University of Alberta

Absorption and Bioavailability of Glucosamine in the Rat

By

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To my beloved country

Egypt

May Allah protect it

ABSTRACT

Glucosamine (GlcN) is an amino monosaccharide that is widely used as a food supplement in the treatment of osteoarthritis (OA). *In vitro* and animal studies strongly support the therapeutic efficacy of the compound; however, clinical reports and meta-analysis are inconclusive. As compared to the concentration used to assess GlcN efficacy *in vitro* or in animal models, the maximum plasma concentration of 0.3-2 μg/ml typically seen following the recommended human oral dose of 1500 mg/day is sub-therapeutic. This is mainly due to the low oral bioavailability of GlcN.

The objectives of this thesis were to investigate the absorption kinetics of GlcN and to determine the different factors that may contribute in decreasing GlcN gut availability. We were able to improve a simple sensitive HPLC assay of GlcN in human and rat plasma with a lower limit of quantification (LLOQ) of 50 ng/mL. The method was further applied in our study.

In the current thesis we revealed that capacity-limited intestinal absorption was not behind the low oral bioavailability of GlcN, as both *in vivo* and *in vitro* results demonstrated linear absorption kinetics. GlcN intestinal absorption was found independent of glucose levels and food co-administration. The glucose transporter (GLUT2) is involved in GlcN intestinal absorption; however, passive diffusion may co-exist. Our *in vitro* studies confirmed that GlcN is absorbed throughout the intestinal tract with the highest permeability from the duodenum. An average of 14.52 ± 6% could not be accounted for in the mass-balance determination after GlcN was incubated with the everted rat segments, indicating

that part of the administered dose is either degraded or utilized by the intestinal tissue. Moreover, treating the rats with antibiotics prior to GlcN administration led to a pronounced increase in the compound bioavailability accompanied by a significant increase in the percent of the oral dose excreted unchanged in the rat feces ($p < 0.05$), from $0.11 \pm 0.15\%$ in the control rat to $11.18 \pm 4.9\%$ in the antibiotic treated rats. This points to a significant degradation by the intestinal flora that may at least in part explain the low oral bioavailability of GlcN.

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LIST OF ABBREVIATION AND SYMBOLS

AA	Adjuvant arthritis
AAOS	American Academy of Orthopedic Surgeons
ACR	American College of Rheumatology
ADAM	1-Aminoadmantan
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under plasma concentration-time curve
BBB	Blood brain barrier
$CaCl_2$	Calcium chloride
CL	Clearance
CL_r	Renal clearance
Cmax	Maximum plasma concentration
CO_2	Carbon dioxide
COX	Cyclooxygenase
CS	Chondroitin sulphate
CV%	Coefficient of variation
CYP	Cytochrome P450
DMARD	Disease modifying anti-rheumatic drugs
DMSO	Dimethylsulfoxide
F	Bioavailability
FDA	Food and Drug Administration
FMOC-Cl	9-Fluorenylmethoxycarbonyl chloride

Fr	Fraction of dose eliminated by renal route
Fructose-6-P	Fructose-6-phosphate
GAGs	Glycosaminoglycans
GFAT	Glutamine:fructose-6-phosphate amidotransferase
GAIT	Glucosamine/chondroitin Arthritis Intervention Trial
GIT	Gastrointestinal tract
GlcN	Glucosamine
GlcN-6-P	Glucosamine-6-phosphate
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GLUT	Glucose transporters
GNPDA	Glucosamine-6-phosphate deaminase
HA	Hyaluronic acid
HBP	Hexosamine biosynthetic pathway
HCQ	Hydroxychloroquine
HPLC	High performance liquid chromatography
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
INR	International normalized ratio
IL	Interleukin
IL-1β	Interleukin-1-beta
iNOS	Inducible nitric oxide

