PLASMA CONCENTRATION OF GLUCOSAMINE AND CHONDROITIN SULFATE IN HORSES FOLLOWING AN ORAL DOSE

A Thesis

by

COURTNEY ANN WELCH

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

MASTER OF SCIENCE

December 2004

Major Subject: Animal Science

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Approved as to style and content by:

_____	_____
Gary Potter (Co-Chair of Committee)	Pete Gibbs (Co-Chair of Committee)
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David Hood (Member)	
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John McNeill (Head of Department)	

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ABSTRACT

Plasma Concentration of Glucosamine and Chondroitin

Sulfate in Horses Following an Oral Dose. (December 2004)

Courtney Ann Welch, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Gary D. Potter
Dr. Pete Gibbs

This study was conducted to study absorption of glucosamine and chondroitin sulfate and to measure any changes in blood concentration of these compounds following feeding them to horses in different amounts. Six mature mares were used in a replicated 3x3 Latin square designed experiment. The experiment consisted of three 15-day periods, which included 10 days of diet adaptation followed by a 5-day sampling period. Blood was drawn on one day during each sampling period. Horses were fed a control diet (40% hay, 60% concentrate) balanced to meet NRC (1989) requirements for maintenance of mature horses. In one experimental diet, 2.0 g chondroitin sulfate and 5.5 g glucosamine were added to the basal ration at each feeding. In the other experimental diet, 3.5 g chondroitin sulfate and 8.5 g glucosamine were added to the basal ration at each feeding. Following total collections, blood was centrifuged and plasma was harvested and data analyzed for the presence of each compound. Analyses for plasma glucosamine were performed in the Protein and Chemistry Lab at Texas A&M University using HPLC. Chondroitin sulfate in the plasma was analyzed using a color reagent, dimethylmethylene blue, followed by UV spectrophotometry.

There were no significant differences ($P<0.05$) in the concentration of chondroitin sulfate or glucosamine concentrations in plasma when comparing the three different diets. This leads to a conclusion that these compounds were not absorbed through the intestinal wall into the bloodstream in the same form as they were fed. This poses a question as to whether or not oral forms of these compounds are absorbed and are able to migrate to joints through the blood to improve joint function. With the significant economic impact that products containing chondroitin sulfate and glucosamine are making in the animal nutrition industry, more research is needed to further elucidate actual efficacy of these compounds in diet supplements for horses.

ACKNOWLEDGEMENTS

I would like to thank my committee, Dr. Gary Potter, Dr. Pete Gibbs and Dr. David Hood for their wisdom and guidance throughout my graduate career. Dr. Potter has always held his graduate students in high regard, and it has made me a better person for trying to rise to his standards. There is no where else in the world that a student could go to study and expect to receive a more fulfilling education in equine science than at Texas A&M because of these three men and their amazing depth of knowledge.

Although her name is not on the list of my committee co-chairs, Elena Michael has been as much a part of my fulfilling this degree as anyone else. During my research project, I made it a goal to model Elena's attitude and work ethic because she knew how to manage such a task efficiently and effectively. There was never a time when she wasn't willing to give up her personal time to help a fellow graduate student. From mucking stalls to calculating statistics, I could not have made it through without her endless support.

Thanks to all my fellow Aggies without whose help my project would have never succeeded. The endless hours of total collections, early morning feeding and rather tedious lab work was all accomplished because of your commitment to Texas A&M and their equine science program. Your help was appreciated more than you know.

To all my friends and family, I express my sincere gratitude for all your support throughout my life and especially during my graduate career. To my husband, thank you so much for looking out for your pregnant wife and helping do things you probably thought you would never do in your lifetime with animals that weren't very cooperative.

To my parents, whom I know thought I was crazy for pursuing agriculture, but never said so because they are so supportive of me in everything. Thanks for your never-ending love and support. And finally, thanks goes out to all my teachers over the years for instilling in me the love of the sciences and the desire to live a life of constant learning.

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CHAPTER I

INTRODUCTION

Numerous dietary supplements containing glucosamine and/or chondroitin sulfate are currently being marketed as a way to help support, improve or restore the health of horses' joints (Ramey et al., 2002). Glucosamine and chondroitin sulfate are fairly new products to the horse industry, and currently studies of the efficacy of these products in equine joint disease have produced mixed results (White et al., 2003). Most of the research done thus far has been performed on more typical lab animals such as dogs and rats. There is little if any accurate data on the absorption of orally dosed chondroitin sulfate and glucosamine in the equine.

Understanding how glucosamine and chondroitin sulfate work as chondroprotective agents ultimately could translate into improved prevention or management of cartilage degeneration in athletic and performance horses (Orth et al., 2002). A well-performed study on the absorption of glucosamine and chondroitin sulfate from the gastrointestinal tract of the equine is needed to provide a base for future research on the efficacy of these products in the equine industry.

This thesis follows the style and format of the Journal of Animal Science.

CHAPTER II

REVIEW OF LITERATURE

Degenerative joint disease and its associated joint pathology contribute significantly to musculoskeletal lameness and loss of function in performance and pleasure horses (Hanson, 1996). Numerous dietary supplements containing glucosamine and/or chondroitin sulfate are marketed as a way to help support, improve or restore the health of horse's joints (Ramey et al., 2002). The main goal of the medical therapy in treatment of degenerative joint disease is to restore and maintain normal joint function by alleviating pain, decreasing joint inflammation, and protecting the cartilage from further injury (Anderson et al., 1999). Recent research in the medical management of these joint problems has focused on slowing the process of cartilage degradation and promotion of cartilage matrix synthesis (Hanson, 1996). Glucosamine and chondroitin sulfate are macromolecules endogenous to cartilage and have been used in veterinary medicine for several years with mixed results. Benefits of oral supplements containing glucosamine and chondroitin sulfate have been shown in many different types of animal models and some human clinical studies (McLaughlin, 2000). Studies of the efficacy of these products in equine joint disease have produce mixed results (White et al., 2003). Elucidating their modes of action at the cellular level is becoming an important area of study in arthritis research (Orth et al., 2002). Understanding how glucosamine and chondroitin sulfate work as chondroprotective agents ultimately could translate into improved prevention or management of cartilage degeneration in athletic and

performance horses (Orth et al., 2002). The first step in this line of research is to determine the absorption of oral products from the gastrointestinal tract in the equine.

The reported combination of glucosamine and chondroitin sulfate has been used in veterinary medicine for several years in the USA to treat degenerative joint disease in small (Moore, 1996) and large (Hanson et al., 1997) animals with some favorable clinical results and no reported clinical side effects in healthy dogs. A survey of veterinary practices revealed that intra-articular lesions account for 33% of all diagnosed equine conditions. Traditional treatments, including surgery, nonsteroidal anti-inflammatory drugs and corticosteroids, have been augmented by additional medications possessing the potential to slow the progression of the disease in addition to treating its symptoms (Fenton et al., 2002). Among these new methods of disease prevention are a class of orally administered “nutraceuticals”, the most common of which contain glucosamine and/or chondroitin sulfate (Fenton et al., 2002). Since chondroitin sulfate and glucosamine are considered nutritional supplements, they are not subject to the same stringent requirements for quality as are pharmaceutical products (Ramey et al., 2002). Currently there is a lack of experimental evidence proving the efficacy of these products on joint health when given orally. Without good, controlled clinical trials, practitioners have had to rely on sporadic anecdotal information coupled with their professional judgment on the worthiness of many “nutraceutical” agents available in the market to treat degenerative joint disease in animals (Anderson et al., 1999).

A mail survey was sent to 3080 small-animal practitioners on the perceived clinical efficacy and safety of oral “nutraceuticals” such as chondroitin sulfate and glucosamine. Sixty four percent of the practitioners reported that they were

recommending the oral nutraceutical to their clients (Anderson et al., 1999). Most practitioners (83%) believed response to treatment with the studied product occurred within four weeks and they rated the clinical efficacy to be either 'good' or 'excellent' in improving mobility, alleviating pain and improving attitude in the majority of treated animals (Anderson et al., 1999). With the rate of distribution and the highly perceived clinical efficacy, the potential importance of these products is great and warrants much scientific research to be conducted.

