This situation could be corrected by calculating sugar concentrations using a narrower range for the standard curves used.

Sucrose showed the lowest recovery percentage. Low accuracy for detection and quantification of sucrose at small concentrations compared with higher concentrations has been reported using gas chromatography.[119] Consequently, recovery can be influenced by loss of the analyte during extraction and oximation derivatization. Also, sucrose degradation may occur as a result of the extraction method and oxime derivatization.[120]

The lower detection limit was 0.03 mg/L for L, R, M, and X, and 0.12 mg/L for S. The accuracy and precision for the five sugars was deemed sufficient to allow further measurement of sugar probes for the assessment of gastrointestinal permeability and intestinal absorptive capacity in dogs. Since this is the first validated method of analysis for five sugars in serum samples using GC-MS, there is no other method available for comparison of accuracy and precision. The %CVs for the intra-assay variability were 6.8% to 12.8% and for the inter-assay variability 6.4% to 15.9% for all five sugars. The coefficients of variation obtained were lower than 16%, indicating a good performance of this method. These values, which are representative of precision and reproducibility, respectively, are in agreement with the general accepted criterion for gas chromatography-mass spectrometry established for the detection of substances in biological matrices.[162]

For the second part of this study, the described GC-MS method was used to establish serum kinetic profiles for L, R, M, X, and S in dogs after a solution containing these sugars was administered by orogastric intubation.

Currently, available methods for assessment of intestinal permeability involve evaluation of the mucosal transport of saccharides, radioactive markers (i.e., ^{51}Cr-EDTA), and polyethylene glycol or by a combination of these markers. Radioactive markers are easily measured and are inexpensive to analyze.[163] Furthermore, they can also be detected in blood samples.[44] However, using radioactive markers require exposure of both patients and personnel to ionizing radiation.[164] In addition, diagnostic specificity may be lacking since increased absorption of some of these markers may occur in a variety of intestinal diseases, including gluten enteropathy,[165] iatrogenic

enteropathy due to nonsteroidal drug administration,[166] and Crohn's disease.[167] In contrast, sugar probes cross the intestinal mucosa via specific pathways, often limited to a specific segment of bowel.[163]

Polyethylene glycol exists in a variety of sizes and can be used as a marker for intestinal permeability. Smaller polymers permeate the mucosa faster than rhamnose, while the permeation rate for the larger polymers is higher than those for ^{51}Cr-EDTA or lactulose.[42,168] In addition, low urine excretion levels of PEG-400 in relation to saccharides and ^{51}Cr-EDTA suggested that the probe may not be sufficiently sensitive to assess intestinal permeability in more subtle disorders of barrier function.[43] Therefore, differences in permeation rates and their tendency to be retained by tissues make PEG less ideal for the evaluation of intestinal permeability.[42] All these markers are orally administered and are mostly measured in urine. However, the above methods are cumbersome and too laborious for use as a routine test because they require the collection of urine over long periods and/or the use radioisotopes. Therefore, we developed a new method to assess intestinal permeability by sampling blood instead of urine.

GI permeability tests using serum have not been published extensively. Most of these use only one sugar probe such as sucrose,[169,70,138,94] xylose,[170,171,172] 3-*O*-methylglucose,[173] and lactulose,[174] or two sugar markers such as lactulose and rhamnose,[71] or lactulose and mannitol[72,93] to evaluate GI permeability and/or intestinal absorptive capacity. In addition, some of these studies in serum have been characterized by one or two randomly timed blood samples,[138,170] or blood samples taken a long time after the test solution had been administered.[175] Therefore, accurate inferences related to gastrointestinal permeability have been absent due to a lack of consecutive measurements of sugar concentrations in serum. So far, the most advanced contribution for the assessment of GI permeability and mucosal function testing using serum have been tests in which sugar probes are measured simultaneously in serum and urine collected between two to six hours after sugar markers were orally administered.[97,175,176] In a comparative study performed for the assessment of intestinal permeability in human

patients with inflammatory bowel disease, in which lactulose was measured in serum and urine, urinary lactulose recoveries changed in parallel with the blood values.[174]

These considerations prompted us to analyze the kinetics of the sugar probes in healthy dogs in serum instead of urine. In this experiment, we measured the concentrations of L, R, X, M, and, S in serum using the validated GC-MS method. After their administration by orogastric intubation, a rapid appearance of the monosaccharides xylose, methylglucose and rhamnose in serum was observed. The serum concentrations of rhamnose, methylglucose, and xylose were significantly increased compared to baseline concentrations. Overall, serum concentrations for lactulose and sucrose were a lot lower than those for the other sugars. Intestinal mucosal permeation of disaccharides is influenced to a certain extent by the molecular size, which dictates the pathways used for these saccharides.