IS	Internal standard
JSW	Joint space width
K	Elimination rate constant
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
Km	Substrate concentration at half the maximum velocity
Kr	Renal elimination rate constant
LC	Liquid chromatography
LC- MS/MS	Liquid chromatography-mass spectroscopy
LLOQ	Lower limit of quantification
LPS	Lipopolysaccharide
$MgSO_4$	Magnesium sulphate
mM	Millimolar
MMPs	Matrix metalloproteinase
mL	Milliliter
mRNA	Messenger ribonucleic acid
MSM	Methylsulfonylmethane
MTX	Methotrexate
MW	Molecular weight
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide

NIDDM	Non insulin dependent diabetes mellitus
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OGT	O-linked GlcNAc transferase
PAI	Plasminogen activator inhibitor
PEG	Polyethylene glycol
PEG_2	Prostaglandin E_2
RA	Rheumatoid arthritis
SGLT	Sodium/glucose co-transporters
SD	Standard deviation
SSZ	Sulfasalazine
Tmax	Time to reach maximum concentration
TNF-α	Tumor necrosis factor-alpha
TP	Total protein
$t_{1/2}$	Half life
UDP-GlcNAc	Uridine diphosphate-N-acetyl-glucosamine
UDP-GalNAc	Uridine diphosphate-N-acetyl galactosamine
µg	Microgram
µM	Micromolar
u-PA	Urokinase plasminogen activator
UV	Ultraviolet
Vd	Volume of distribution

Vdss	Volume of distribution at steady state
Vmax	The maximum velocity of a transporter or enzyme
WBC	White blood cell count
WOMAC	Western Ontario and McMaster Universities Osteoarthritis index

CHAPTER 1

Introduction

Glucosamine (GlcN) is an amino monosaccharide widely used as a dietary supplement for treatment of osteoarthritis (OA). GlcN is endogenously formed in the body from glucose through a minor metabolic pathway known as the hexosamine biosynthetic pathway (HBP). This pathway converts fructose-6-phosphate (fructose-6-P) to GlcN-6-phosphate (GlcN-6-P), which then enters into several metabolic cascades leading to the formation of proteoglycan, glycoprotein and glycolipids. Proteoglycans or glycosaminoglycans (GAGs) is the main constituent of the extracellular matrix of the cartilage; hence, GlcN is considered the main building block for cartilage formation (Anderson *et al.*, 2005). It is assumed that administering GlcN to OA patients can help regenerate cartilage and restore normal joint function by stimulating GAG production.

GlcN is not normally present in diet; it is present in the shells of shellfish, animal bones and bone marrow. Exogenous GlcN (sulphate and hydrochloride salts) is mainly obtained by acid or enzymatic hydrolysis from chitin present in the shell of the shellfish (Miller & Clegg, 2011). Vegetarian GlcN (shellfish-free GlcN) is the hydrochloride salt of the compound obtained from corn fermentation, and is believed to be safe for people allergic to shellfish.

GlcN is available in the marketplace as GlcN sulphate, GlcN hydrochloride (GlcN HCl) and N-acetylglucosamine (GlcNAc) (Figure 1.1). It is usually formulated alone or in combination with chondroitin sulphate (CS) and/or methylsulfonylmethane (MSM). It is mainly administered orally in the form of

tablets, caplets, capsules, or powder; however, intra-articular and transdermal preparations are also available. The recommended oral dose is 1500 mg/day taken once or divided into three equal doses. GlcN sulphate is highly hygroscopic and easily degraded, which requires stabilization by co-crystallization with sodium or potassium salts. Crystalline GlcN sulphate sodium chloride (Dona®, Rotta Pharmaceutics, Monza, Italy), is the original form of GlcN that has been studied thoroughly for its clinical efficacy. In these tablets, GlcN exists with sulphate, sodium and chloride in a stoichiometric ratio of 2:1:2:2 (Herrero-beaumont & Rovati 2006).