Glucosamine and chondroitin sulfate are macromolecules endogenous to cartilage. Articular cartilage is composed of chondrocytes, which synthesize and deposit around themselves a watery matrix. This matrix gives cartilage its resiliency and tensile strength and is composed of collagen and proteoglycans (Hanson, 1996). Glycosaminoglycans are highly negatively charged long chains of repeating disaccharides that attach to a core protein (MacLeod, 2001). Glycosaminoglycans (GAG) aggregate with hyaluronic acid to form proteoglycan macromolecules (Davidson, 2000). Healthy chondrocytes can replenish up to a 50% loss in matrix volume (Davis, 1998). Any insult to cartilage can cause a loss of GAG content and therefore a loss of elasticity and ability to bear and transmit forces efficiently, resulting in a cascading cycle of more cartilage insults. Chronic overuse can increase the rate of cartilage breakdown and exceed the chondrocytes' ability to replace the matrix (MacLeod, 2001). Conditions such as this result in a need to supply the raw materials (nutrients) to the cartilage, so that the synthesis process is not impaired and cartilage may replenish itself (Hanson, 1996).

Chondroitin Sulfate

Chondroitin sulfate (CS), the most abundant GAG in the body, is a long chain polymer of a repeating disaccharide unit galactosamine sulfate and glucuronic acid (Hanson et al. 1997). Glycosaminoglycans are present in various mammalian organs and tissues such as cartilage, blood vessels, cell surfaces and intracellular organelles and are also normally found in plasma. The mass of CS is variable ranging from 14,000 Da to 30,000 Da. The major site of metabolism for circulating chondroitin sulfate is the liver, where the GAG, depending on the animal species, may be partly degraded to oligosaccharides that subsequently lose their sulfate groups as inorganic sulfate (Baici et al., 1992). Inorganic sulfate and intact polymeric chondroitin sulfate are excreted in the urine (Wood et al., 1973). Parts of the glycosaminoglycans are taken up by the cells, where they are degraded to low molecular mass products (Revell and Muir, 1972).

The bioavailability of oral chondroitin sulfate in animals is a subject debated in the literature. Palmieri et al. (1990) found more than 70% absorption when using ^{3}H-labeled CS fed to rats and dogs. Andermann and Dietz (1982) investigated the absorption of chondroitin sulfate in rabbits after oral administration and found neither absorption nor release of a characteristic-clearing factor into the blood stream. One factor that may affect the absorption of CS is the chain length of the molecule. Current theory supports higher permeability for CS with lower molecular weight (Eddington and White, 2001). The mammalian intestinal epithelium is a highly effective barrier, which hinders the diffusion of a wide variety of compounds, especially those that are charged and/or have a high molecular mass (Baici et al., 1992). The most important factor determining the intestinal absorption of an organic electrolyte is its degree of ionization (Schanker et al., 1958).

Oral intake of chondroitin sulfate implies exposure to both the enzymes of the stomach and of the intestine (Baici et al., 1992). Considering all the above-mentioned experimental evidence, it is of great importance that absorption of chondroitin sulfate is studied in the equine.

Absorption of chondroitin sulfate has been reported in studies in which radiolabeled compounds were used. Pinocytosis is the main mechanism of GI absorption of GAG's (Conte et al., 1995). Palmieri et al. (1990) demonstrated that more than 70% of the radioactivity administered orally to rats and dogs has absorbed. The labeling methods used by the authors suggest that each chondroitin sulfate molecule bear a single label at the reducing end of the molecule (Baici et al., 1992). The in vivo enzymatic degradation of the polymer proceeds, step by step from the non-reducing end toward the reducing end (Conte et al., 1995). This sort of labeling is not representative of the whole molecule. Conte et al. (1991) reported on the plasma concentration of chondroitin sulfate given orally to man and concluded that the absolute bioavailability of the glycosaminoglycan was 13.2% of the administered dose. Judging from the amplitude of the standard deviation reported for total chondroitin sulfate, it must be concluded that the authors also observed large variations, at least among different volunteers (Baici et al., 1992). Conte et al. (1995) reported that when feeding ^{3}H-chondroitin sulfate to rats and dogs, more than 70% of radioactivity was absorbed. They found low molecular mass CS in synovial fluid of these animals, but to date there are no enzymes currently known to catalyze reoxidation of the monosaccharides of GAG chains (Conte et al., 1995). The experimenters also found high molecular mass CS, higher than what was fed. This may be due to the binding of the polysaccharide and its derivatives with proteins (Conte et al.,

1995). In a study of absorption of sulfated glycopeptides (GLPS), it was reported that when rats and dogs were fed a ^{33}S GLPS almost all of the radioactivity resulting from a single oral dose was excreted in the feces with in five days (Chasseaud, 1972). More specifically, 88.3% of the 89.3% recovered GLPS was found in the feces of rats within five days, and 96.0% of the 97.0% recovered GLPS was found in the feces of dogs within five days. Oral dosing of GLPS in humans was also studied and it was reported that most of the radioactivity was rapidly eliminated in four days (Chasseaud, 1972).

Glucosamine

Glucosamine (GlucN), an amino sugar synthesized by chondrocytes from glucose and glutamine, is an important intermediate for the formation of numerous compounds including GAGs (Davidson, 2000). Glucosamine is a small molecule (m.w. = 179.17) and very soluble in water. The pKa of GlucN is very favorable for absorption from the small intestine and in general for the crossing of biological barriers in the body (Setnikar et al., 1986). As a result of their structure, GAGs bind large amounts of water, thereby allowing them to function in support of the cellular and fibrous components of tissue (Johnston, 1997). Certain articular disorders are due to a “degenerative” process affecting the cartilage. The causes of this process are unknown, but an important factor is represented by a defect of the biosynthesis and the turnover of glucosamine and of other aminosugars (Setnikar et al., 1986). Studies have shown that administration of GlucN tends to normalize cartilage metabolism and stimulate the synthesis of proteoglycans so that articular function is partially restored (Hanson, 1996).

Glucosamine has been widely studied as an agent to reduce or alleviate symptoms of arthritis. Glucosamine at concentrations as low as 0.5 mg/ml has been shown to

inhibit nitric oxide production (Orth et al., 2002). Patients with arthritis have higher concentrations of nitric oxide catabolites in their serum and urine than age-matched individuals with no clinical signs of arthritis (Grabowski et al., 1996). Articular cartilage explants from horses with moderate osteoarthritis produce more nitric oxide than normal cartilage (Orth et al., 2002). Glucosamine was also found to inhibit PGE_2 production. Prostaglandin E_2 is upregulated during an inflammatory response and is found in increased concentrations in the synovial fluid of patients with arthritis (Orth et al., 2002). Horses with degenerative joint diseases have elevated concentrations of PGE_2 (May et al., 1994). Therefore, the ability of glucosamine to alleviate clinical signs of osteoarthritis could be due to its ability to inhibit PGE_2 synthesis (Orth et al., 2002).

Glucosamine absorption from the gastrointestinal tract has been studied on many occasions in many animals. In a study of intestinal absorption of GlucN in rats, it was reported that GlucN is easily absorbed through a simple carrier mediated transport across the intestinal wall. GlucN was found to be absorbed without modification to its molecular structure (Tesoriere, 1972). Glucosamine has also been found to concentrate in certain organs and tissues such as the liver, kidney and cartilage. The liver is the main organ responsible for the metabolism and biotransformation of exogenous GlucN (Setnikar et al., 1984). The kidney concentrates GlucN and excretes it in the urine (Setnikar et al., 1986). In a study using uniformly labeled GlucN, it was found that GlucN is rapidly and well absorbed from the GI tract of dogs. It was reported that the absorption in dogs was 87% of the administered dose (Setnikar et al., 1986). In another study using labeled GlucN fed to rats, it was reported that the radioactivity found was not

due to GlucN, but was from chemical entities which changed over time (Setnikar et al., 1984).

A synergistic rather than an additive effect would be expected by combining glucosamine and chondroitin sulfate, since both agents are endogenous to chondrocytes (Hanson et al., 1997). The combination of the two was studied and was found able to decrease proteoglycan degradation (Orth et al., 2002). With increased popularity of CS, glucosamine and other "nutraceuticals" for treatment of joint problems, bioavailability of these compounds becomes even more important. A better understanding of the bioavailability of these products can alleviate confusion in the horse industry and provide much needed data on a new and exciting subject.