In general, serum concentrations of sugar probes showed a well defined profile over the entire sampling period until they reached the limit of resolution. Kinetic profiles for the monosaccharides xylose and methylglucose in serum were consistent throughout the experiment. More over, they reached the maximum peak concentration at 90 min post dosing followed by a gradual decline. Both sugars showed identical appearance and clearance patterns in serum after the ingestion of the sugar solution. These findings compare well with results of kinetic studies of intestinal permeability in humans using xylose and methylglucose, which showed similar kinetic characteristics in plasma.[52] Both sugars are absorbed rapidly in the upper jejunum.[177] Also, both sugars reach their maximum concentration at 90 min after ingestion. It is at this time point when absorption and excretion of a substance are in balance.[178]

In a study, which evaluated xylose absorption in human patients, absorption of this sugar averaged 81% of the oral dose in normal subjects, compared to less than 60% of the oral dose in patient with various absorptive pathologies. In both cases the maximum serum concentrations of xylose were found at 90 minutes after oral administration.[179] Also, in studies of xylose absorption in polygastric species, serum kinetic profiles of xylose showed a maximum peak concentration at 90 min.[180] Studies in

different animal species that evaluated the rate of xylose absorption suggested that absorption is influenced primarily by the gastric empting rate.[181-183] Also, small intestinal motility, the surface area available for absorption, and the microbiota of the small intestine influence the kinetics of xylose absorption.[180,182,184] The oral bioavailability of xylose has been reported to be 90% in rats and 81% in humans. In horses only 30% of xylose is absorbed, and is further metabolized in the large intestine.[185]

The inclusion of an actively transported but non-metabolizable marker in an intestinal permeability tests, such as methylglucose, should reflect the overall absorptive capacity of the small bowel (i.e., surface area),[186] but it would also reflect alterations in gastric emptying, small intestinal motility patterns, the mucosal barrier with the unstirred water layer of the small intestine, and finally the transport capacity of the enterocytes.[187] Several studies have shown that the small intestine has a large reserve capacity for the active mediated absorption of methylglucose in contrast to xylose, anticipating a relative insensitivity of methylglucose for intestinal damage when compared to xylose.

On the other hand, serum concentrations of lactulose and rhamnose were significantly different between baseline and 90 to 240 and 60 to 300 min post dosing, respectively. Such values are in close agreement with those found in a control group of dogs in a study reported by Sørensen et al..[97] Mean maximum concentrations of lactulose (mean ± SD: 8.2 ± 4.9 mg/L) and rhamnose (mean ± SD 35.6 ± 4.0 mg/L) were obtained at 180 minutes, followed by a gradual decline. The sugar profiles of both sugars are a reflection of their permeation characteristics. According to Maxton et al., two distinct pathways are hypothesized for unmediated mucosal permeation: one consisting of small pores in the cell membrane of enterocytes through which rhamnose passes and the other of large channels related to the tight junctions and/or extrusion zones.[41]

Monosaccharides, such as rhamnose or mannitol reflect the degree of transcellular permeation of small molecules (0.65 nm). Disaccharides, such as lactulose or cellobiose, reflect paracellular permeation of larger molecules (0.93 nm).[188] Thus, the kinetic profiles of serum concentrations displayed by lactulose and rhamnose follow these permeation principles, with rhamnose being the smaller molecule and lactulose

being the larger molecule, which is only able to permeate through the relatively infrequent paracellular pores.

Several studies have assessed the passive permeability characteristics of the small intestine using water-soluble probes that differ in molecular size, such as carbohydrates[168,189] or polyethylene glycol (PEG) of varying molecular weights.[40,190] In these studies, absorption declined as the increasing molecular weight of probes more rapidly than the free aqueous diffusion coefficients of the probes, consistent with movement. A phenomenon called solvent drag generated by water movement has been used to explain differences in the permeation of compounds with similar characteristics but a different molecular size. Thus, the solvent drag affects, presumably, the flux through pores that selectively allow the passage of smaller molecules and are restrictive to larger-sized molecules.[191] Pioneers in the intestinal permeability field stated that the osmotic pressure gradient between plasma and luminal content is not consistent from the upper to the lower small intestine.[192]Also, the ability of a given concentration gradient to promote the movement of water varies with the molecular size of substances used. As a result, the ability of a non-lipid soluble solute to generate effective osmotic pressure is inversely related to its ability to penetrate the membrane, which in turn is dependent upon the molecular size of the substance in question. Such an observation was markedly expressed from the jejunum to the ileum because the pore radius in the jejunum is twice the size of the effective pore radius in the ileum.[192] Not surprisingly, serum profiles of lactulose and rhamnose (Figure 15) give an indication of the size selectivity of the different pathways.