The compound is thought to improve OA by enhancing the production of GAG and helping to regenerate normal cartilage as suggested by Jason Theodasakis, in his famous book (The arthritis cure) that published in 1997 and arouse the attention about GlcN (Theodosakis, 1997). Nevertheless, recent studies indicated that GlcN has anti-inflammatory activity that mediates its therapeutic benefits for treating OA and, at the same time, makes it a suitable candidate to treat other inflammatory disorders (Chan *et al.*, 2005a; Gouze *et al.*, 2006; Kim *et al.*, 2007). GlcN may be useful in rheumatoid arthritis (RA), as administration of 1500 mg GlcN HCl to RA patients for 12 weeks was able to improve the symptoms to an acceptable level compared to the placebo group (Nakamura *et al.*, 2007). In rat model of adjuvant arthritis, daily administration of 300 mg/kg GlcN, significantly ($p < 0.05$) inhibited the progression of arthritis (Hua *et al.*, 2005). GlcN can be useful in systemic inflammatory complications including inflammation–associated aortic lesions and atherosclerosis (Duan *et al.*,

2005; Largo *et al.*, 2009; Wu *et al.*, 2010). It also showed a neuroprotective activity in a rat middle cerebral artery occlusion model via its anti-inflammatory properties (Hwang *et al.*, 2010). Both GlcN and GlcNAc showed a promising activity in treating inflammatory bowel disease as Crohn's and ulcerative colitis (Russell, 1999; Salvatore *et al.*, 2000). Moreover, it is reported that GlcN can improve cardiac function and organ perfusion, and reduce circulatory inflammatory cytokines if administered intravenously during resuscitation after trauma hemorrhage (Yang *et al.*, 2006).

GlcN is a highly promising medication. Its anticancer activity was originally reported by Quastel and Cantero, who noticed the ability of D-GlcN to inhibit tumor growth (Quastel & Cantero, 1953). Subsequent *in vitro* studies confirmed the cytotoxic activity of GlcN in different experimental tumors with little harmful effect on the normal host cells (Bekesi & Winzler, 1969; Bekesi & Winzler, 1970). Concurrent administration of membrane active drugs (local anesthetics) was able to enhance GlcN cytotoxicity (Friedman & Skehan, 1980). Recent studies showed that GlcN at a concentration of 2 mM can induce cell cycle arrest and stimulate apoptosis on human prostate cancer DU145 and PC3 cell lines through inhibition of STAT3 signaling (Chesnokov *et al.*, 2009). The anticancer activity was also confirmed *in vivo*. The infusion of GlcN for 40 h in rats with Walker 256 carcinoma led to a complete necrosis of tumor cells and the regeneration of the fine structure of parenchymal cells of the rat liver and kidney (Molnar & Bekesi, 1972). Orally administered GlcN HCl in a dose of 250 mg/kg significantly inhibited Sarcoma 180 tumor growth and promoted T-lymphocyte