CHAPTER III

MATERIALS AND METHODS

Management of Animals

Six mature mares were used in a replicated 3x3 Latin square designed experiment. Their ages and weights ranged from 3-10 years and 472-557 kg, respectively. The horses were maintained at the Texas A&M University Horse Center following a protocol approved by the Institutional Agricultural Animal Care and Use Committee. Prior to the experiment, the horses were dewormed, vaccinated and their teeth were floated where necessary.

Experimental Diets

For each period of the Latin square, the horses were randomly assigned to one of three treatment groups, with two horses per group. The control diet (diet1) was fed at 1.5% of body weight and was balanced to meet or exceed NRC (1989) requirements for maintenance of mature horses. It contained a ratio of 40% bermudagrass hay and 60% concentrate[a]. When the horses were on the level one experimental diet (diet 2) they were administered a dose of 2.0g chondroitin sulfate and 5.5g glucosamine, top dressed on the control diet, at 7:00am and 7:00pm. When the horses were on the level two diet (diet 3) they received 3.5g chondroitin sulfate and 8.5g glucosamine fed at the same time and in the same manner as above. Chondroitin sulfate and glucosamine dosages were determined by review previous literature using these compounds fed in an oral form (Fenton et al., 1999; White et al., 2003; Conte et al., 1995; Baici et al., 1992).

[a] 12% Horse Feed, Producers Cooperative, College Station, Texas

Horses were put into individual stalls to be fed. They were allowed two hours to consume the meal, after which, any refusals were weighed back and discarded. Horses had free access to water. The chemical grade glucosamine-HCl used was obtained from Sigma Chemical Co., St. Louis, Missouri. The chemical grade chondroitin-4-sulfate used was obtained from CarboMer, Inc., Westborough, Massachusetts.

Sample Collection

In each treatment period, horses were managed in dry-lot pens during a 14-day diet adaptation period with ad libitum access to water. On day 15, blood samples were taken via indwelling jugular catheters. The blood was drawn from the catheter and injected into heparinized tubes and immediately taken to the lab to be spun down and separated. The plasma was then stored at -20°C until later analysis. Blood samples were taken on one day during each period around the feeding at -30min, 30min, 60min, 90min, 2 hr, 2.5hr, 3hr, 3.5hr, 4 hr, 4.5 hr, 5 hr, 5.5 hr, 6 hr, 7 hr, and 8 hr. GAGs are cleared from the circulation within 4 hours (Revell and Muir, 1972). The absorption of GlucN has been proven to be relatively fast with a T_{max} range of 1.1-1.6 h after oral treatments in dogs (Adebowale et al., 2002).

Analytical Methods

Chondroitin sulfate in the plasma was analyzed using a color reagent, dimethylmethylene blue (DMMB), followed by UV spectrophotometry (Baici et al., 1992). The DMMB assay for sulfated glycosaminoglycans (GAG) has found wide acceptance as a quick and simple method of measuring the sulfated GAG content of tissues and fluids (Farndale et al., 1986). Plasma samples were analyzed for glucosamine in the Laboratory for Protein Chemistry at Texas A&M University.

Chondroitin Sulfate Analyses

Preparation of Plasma

After centrifugation of non-coagulated blood, plasma was retrieved and frozen at -20°C until analyses. One mL of plasma was mixed with 0.94 mL of 20mM sodium phosphate buffer and 60 uL of papain solution (10mg/mL) in the same buffer to eliminate proteins. The sodium buffer contained 2mM EDTA and 2mM dithiothreitol at pH 6.8. The samples were incubated in a shaking water bath for 30 minutes at 45ºC, followed by 30 minutes at 55ºC and finally at 120 minutes at 62ºC. Cloudy samples were incubated further to eliminate as much protein as possible. After centrifugation for 30 minutes at 3000g, the supernatants were analyzed the same day using UV spectrophotometry (Baici et al., 1992). The assay was calibrated by use of blanks and standards containing 1 to 5μg of the same CS as fed to the horses.

Glycosaminoglycan assay

To detect chondroitin sulfate in the plasma, a DMMB reagent was used; the same DMMB reagent as described above. Two mL of the DMMB reagent was pipetted into a disposable polystyrene cuvette and was read at A_{525nm}. One hundred μL of sample was added and the sample was read again at A_{525nm} 15s after mixing (Baici et al., 1992).

Glucosamine Analysis

Plasma was obtained by centrifugation of non-coagulated blood and kept frozen at -20ºC until analysis. Plasma was treated with acetonitrile to precipitate most of the proteins. About 500 uL of the plasma/acetonitrile mixture were taken to the Texas A&M Protein Chemistry Lab for analyses.

Reagents

Glucosamine (GlucN) standards of 2ugm/mL to 1000ugm/mL GlucN, in water, were prepared. Norvaline was used as the internal standard for the assay. Free amino acid standards were obtained from Sigma Chemical Co. and Agilent, Palo Alto, California.

Standard and Sample Preparation

A duplicate set of standards between 2 and 8 ugm/mL GlucN were prepared for every assay. Five nanomoles of 24 amino acids found in serum were added to 200uL of GlucN standard to further simulate the conditions found in the serum. Additionally five nanomoles of internal standard were added to all samples and standards.

Samples were then spun in a centrifuge to further remove any precipitate before analyses, and a 200uL aliquot was taken per sample.

Method

All samples and standards were taken to dryness in a Savant Speed-Vac with radiant cover then reconstituted in 50uL of 0.4 M Borate buffer. One microliter of re-suspended sample was injected into the HPLC after automated pre-column derivitization with orthophthalaldehyde (OPA) in the presence of 3-mercaptopriopionic acid (3-MPA) to produce the isoindole derivative of the amino acids. This derivative is detected at 338 nm by an UV detector. The amino acids and GlucN were separated on a Hypersil C-18 column by gradient of a sodium acetate eluant. Chromatographic conditions to be used were identical to those used for routine amino acid analysis in the Texas A & M Protein Chemistry Lab except that the eluants contained a 3/4 strength Sodium Acetate concentration to allow separation in the middle of the chromatogram where Alanine, the

first anomer of GlucN and Arginine elute. (Eluant A: 14.5 mM sodium acetate, 0.05 mM EDTA with 180 uL TEA and 3 mLs THF per liter. Eluant B: 14.5 mM sodium acetate, 40% acetonitrile, 40% methanol, 20% water.) Derivitized amino acids were separated on a Hypersyl AA from Agilent.

To further confirm the accuracy of our GlucN analysis, spiked samples were analyzed. Different methods were used to spike the plasma samples. The first method included adding GlucN to the plasma before acetonitrile was added. The second method included adding GlucN after the acetonitrile was added to the plasma and the proteins were precipitated and removed.

Statistical Analyses

All data were analyzed by analyses of variance appropriate for the Latin square design, using STATA statistical software, Stata Corp, 2001. When necessary, means were further separated using a Fisher-Hayter means comparison test. Differences were considered significant at $P<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

Glucosamine

Calibration

Glucosamine elutes as a broad peak (Figure 1). The first and last peaks represent the alpha and beta conformers and the area between these two peaks represents the linear form of the molecule. While it is not possible to integrate the whole area under the curve due to the elution of arginine and taurine (Figure 2), it is possible to quantitate by either or both (simultaneously) of the anomeric peaks with linear results. In a blind study performed by the Texas A&M Protein and Chemistry Lab (PCL), spiked plasma samples were submitted for analysis in the range of 2 to 15 ugms/mL. These samples were analyzed using both the alpha and beta peaks, averaging the results. Recovery of the GlucN in the spiked samples averaged at 98% (95-100%) (Figure 2). This analysis resulted in very accurate results. Quantitation threshold was determined to be 1 ugm/mL being 8 times the signal to noise ratio for these conditions.