Under normal circumstances, ingested sucrose passes into the small intestine where it is rapidly degraded by brush border disaccharidases.[136] Sucrose, being a disaccharide, is believed to be absorbed via the paracellular route and reflects the permeability of the intercellular tight junctions.[43] In the present study the sucrose probe showed a serum profile with a small increase in the serum concentration (mean ± SD: 4.1 ± 1.7 mg/L). Low serum sucrose concentrations under physiologic conditions are due

to a much smaller surface area and shorter contact time of the sugar probe in the stomach.[83]

In theory, sucrose should not permeate the gastric mucosa in healthy individuals. In a screening test to detect sucrosemia in celiac disease developed by Cox et al., sucrose was not found in normal control subjects in serum samples taken at 45 min and 2 hours after ingestion of 50 g of sucrose. In contrast, other data showed sucrose in urine collected from normal subjects after ingestion of 100 g of sucrose.[138] The results of this study assumed differences in the administered dose, as well as differences in the sensitivity of HPLC analysis of urine collected over a period of 6 hours in comparison to a single serum sample. Furthermore, in this study, the serum kinetic profile for sucrose compared well with the sucrose profile obtained in normal human volunteers.[70]

In summary, we collected blood specimens from 8 dogs at 13 different sampling times during 24 hours and examined the changes in serum lactulose, rhamnose, methylglucose, xylose, and sucrose concentrations in order to determine a time point appropriate for collection of a serum sample for gastrointestinal permeability and mucosal function testing. There was no significant difference of the sugar concentration of any of the 5 sugars between 90 and 180 minutes after administration. Thus, it may be possible to use a single serum sample collected at any point between 90 and 180 min after the administration of the sugar probes.

CHAPTER V

CONCLUSIONS

In this study, a method for the simultaneous quantitation of lactulose, rhamnose, methylglucose, xylose, and sucrose in canine serum was developed and analytically validated. The method included deproteinization of serum samples and a two-step derivatization process combined with gas chromatography mass spectroscopy analysis.

The results obtained from the present study extend the possibilities for gastrointestinal permeability and mucosal function testing. The described GC-MS method was used to determine serum kinetic profiles for lactulose, rhamnose, methylglucose, xylose, and sucrose in dogs after administration of a solution containing these sugars by orogastric intubation. Measureable serum concentrations of the monosaccharides xylose, methylglucose, and rhamnose appeared in the serum shortly after administration. In contrast to the concentrations of the monosaccharides, the disaccharides lactulose and sucrose only reached much lower concentrations in the serum, suggesting a low level of gastrointestinal permeation.

For all of the 5 sugar probes, no statistically significant differences were found between concentrations measured at 90, 120, and 180 min after administration. Thus, it may be feasible to detect gastrointestinal injury (reflected by alterations in permeability and absorptive capacity) using serum obtained by only two blood samples: one sample taken at baseline and a second sample taken between 90 to 180 min post-dosing of sugar probes.

Results generated from this study provide a new method for the evaluation of the integrity of the gastrointestinal mucosa, which can not be achieved by conventional diagnostic techniques. Furthermore, information about gastrointestinal permeability and mucosal absorptive capacity might complement the information provided by conventional techniques used for the characterization and distribution of gross and histopathologic lesions. Also, the minimally-invasive nature of permeability and

absorption tests would allow these tests to be used routinely for diagnostic screening of clinical patients and also as a research tool, and could possibly replace the need for more invasive endoscopy and biopsy procedures. Consequently, the use of such minimally-invasive methods provides the option for clinicians and researchers in gastroenterology to test intestinal permeability and absorptive capacity, which allows for assessment of an important function of the gastrointestinal mucosa.

As described earlier, most of the assays described in the literature for permeability testing in serum used methods other than GC-MS. The major improvement of our method compared to those previously described is the simultaneous determination of five sugars in serum. Furthermore, the use of GC-MS has led to a more sensitive method, compared to the published protocols. The use of this sensitive method for the measurement of serum concentrations of sugar markers to predict GI permeability and absorptive capacity in a clinical setting would be the next step for evaluation of the clinical usefulness.

For example, these measurements could provide information complementary to the results of other diagnostic tests, such as histopathology or laparoscopy. It would seem reasonable to assume that having the ability to detect low concentrations of sugars in serum would substantially enhance the power of GI permeability and absorption tests. Therefore, it may be plausible that the current GC-MS assay, capable of detecting low concentrations of sugars of 0.03 mg/dl, would be clinically more useful than previously described. Ultimately, the cost, applicability, practicality, and availability of the GC-MS assay will need to be compared with the methods previously described to determine which assay might be preferable for wide-scale application.

In conclusion, we described the development and analytical validation of a GC-MS method for the simultaneous determination of five sugar probes in canine serum. Also, the kinetic study showed no statistically significant differences for all of the 5 sugar probes when measured at 90, 120, and 180 min after administration, suggesting that a simple sample post-dosing may be sufficient to evaluate permeability and absorptive capacity for all five sugar probes.

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