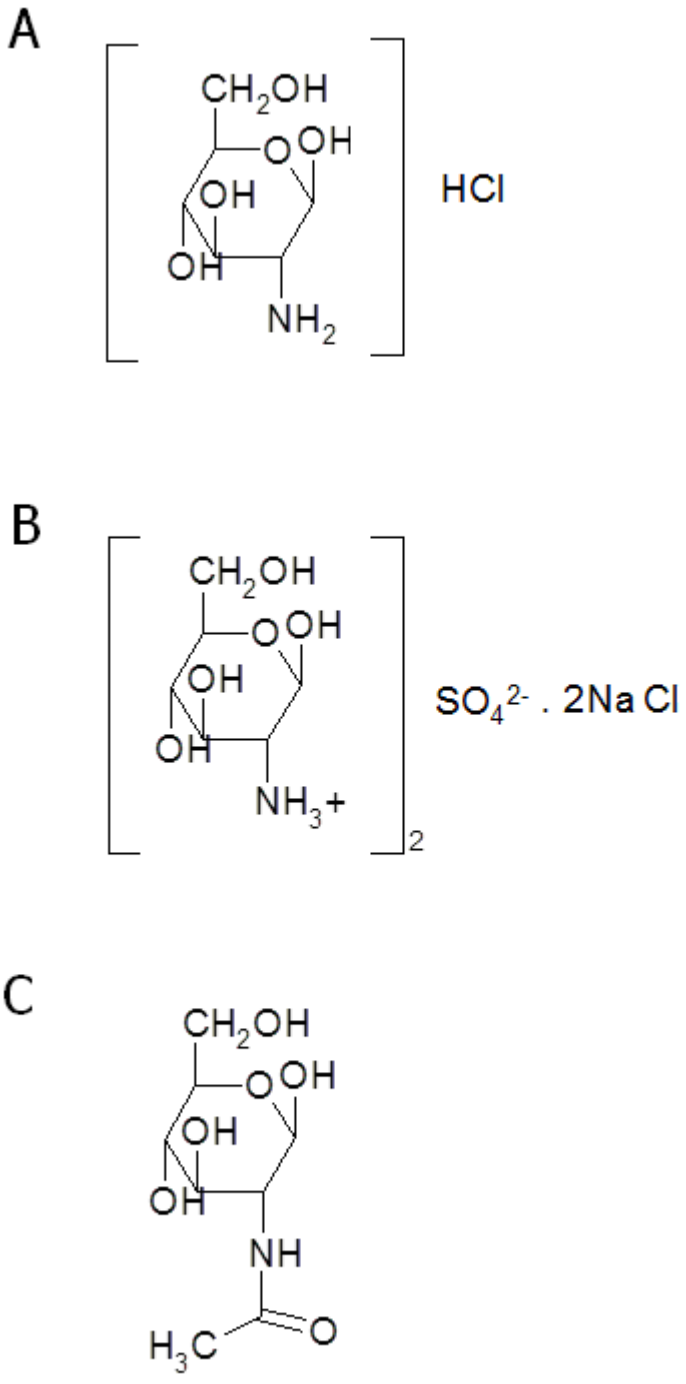


Fig 1.1. Chemical structures of the different forms of GlcN available in the market. GlcN HCl (A); Crystalline GlcN sulphate (B); and N-acetylGlcN (C).

proliferation, thymus index, and spleen index in Kunming male mice (Zhang *et al.*, 2006a). Moreover, because of its anti-inflammatory properties, administering GlcN regularly was able to decrease the risk of lung and prostate cancer (Tsai *et al.*, 2009; Brasky *et al.*, 2011). The antitumor activity of GlcN is still under investigation, but if proven it could provide a very safe alternative for cancer patients.

1.1. GlcN pharmacokinetics

1.1.1. Absorption

GlcN is an amino sugar with a small molecular weight (MW = 179.17). It is highly water soluble, slightly acidic compound with pka value of 6.91; which indicates complete ionization in the stomach at pH 1-3, while in the small intestine (pH = 6.8) 46% of it is ionized and 54 % is in the unionized form. This degree of ionization promotes its absorption from the small intestine. (Setnikar *et al.*, 1986).

GlcN is mainly administered orally; however, it has low oral bioavailability. The absolute oral bioavailability of the unchanged compound was measured in dogs, horses and rats and found to be 12%, 2.5%, and 19%, respectively (Adebowale *et al.*, 2002; Aghazadeh-Habashi *et al.*, 2002b; Du *et al.*, 2004).

In human, due to the lack of a sensitive assay, the absolute oral bioavailability was determined using the radiolabeled compound. After orally administering 314 mg labeled crystalline GlcN sulphate to human, no radioactivity was detected in the deproteinized plasma. After 1.5 h lag time, the radioactivity started to appear

in plasma protein (globulin) to reach a maximum level in 9 h followed by a slow decline. By measuring the area under the plasma concentration-time curve (AUC) of globulin-incorporated radioactivity and comparing it with that obtained after an intravenous (i.v.) dose, the absolute oral bioavailability was found to be 44%. However, this value cannot be considered as the true oral bioavailability of GlcN since it did not differentiate between GlcN and its metabolic products (Setnikar & Rovati, 2001).