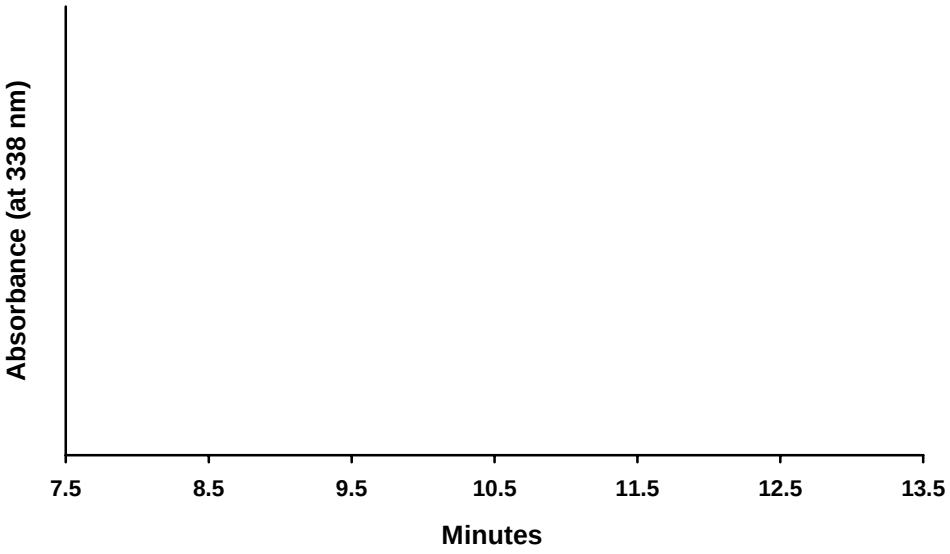

Figure 1. Elution of glucosamine

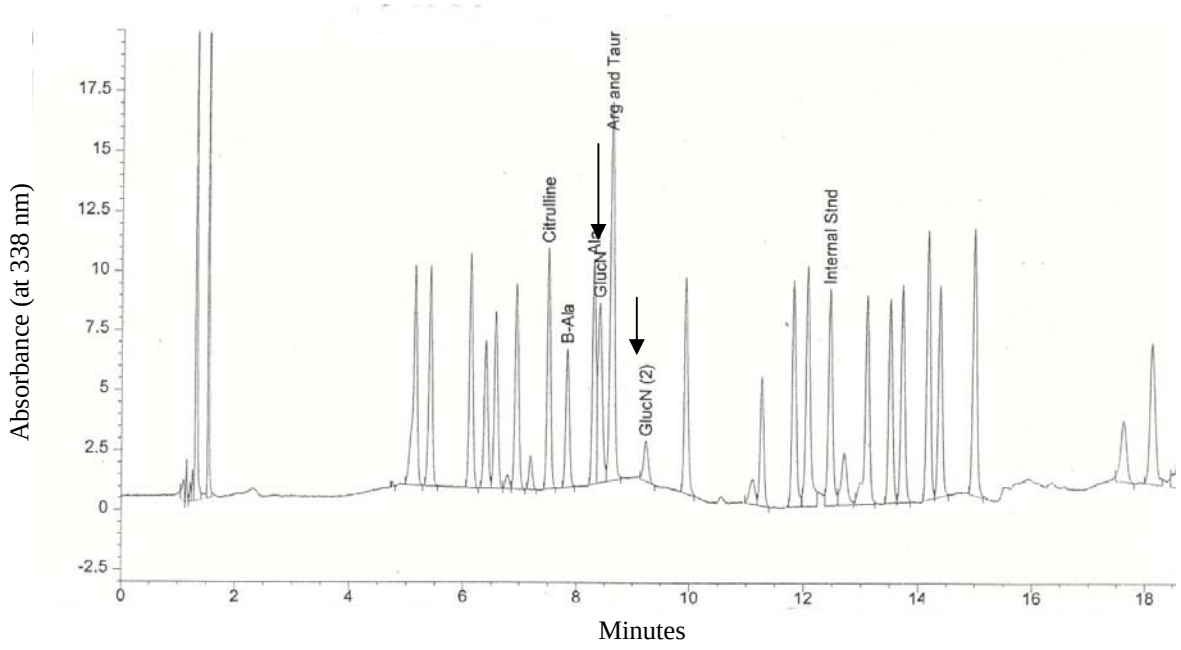

Figure 2. Chromatogram of horse plasma spiked with 10 μg/ml glucosamine-HCl

GlucN Results

In no case out of 250 samples was any glucosamine detected in the horse plasma (Figures 3-5). As shown in Figure 6, if glucosamine were present in the plasma samples, it would elute in two peaks. Previous studies on absorption of GlucN have much higher reported values than the current study. It is not clear whether the glucosamine molecule is absorbed in its entirety or is degraded prior to absorption (Brief et al., 2001). In the current study, only whole glucosamine was analyzed for in the blood. Previously it has been shown that GlucN from exogenous sources is incorporated into the metabolic pathway of GAG synthesis (Barclay et al., 1998). After entering the cells, GlucN may be phosphorylated to give glucosamine-1-phosphate and subsequently N-acetylated, yielding acetylglucosamine-1-phosphate (GlucNAc-1-P). N-acetylglucosamine is one of the intermediates in this GAG synthesis pathway. These events could possibly account for the fact that no free GlucN was detected in the blood (Dr. Lennart Roden, personal communication, 2004). In an experiment in which D-glucosamine was given to rats via stomach tube, it appears likely that GlucN is phosphorylated, converted to fructose-6-phosphate and metabolized (Kohn et al., 1962). Also in previous studies, the bioavailability of GlucN has been studied in rats, dogs and man using radio-labeled GlucN (Setnikar et al., 1986; Tesiore, 1972). In a study performed by Setnikar et al, in 1984, using labeled GlucN fed to rats, it was reported that the radioactivity found was not due to GlucN, but was from chemical entities, which changed over time.

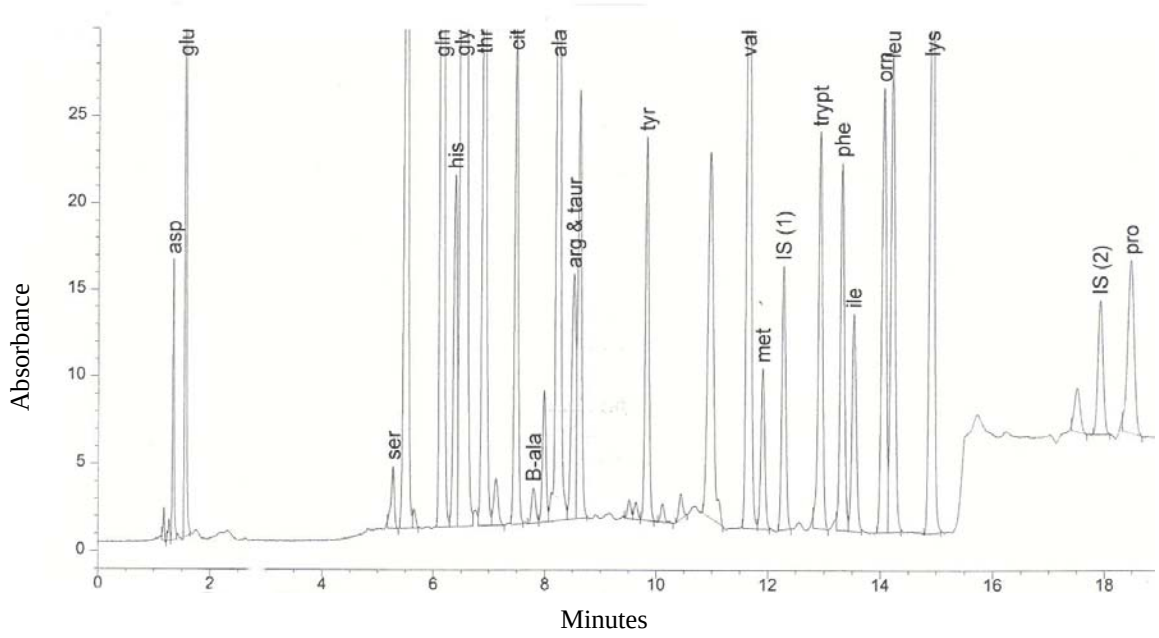

Figure 3. Chromatogram of plasma sample of horse 2C taken during period 1 at hour 4