The study also reported that 11.3% of the administered dose of radioactivity was detected in the feces over 120 h post dose. Subtracting this value from the total administered dose radioactivity leads to the assumption that 88.7% of the dose is absorbed from the gastrointestinal tract (GIT). Since the percentage that reaches the systemic circulation is only 44%, not 88.7%, Setnikar *et al.* assumed that 45% of the absorbed dose was lost during the hepatic first pass, and that the liver most probably metabolized GlcN into CO_2, water, and urea (Setnikar *et al.*, 1993; Setnikar & Rovati, 2001).

GlcN pharmacokinetics was studied in the rat after i.v., intraperitoneal (i.p.), and oral administration. A complete absorption of the compound was observed from its i.p. dose, while the absolute oral bioavailability was only 19 ± 21%. Complete absorption after the i.p. dose indicates that GlcN is not subjected to extensive hepatic metabolism, as i.p. administration delivers the compound directly to the liver through the portal vein before reaching the systemic circulation. If GlcN is extensively metabolized or biotransformed by the liver, it would be expected that the absolute bioavailability after i.p. administration is less

than unity. However, this was not the case, hence; the gut, not the liver, should be responsible for the observed low oral bioavailability of GlcN. Intestinal degradation, metabolism, and/or limited absorption could be the cause (Aghazadeh-Habashi *et al.*, 2002b).

GlcN is almost completely and rapidly absorbed after intramuscular (i.m.) administration with 96% absolute bioavailability (Setnikar *et al.*, 1993), however, both i.v. and i.m. dosage forms are not available in the market, due to their inconvenience and the risk of insulin resistance (Anderson *et al.*, 2005).

Although, GlcN is highly hydrophilic, it has good skin permeability (13.27 $\mu g/cm^2/h$ at 5% concentration), which makes it suitable to be formulated in transdermal dosage forms (Kanwischer *et al.*, 2005). *In vitro* studies showed that the highest skin permeation of GlcN HCl through rat skin occurs when it is formulated in a liquid crystalline vehicle, then oil in water (o/w) cream and liposomal vehicle (Han *et al.*, 2010). In spite of the reported low oral bioavailability of GlcN, which encourages a non-oral route of administration, to our knowledge, no studies have been conducted to investigate GlcN pharmacokinetics and efficacy from its transdermal preparation.

Pharmacokinetic parameters of orally administered GlcN in human

For a long time, the lack of a sensitive analytical method in plasma and biological fluid was an obstacle towards studying the oral pharmacokinetics of GlcN in human; hence, most of the studies using non-labeled GlcN were done on animals (rat, rabbit, and dog). Most of the animal studies were using high oral

doses ranging from 100-375 mg/kg (Lippiello *et al.*, 2000; Adebowale *et al.*, 2002; Aghazadeh-Habashi *et al.*, 2002b; Oegema *et al.*, 2002).

The first human pharmacokinetic study on the non-labeled compound was reported in 2005 by Persiani *et al.*, who used the liquid chromatography-mass spectroscopy (LC-MS/MS) method to analyze plasma samples. Before dosing, the average endogenous GlcN plasma concentration was 0.063 ± 0.05 μg/mL (range 0.01- 0.20 μg/mL). After an oral administration of 1500 mg GlcN sulphate (Rotta Pharmaceutics), a rapid increase in the plasma level was observed to reach a maximum level of 1.60 ± 0.42 μg/mL in 3-4 h post dose. The serum level of GlcN was also studied in healthy human volunteers after ingestion of a single oral dose of 1500 mg GlcN sulphate (Rotta Pharmaceutics). The endogenous GlcN level in the serum was below the detection limit. The serum level started to increase in 17 out of 18 subjects after dosing, with a lag period range of 30-45 min. The maximum serum level (Cmax) was 0.34-2 μg/mL and the time to reach maximum concentration (Tmax) was achieved in 1.5-2.5 h. No correlation was found between age, sex, or weight and the Tmax or Cmax (Biggee *et al.*, 2006). The study reported that GlcN appears faster in the serum of the subjects who had previously taken GlcN, and reaches a significant higher serum levels ($p = 0.03$) than those who did not previously taking GlcN (Biggee *et al.*, 2006).

Since then, several studies were conducted to determine GlcN pharmacokinetics in human plasma (Zhang *et al.*, 2006b; Zhong *et al.*, 2007; Zhu *et al.*, 2009), and almost every published study used a different method of analysis. The difference in the analytical method, age, weight, and gender of the

volunteers led to great variability in the reported pharmacokinetic parameters (Table 1.1). Nevertheless, the average Cmax of GlcN after an oral administration of 1500 mg in human was around 10 μM (range 2.7-19 μM) or 1.8 μg/mL (range 0.9-3.36 μg/mL). This value is much lower than the concentration used in human cell lines and OA animal models that have shown pharmacological activities (Block *et al.*, 2010; Aghazadeh-Habashi & Jamali, 2011).

GlcN sulphate versus GlcN HCl

GlcN is available on the market as crystalline GlcN sulphate ($[GlcNH^{3+}]_2$ $2Na^{+}.SO_4^{2-}.2Cl^{-}$) and GlcN HCl. Clinical studies sponsored by the sulphate salt suppliers reported a superior pharmacological effect as compared with those reported for the HCl salt (Noack *et al.*, 1994; Pavelka *et al.*, 2002; Herrero-Beaumont *et al.*, 2007). The claim triggered much debate, since both salts are expected to be completely dissociated in the stomach (pH 1-2) to generate a GlcN free base. When the same dose of the free base is available for absorption, the same pharmacological effect should occur. If the difference is valid, there should be a difference in the nature (purity) of the compound or the claimed amount of the amino sugar used in both formulations. The formulation additives may enhance the bioavailability of the sulphate form and further explain the observed superior activity. Another possibility is the synergistic or additive effect of the sulphate moiety, which is important for GAG synthesis and cartilage regeneration. In fact, all of the above possibilities were investigated thoroughly. Meulyzer *et al.*

Table 1.1. Pharmacokinetic parameters of orally administered GlcN in human

Type	Dose (mg)	Subject No.	Gender	Age	Analytical method	Cmax (µg/mL)	Tmax (h)	AUC_{0-t} (µg.h/mL)	$t_{1/2}$ (h)	Ref.
SO_4	1500	12	M + F	55.2 ± 21	LC- MS/MS	1.6 ± 0.42	3	nd.	nd.	(Persiani *et al.*, 2005)
SO_4	1500	18	M	22.2 ± 0.6	LC- ESI-MS	3.2 ±1.3	1.8 ± 0.4	9.5 ± 3.3 (t = 6 h)	1.1± 0.3	(Huang *et al.*, 2006a)
SO_4	1500	18	M + F	59. 6 ± 9.8	amperometric	0.34 ± 0.2	nd.	nd.	nd.	(Biggee *et al.*, 2006)
SO_4	1500	20	M		fluorescence	3.11 ± 2.14	1.76	9.82 ± 4.05	1.57 ± 0.28	(Zhang *et al.*, 2006b)
SO_4	1500	2			fluorescence	4.74	1.5	13.45 (t = 8 h)	1.18	(Huang *et al.*, 2006b)
SO_4	1500	12	M + F	25 ± 4.3	LC-ESI-MS/MS	0.98 ± 0.27	3	nd.	nd.	(Roda *et al.*, 2006)
SO_4	750	22			LC- ESI/MS	0.31 ± 0.09	2.64 ± 0.79	1.64 ± 0.45 (t = 24)	4.73 ± 1.32	(Zhong *et al.*, 2007)
SO_4	500	22	M	24 (range 22-26)	LC-MS/MS	0.37 ± 0.17	2.48 ± 1.3	1.77 ± 0.72 (t =14 h)	2.19 ± 0.87	(Zhu *et al.*, 2009)
HCl	1500	8	M + F	41.9 ± 17.9	FACE	0.49 ± 0.16	2.31 ± 1.19	2.38 ± 0.93	2.51 ± 1.84	(Jackson *et al.*, 2010)