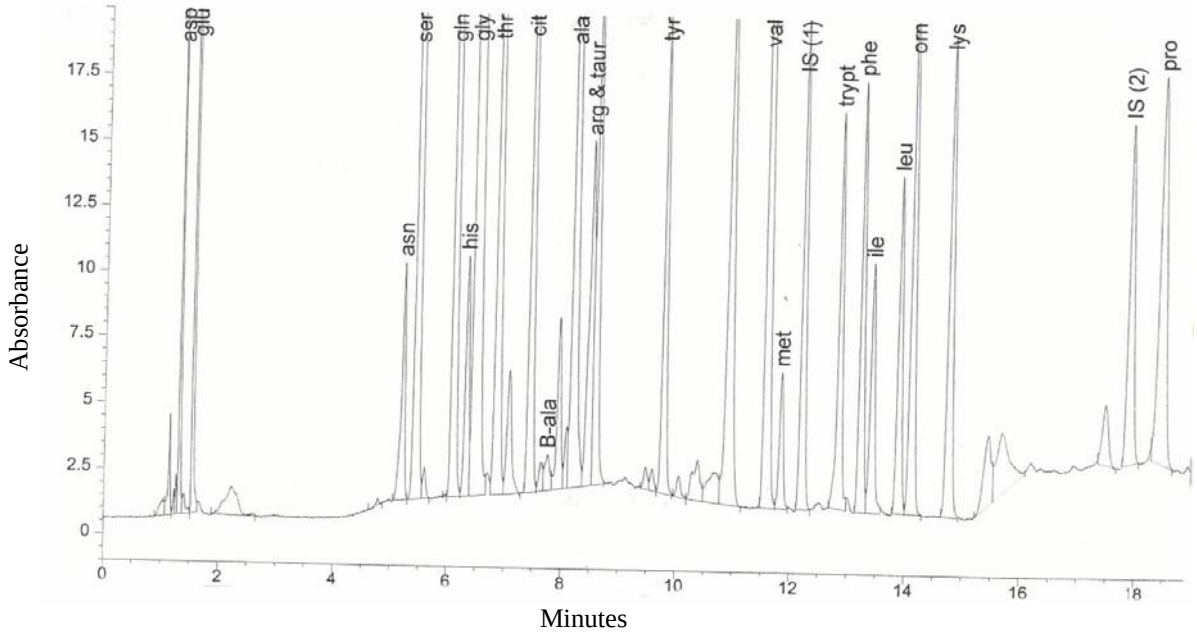

Figure 4. Chromatogram of plasma sample of horse 2C taken during period 2 at hour 4

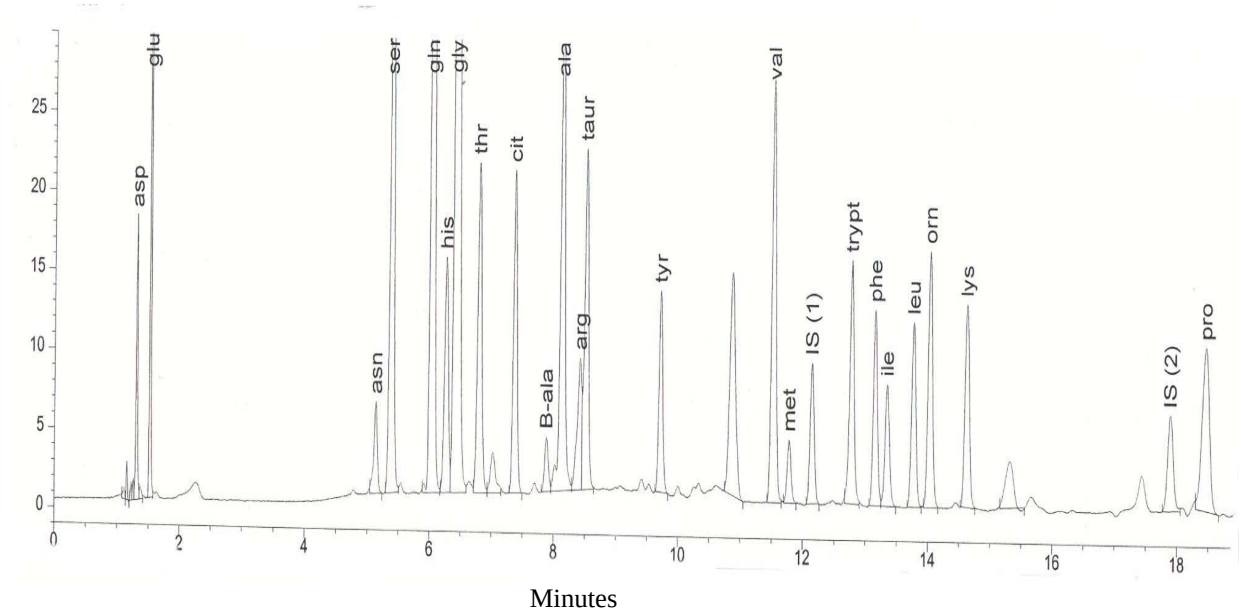

Figure 5. Chromatogram of plasma sample of horse 2C taken during period 3 at hour 4

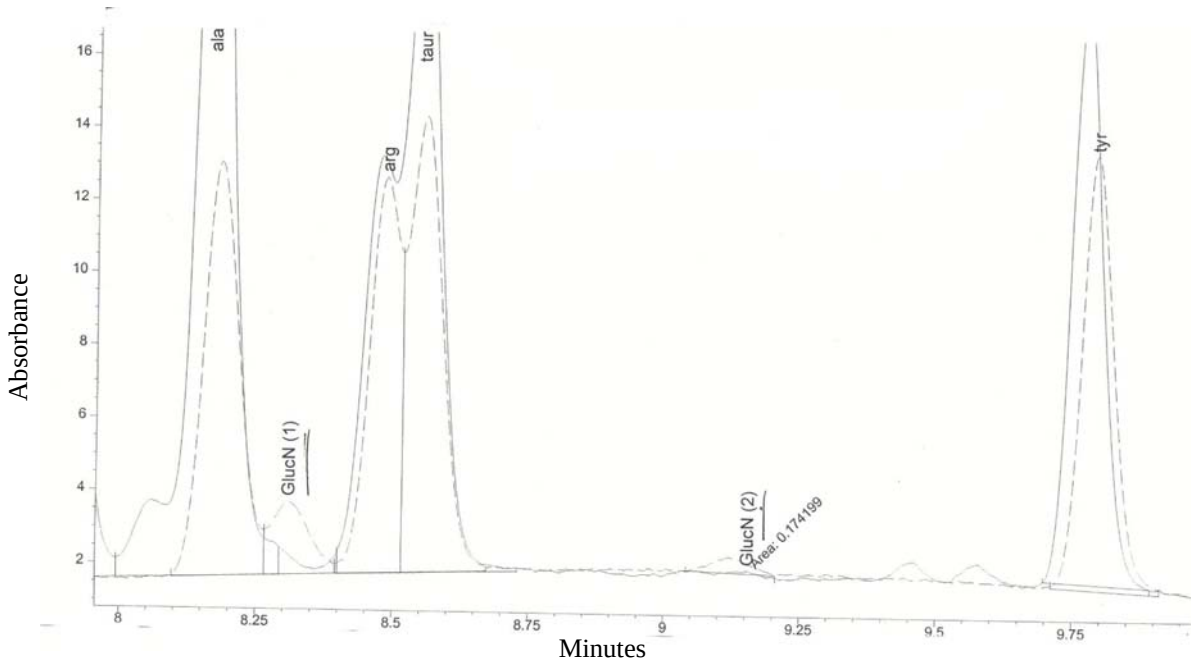

Figure 6. Sample of horse plasma overlayed with 10 μg/ml standard

Chondroitin Sulfate

Diet and Period Effects

There were no significant differences between treatments in the amount of CS found in the blood based on diet (Table 1). There was, however, a significant ($P<0.05$) period effect. Mean plasma concentrations of CS were significantly lower in period 3 than in the other periods (Table 2). Chondroitin sulfate concentrations were lower for all three diets during period 3 (Table 3). In order to further examine period effects, an ANOVA was done to examine any effects of the sequence in which horses received the treatments (Appendix 2). There was no noted sequence effect ($P<0.05$) indicating that the lower plasma concentrations of CS in period 3 were not due to adaptation.

Table 1. Mean Plasma Concentrations of Chondroitin Sulfate by Diet Over All Periods

	Chondroitin Sulfate	
	Mean (mg/mL)	SE
Control	62.4	6.7
Level 1Diet	79.9	7.4
Level 2 Diet	75.1	7.3
Total	72.2	4.1

Table 2. Mean Plasma Concentrations of Chondroitin Sulfate by Period Over All Treatments

	Chondrotin Sulfate	
	Mean (mg/mL)	SE
Period 1	81.5	7.4
Period 2	87.8	6.3
Period 3	48.4*	6.5
Total	72.2	4.1

*****Period 3 significantly different from other periods ($P<0.05$)

Table 3. Mean Plasma Concentrations of Chondroitin Sulfate by Diet and Period

	Chondroitin Sulfate	
	Mean (mg/mL)	**SE**
Period 1		
Diet 1	88.5	15.3
Diet 2	87.3	10.9
Diet 3	65.8	11.7
Total	81.5	7.4
Period 2		
Diet 1	68.9	8.3
Diet 2	99.6	14.4
Diet 3	96.8	9.1
Total	87.8	6.3
Period 3		
Diet 1	37.1	9.4
Diet 2	55.6	10.3
Diet 3	53.8	14.4
Total	48.4	6.5

Time Effects

To better visualize and further examine the change in chondroitin sulfate over time, the data points were normalized to baseline values. When the blood samples were taken following feeding, there was no noted significant ($P<0.05$) change in the blood concentration due to feeding CS (Appendix 3). Never at any point was there a significant increase in plasma CS levels over the baseline value during the eight hour sampling period post-feeding (Figure 7).

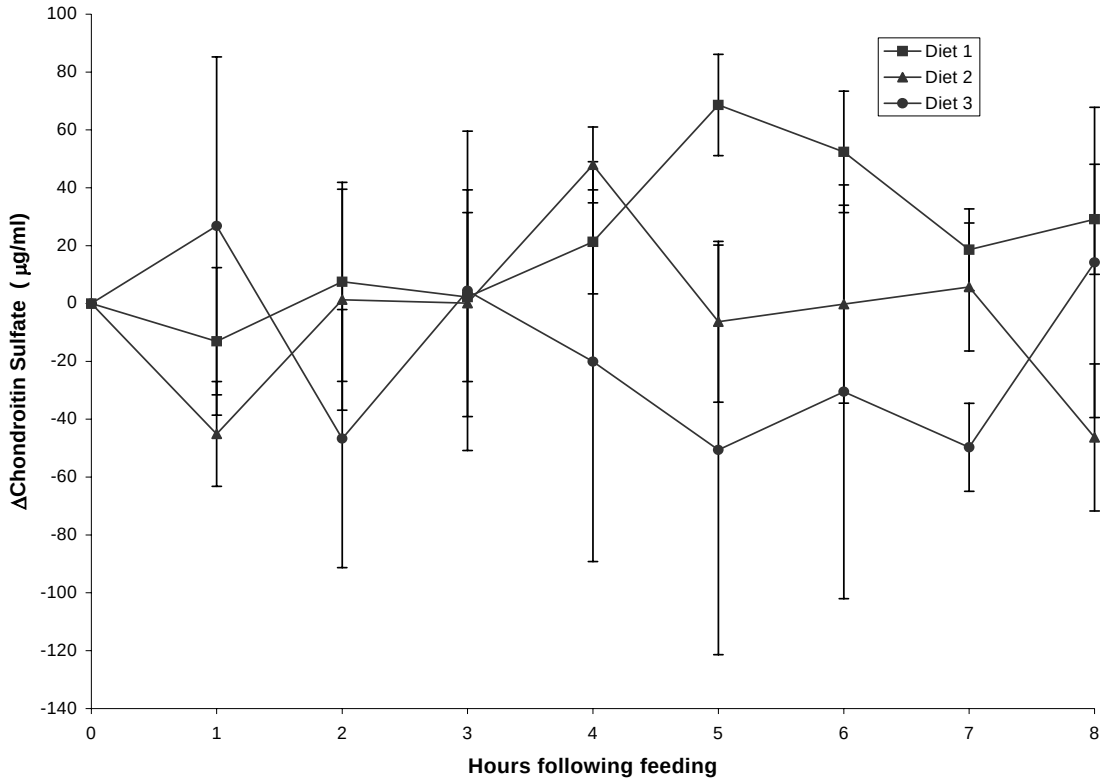

Figure 7. Postprandial curve – CS levels in plasma around feeding

Figure 7 illustrates that there was no significant postprandial response to feeding CS in diets 2 and 3. If chondroitin sulfate was present in the plasma after being fed, there should have been an increase in concentration of CS when horses were fed diets 2 and 3 compared to diet 1. The results obtained here seem logical when looking at the chemical make-up of chondroitin sulfate. Mass of CS can range from 14,000 Da to 30,000 Da. Its weight would suggest that absorption across the gastrointestinal mucosa, which contains a variety of GAG-degrading enzymes, is low (Eddington and White, 2001).

In the current study, there was no effect on plasma CS concentrations due to orally administering CS to horses. Previous studies have used radiolabeled chondroitin

sulfate and absorption results were based on recovery of radioactivity. Conte et al. (1995) reported over 70% absorption of label from CS when fed to rats and dogs. Total radioactivity in that study was based on the sum of exogenous chondroitin sulfate and of several labeled molecular species which derive from its depolymerization and catabolism (Conte et al., 1995). This study and one performed by Palmieri et al. (1990) used a labeling method that produces a chondroitin sulfate molecule with a single label at the reducing end of the molecule. This sort of labeling is not representative of the whole molecule, and measuring the radioactivity of any absorbed material actually means following the metabolic fate of the very last residue in the polymer backbone (Baici et al., 1992).

CHAPTER V

GENERAL DISCUSSION

With degenerative joint disease (DJD) and osteoarthritis posing such a major problem in the equine industry today, many different treatments, such as administering glucosamine (GlucN) and chondroitin sulfate (CS), have been designed to reduce pain and inflammation along with improving joint function. GlucN and CS are endogenous molecules to the equine system and are essential for proper cartilage formation and joint function (Brief et al., 2001). To fully elucidate the effects of oral forms of these products on joint health in animals such as horses, more information must first be known about their bioavailability.

Glucosamine

The pharmacokinetics of GlucN are difficult to investigate because it is an endogenous substance which is rapidly utilized by the body for the biosynthesis of other normal constituents (Adebowale et al., 2002). The majority of the previous studies conducted on animals involved feeding uniformly labeled GlucN. The use of radiolabeled GlucN to determine total radioactivity in biological fluids fails to detect presystemic metabolism in the gut or liver during absorption since drug and metabolites are not differentiated (Adebowale et al., 2002). In a previous study, it was stated that indirect evidence was available to support the fact that GlucN may be degraded by intestinal flora. It was also noted that their results did not exclude the possibility that some GlucN was converted to a readily absorbed degradation product (Capps et al., 1966). According to Setnikar et al. (1984) more than 81% of the administered

radioactivity was recovered in CO_2. This shows the high degree of metabolism of ^{14}C GlucN after oral administration (Setnikar et al., 1984). It has also been stated that glucosamine absorbed by the gastrointestinal tract undergoes significant first-pass metabolism in the liver (Barclay et al., 1998). As a result of hepatic metabolism, GlucN may be incorporated into plasma proteins (Setnikar et al., 1986; Setnikar et al., 1993). This along with the fact that systemic availability is always overestimated when radioactivity is used may explain the difference found between the amount of GlucN absorbed in this study compared to previous studies.

Chondroitin Sulfate

The actual metabolic uptake of orally administered chondroitin sulfate has been found to be inconsistent, possibly because of variation in the structure, biochemical properties, and molecular weights of the various preparations (Brief et al., 2001). According to Dohlman, (1956) CS is degraded in the gastrointestinal tract and no intact CS can be absorbed through the intestinal wall. It is known that when given intravenously, CS is degraded mainly if not solely by the liver. The sulfate groups are lost as inorganic sulfate (Baici et al., 1992; Wood et al., 1973; Revell and Muir, 1972). The question that remains is whether CS makes it out of the gut to the liver to be degraded. One study was conducted to investigate the impact of oral chondroitin sulfate on the concentration of glycosaminoglycans in human serum. It was found that CS was not absorbed either in an intact form or as a sulfated oligosaccharide and therefore, did not produce any measurable change in the total serum concentration of glycosaminoglycans (Baici et al., 1992). The authors of this study concluded that the

theory that orally administered CS alone offers chondroprotection is biologically and pharmacologically unfounded (Brief et al., 2002).

To offer an explanation of why the present study did not reveal a postprandial curve when measuring whole CS after it was fed orally, first it is important to understand the metabolism of CS as it travels through the gastrointestinal tract (Lualdi, 1993). In its native form in the cartilage, CS is part of a huge proteoglycan core protein with up to 100 chondroitin sulfate chains attached. Many of these macromolecules are bound noncovalently to hyaluronan to yield complexes with a mass of up to 200 million daltons (Dr. Lennart Roden, personal communication, 2004). The intestinal epithelium is a highly effective barrier, which hinders the diffusion of a wide variety of high molecular mass compounds, such as CS. It has been shown that in humans, CS is not absorbed in the intestine, but the bacteria of the large intestine utilize CS as an energy source (Salyers, 1979). These microorganisms metabolize CS by the action of three enzymes. First, a periplasmatic CS-lyase releases unsaturated disaccharides from the CS polymer. Next, the disaccharide is desulfated by an intracellular sulfatase, and finally an intracellular glucuronidase splits the disaccharide into monomers. The monomers are then used as an energy source and no evidence has been found for the production of CS fragments larger than the disaccharide (Salyers and O'Brien, 1980). This information offers enough evidence to assert that neither intact, nor depolymerized CS is absorbed by the mammalian gastrointestinal tract (Lualdi, 1993).

The possibility that low molecular mass desulfated oligomers and monomers may be produced from the breakdown of CS cannot be ruled out on the basis of this sudy. Without the existence of a polymer chain and the presence of sulfate groups, CS would

not retain the biochemical and biological properties attributed to the intact molecule (Baici et al., 1992). Because all of the claims regarding pharmacological effects of CS refer to the properties of this molecule as a whole molecule, not to the properties of any degradation product, any direct action of orally administered CS on cartilage and chondrocytes is not possible (Lualdi, 1993).

Many unanswered questions remain surrounding the most effective dosage and route along with any actual beneficial effects of glucosamine and chondroitin sulfate. GlucN and CS, when administered in a pure bioavailable form, have been show to be safe (Adebowale et al., 2000). When fed to rats at the high level of 5g/kg body weight, they proved to be non-toxic (Davidson, 2000). A well-designed prospective study of the metabolism of these products as they travel through the gastrointestinal tract of the equine needs to be conducted.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Many compounds are commercially available which claim to “improve joint health”. This study was conducted to further elucidate unanswered questions surrounding “nutraceuticals”, specifically glucosamine and chondroitin sulfate. Six mature horses were fed diets with pure GlucN and CS top dressed on their feed. Blood samples were taken and appropriate analyses were run to determine if either whole GlucN or whole CS was present or increased in the blood due to oral dosing.

When feeding pure GlucN to mature horses at two different amounts, there was no detectable GlucN measured in the plasma. These data are obviously different than previous reports, but a lot of questions about the exact fate of exogenous GlucN in the equine gastrointestinal tract remain to be answered.

Chondroitin sulfate is a molecule known to be endogenous to horses. When horses were fed an exogenous source of CS, plasma samples contained no significant increase in amounts of CS to produce a postprandial curve. It is well documented in the literature that the mammalian gastrointestinal tract is not suited for allowing whole CS to be absorbed.

Due to the lack of significant amounts of either GlucN or CS in the blood, this study does not support the theory that orally dosed GlucN and CS are readily available to the joints through the bloodstream. With the enormous impact that “nutraceuticals” are having on the equine industry, it is important to know whether resources are being used efficiently and effectively. The ability to lessen the effects of DJD and osteoarthritis are

important. However, more research is needed to determine if oral products containing glucosamine and chondroitin sulfate are actually efficacious in that regard.

LITERATURE CITED

Adebowale, A.O., D.S. Cox, Z. Liang and N.D. Eddington. 2000. Analysis of glucosamine and chondroitin sulfate content in marketed products and the caco-2 permeability of chondroitin sulfate raw materials. Journal of the American Nutraceutical Association. 3: 37-33-44.

Adebowale, A., J. Du, Z. Liang, J.L. Leslie and N.D. Eddington. 2002. The bioavailability and pharmacokinetics of glucosamine hydrochloride and low molecular weight chondroitin sulfate after single and multiple doses to beagle dogs. Biopharmaceutics and Drug Disposition. 23: 217-225.

Andermann, G. and M. Dietz. 1982. The influence of the route of administration on the bioavailability of an endogenous macromolecule: chondroitin sulfate (CSA). Eur J Drug Metab Pharmacokinet. 7: 11-16.

Anderson, M.A., M.R. Slater and T.A. Hammad. 1999. Results of a survey of small animal practitioners on the perceived clinical efficacy and safety of an oral nutraceutical. Preventative Veterinary Medicine. 38: 65-74.

Baici, A., D. Horler, B. Moser, H.O. Hofer, K. Fehr and F.J. Wagenhauser. 1992. Analysis of glycosaminoglycans in human serum after oral administration of chondroitin sulfate. Rheumatology International. 12: 81-88.

Barclay, T.S., C. Tsourounis and G.M. McCart. 1998. Glucosamine. The Annals of Pharmacotherapy. 32: 574-579.

Brief, A., S. Maurer, and P. Di Casare. 2001. Use of glucosamine and chondroitin sulfate in the management of osteoarthritis. Journal of the American Academy of Orthopedic Surgery. 9: 71-78.

Capps, J.C., M.R. Shetlar, and R. Bradford. 1966. Hexosamine metabolism I. The absorption and metabolism, in vivo, of orally administered D-glucosamine and N-Acetyl-D-glucosamine in the rat. Biochimica et Biophysica Acta. 127: 194-204.

Chasseaud, L.F., B.J. Fry, V.H. Saggers, I.P. Sword and D.E. Hathway. 1972. Studies on the possible absorption of a sulphated glycopeptide (GLPS) in relation to its mode of action. Biochemical Pharmacology. 21: 3121-3130.

Conte, A., M. Bernardi, L. Palmierei, P. Lualdi, G. Mautone and G. Ronca. 1991. Metabolic fate of exogenous chondroitin sulfate in man. Arzneimittelforschung/Drug Research. 41: 768-772.

Conte, A., N. Volpi, L. Palmieri and G. Ronca. 1995. Biochemical and pharmacokinetic aspects of oral treatment with chondroitin sulfate. Arzneimittelforschung/Drug Research. 45: 918-925.

Davidson, G. 2000. Glucosamine and chondroitin sulfate. Compendium on Continuing Education for the Practicing Veterinarian. 22:454-458

Davis, W.M. 1998. The role of glucosamine and chondroitin sulfate in the management of arthritis. Drug Topics. April(Suppl): 35-135.

Dohlman, C.H. 1956. The fate of the sulfate group of chondroitin sulfate after administration to rats. Acta Physiologica Scandinavica. 37: 220-234.

Eddington, N.D., and N. White. 2001. Evidence of the oral absorption of chondroitin sulfate as determined by total disaccharide content after oral and intravenous administration to horses. American Association of Equine Practitioners Proc. 47:326-328

Farndale, R., D. Buttle, and A. Barrett. 1986. Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue. Biochimica et Biophysica Acta. 883: 173-177.

Fenton, J.I., M.W. Orth, K.A. Chlebek-Brown, B.D. Nielson, C.D. Corn, K.S. Waite and J.P. Caron. 1999. Effect of longeing and glucosamine supplementation on serum markers of bone and joint metabolism in yearling quarter horses. Canadian Journal of Verterinary Research. 63: 288-291.

Fenton, J.I., K.A. Chlebek-Brown, J.P. Caron and M.W. Orth. 2002. Effect of glucosamine on interleukin-1-conditioned articular cartilage. Equine Veterinary Journal, Supplement. 34: 219-223.

Grabowski, P.S., A.J. England, R. Dykhuizen, M. Copland, N. Benjamin, D. Reid and S.H. Ralston. 1996. Elevated nitric oxide production in rheumatoid arthritis. Arthritis and Rheumatology. 39: 643-647.

Hanson, R.R. 1996. Oral glycosaminoglycans in treatment of degenerative joint disease in horses. Equine Practice. 18:18-22

Hanson, R.R., L. Smalley, G.K. Huff, S. White and T. Hammad. 1997. Oral treatment with a glucosamine-chondroitin sulfate compound for degenerative joint disease in horses: 25 cases. Equine Practice. 19:16-22.

Johnston, S.A. 1997. Osteoarthritis: Joint anatomy, physiology and pathobiology. Veterinary Clinics of North America: Small Animal Practitioners. 27: 703-705

Kohn, P., R.J. Winzler and R.C. Hoffman. 1962. Metabolism of D-glucosamine and N-Acetyl-D-glucosamine in the intact rat. The Journal of Biological Chemsitry. 237:304-308.

Lualdi, P. 1993. Bioavailability of oral chondroitin sulfate. Rheumatology International. 13: 39-43.

MacLeod, A. 2001. Use of Cosequin for the treatment of degenerative joint disease in a 7-year-old Appaloosa. Compendium on Continuing Education for the Practicing Veterinarian. 23: 842-847.

May, S.A., P. Lees, R. Hooke, K. Peremans and F. Verschooten. 1994. Prostagladin E2 in equine joint disease. Vlaams Diergeneesk. Tijdschr. 63: 187-191.

McLaughlin, R. 2000. Management of chronic osteoarthritic pain. Veterinary Clinics of North America: Small Animal Practicioners. 30: 933-949.

Moore, M.G. 1996. Promising responses to a new oral treatment for degenerative joint disorder. Canine Practice. 2: 7-11.

NRC. 1989. Nutrient Requirements of Horses (5^{th} Ed.) National Academy Press, Washington, D.C.

Orth, M.W., T.L. Peters and J.N. Hawkins. 2002. Inhibition of articular cartilage degradation by glucosamine HCl and chondroitin sulfate. Equine Veterinary Journal, Supplement. 34: 224-229.

Palmieri, L., A. Conte, L. Giovannini, P. Lualdi and G. Ronca. 1990. Metabolic fate of exogenous chondroitin sulfate in the experimental animal. Arzneimittelforschung/Drug Research. 41: 768-772.

Ramey, D.W., N. Eddington, E. Thonar and M. Lee. 2002. An analysis of glucosamine and chondroitin sulfate content in oral joint supplement products. Journal of Equine Veterinary Science. 22:125-127

Revell, PA and H. Muir. 1972. The excretion and degradation of chondroitin 4-sulphate administered to guinea pigs as free chondroitin sulfate and as proteoglycan. Journal of Biochemistry. 130: 597-606.

Ronca, F., L. Palmieri, P. Panicucci and G. Ronca. 1998. Anti-inflammatory activity of chondroitin sulfate. Osteoarthritis and Cartilage. 6(Supplement): 14-21.

Salyers, A. 1979. Energy sources of major intestinal fermentative anaerobes. American Journal of Clinical Nutrition. 32: 158-163.

Salyers, A. and M. O'Brien. 1980. Cellular location of enzymes involved in chondroitin sulfate breakdown by *Bacteriodes thetaiotaomicron*. Journal of Bacteriology. 143: 772-780.

Schanker, L.S., D.J. Tocco, B.B. Brodie and C. Hogben. 1958. Absorption of drugs from the rat small intestine. Journal of Pharmacology and Experimental Therapeutics. 123: 81-88.

Setnikar, I., C. Giacchetti and G. Zanolo. 1984. Absorption, distribution and excretion of radioactivity after a single intravenous or oral administration of [14C] glucosamine to the rat. Pharmatherapeutica. 3: 538-550.

Setnikar, I., C. Giacchetti and G. Zanolo. 1986. Pharmacokinetics of glucosamine in the dog and in man. Arzneimittel-Forschung. Drug Research. 36:729-735

Tesoriere, G. 1972. Intestinal absorption of glucosamine and N-Acetylglucosamine. Experientia. 28:770-771.

White, G.W., T. Strites, E.W. Jones and S. Jordan. 2003. Efficacy of intramuscular chondroitin sulfate and compounded Acetyl-d-glucosamine in a positive controlled study of equine carpitis. Journal of Equine Veterinary Science. 23:295-300.

Wood, K.M., F.S. Wusteman and C.G. Curtis. 1973, The degradation of intravenously injected chondroitin 4-sulphate in the rat. Journal of Biochemistry. 134: 1009-1013.

APPENDIX

1. ANOVA Table for Chondroitin Sulfate in the Blood – Overall Diet and Period Effects

Source	df	Partial SS	MS	F-value	P-value
Total	132	297754.3290	2255.7146		
Model	8	59019.9151	7377.4894	3.8300	0.0005
Error	124	238734.4140	1925.2775		
Diet	2	5711.9961	2855.9981	1.4800	0.2309
Period	2	41259.2987	20629.6494	10.7200	0.0000
Diet*Period	4	9362.6296	2340.6574	1.2200	0.3075

2. ANOVA Table for Chondroitin Sulfate in the Blood- Overall Diet, Period and Time Effects

Source	df	Partial SS	MS	F-value	P-value
Total	132	297754.3290	2255.7146		
Model	32	123120.3710	3847.5116	2.2000	0.0016
Error	100	174633.9580	1746.3396		
Diet	2	5761.2211	2880.6105	1.6500	0.1973
Period	2	40866.2953	20433.1477	11.7000	0.0000
Time	8	21532.4499	2691.5562	1.5400	0.1526
Diet*Time	16	41587.4186	2599.2137	1.4900	0.1189
Diet*Period	4	11104.2289	2776.0572	1.5900	0.1829

3. ANOVA Table for Crossover Effects

Source of Variation	df	Partial SS	MS	F-value	P-value
Intersubjects					
Sequence effect	2	46.37	23.19	0.9500	0.4778
Error	3	72.89	24.30	1.2700	0.2880
Intrasubjects					
Treatment effect	2	76.55	38.27	2.0000	0.1398
Period effect	2	431.78	215.89	11.2800	0.0000
Error	123	2354.44	19.14		
Total	132	2977.54			

4. ANOVA Table for CS concentrations in the blood – time effects by diet

Diet 1

Source	df	Partial SS	MS	F-value	P-value
Total	46	95956.6194	2086.0135		
Model	8	16490.1602	2061.2700	0.9900	0.4622
Error	38	79466.4591	2091.2226		
Diet	2	16490.1602	2061.2700	0.9900	0.4622

Diet 2

Source	df	Partial SS	MS	F-value	P-value
Total	43	103609.188	2409.516		
Model	8	24270.9159	3033.8645	1.34	0.2576
Error	35	79338.2722	2266.8078		
Diet	8	24270.9159	3033.8645	1.34	0.2576

Diet 3

Source	df	Partial SS	MS	F-value	P-value
Total	41	90678.1942	2211.6633		
Model	8	22416.9016	2802.1127	1.35	0.2524
Error	33	68261.2929	2068.524		
Diet	8	22416.9013	2802.1127	1.35	0.2524

5. Normalized Mean Table Separated by Diet and Time

Diet 1

Time		Mean	SE
0		0.0000	0.0000
1		-13.0950	25.5474
2		7.5092	34.3538
4		2.2387	29.2316
4		21.3342	18.0408
5		68.6460	17.4979
6		52.4348	20.9948
7		18.6467	14.0895
8		29.1475	18.9774
Total		18.848	8.0475

Diet 2

Time		Mean	SE
0		0.0000	0.0000
1		-45.1250	18.1041
2		1.3060	38.1927
4		0.0837	39.2345
4		47.8600	13.1286
5		-6.3050	27.7502
6		-0.2413	34.2838
7		5.6500	22.0500
8		-46.2567	25.3972
Total		-3.6689	9.2311

Diet 3

Time		Mean	SE
0		0.0000	0.0000
1		26.8.5	58.3850
2		-46.6875	44.5575
4		4.3750	55.1950
4		-20.1375	69.0675
5		-50.5975	70.7875
6		-30.5225	71.5025
7		-49.6800	15.1800
8		14.2250	53.5550
Total		-16.9133	14.7625

VITA

Courtney Ann Welch is the daughter of James and Beverly Drost, the wife of Brandon Welch and the mother of Kathryn Ann Welch.

Following graduation from Yoakum High School in Yoakum, Texas, Courtney attended Texas A&M University. At Texas A&M, she received her B.S. in animal science, graduating Magna Cum Laude in May 2001. Courtney entered graduate school in August 2001 under Dr. Gary Potter at Texas A&M University to earn a Master of Science degree in animal science. Her graduate career has involved research in the field of equine nutrition. While at Texas A&M, Courtney has served as a research and teaching graduate assistant.

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