F, Female; M, male; nd, not determined; FACE, Fluorescence-assisted carbohydrate electrophoresis.

performed a bioequivalent study to investigate the difference in the pharmacokinetic parameters of GlcN salts. Horses were given nasogastric and i.v. doses equivalent to 20 mg/kg GlcN extracted from crystalline GlcN sulphate (Dona®, Rotta Pharmaceutics) and analytical grade GlcN HCl. After the i.v. administration, a non-significant difference in all pharmacokinetic parameters was observed between the two salts. After nasogastric administration, both forms were absorbed rapidly with an average Tmax of 0.86 h and 0.81 h and an average Cmax of 1.08 μg/mL and 0.94 μg/mL for the sulphate and HCl salts, respectively. A non-significant difference was obtained from those parameters; however, the absolute oral bioavailability of the sulphate form was higher than the HCl form (F = 9.4 % and 6.1% for the sulphate and HCl salts, respectively). The difference in the mean $AUC_{0\text{-}12}$ and $AUC_{0\text{-}inf}$ did not reach statistically significant levels, while the median of those values was significantly different ($p < 0.05$). The higher bioavailability was accompanied by a significantly higher synovial fluid level of GlcN from its sulphate salt (Meulyzer *et al.*, 2008).

The authors explained the difference by the effect of formulation additive that exist in Dona® tablets. In the study, GlcN sulphate (Dona®, Rotta Pharmaceutics) and HCl salts were dissolved in 0.9% saline. The pH was then adjusted to 6.0, and GlcN sulphate or HC1 were filtered through a 0.2 μm filter before being administered. The filtration process eliminates bacteria and insoluble additives, but soluble additives including Na^+, Cl^-, aspartame, sorbitol, citric acid, and PEG 400 are able to pass. PEG 400 is commonly used in pharmaceutical preparations to enhance the water solubility of the active ingredients and facilitate their rapid

absorption; hence, it may increase GlcN sulphate bioavailability (Meulyzer *et al.*, 2008).

To confirm the absence of a natural difference between GlcN sulphate and HCl, another cross-over bioequivalent study was conducted in the rat. This time GlcN sulphate extracted from the tablets (Dona®, Rotta Pharmaceutics) and GlcN HCl powder (Sigma-Aldrich Canada, LTD, Oakville, ON) were suspended in PEG 400 before the oral administration of a dose equivalent to 100 mg/kg GlcN base to the rat. There was non-significant difference in the pharmacokinetic parameters (AUC_{0-4}, 10.12 ± 2.54 mg.h/L and 13.59 ± 3.64 mg.h/L for the sulphate and HCl salts, respectively (Aghazadeh-Habashi & Jamali, 2011). Moreover, the same authors carried out a bioequivalent study on human after administering the conventional tablet of crystalline GlcN sulphate (Dona®, Rotta Pharmaceutics) and GlcN HCl (Sigma-Aldrich Canada, LTD, Oakville, ON) dispensed in a soft gelatin capsule. A non-significant difference in the percentage of the dose excreted in urine over 13 h post dose was observed (2.43 ± 0.6% and 3.16 ± 0.18% for the sulphate and HCl salts, respectively), which reflects equivalent body exposure of the two salts (Aghazadeh-Habashi & Jamali, 2011).

Early studied showed that GAGs synthesis by human articular tissues is very sensitive to any changes in the physiological levels of the sulphate ion (van der Kraan *et al.*, 1990). The possibility that a sulphate ion is mediating the superior activity of GlcN sulphate over the HCl salt by increasing the production of sulphated GAGs was investigated. A reported increase in the serum sulphate level was observed 3 h after the administration of 1 g GlcN sulphate to healthy human

volunteers, while a similar increase was not observed when sodium sulphate was given alone to the same volunteers. The results indicated that GlcN may enhance the intestinal absorption of the sulphate ion (Hoffer *et al.*, 2001). The authors also studied the correlation between the serum and synovial levels of the sulphate ions in 15 OA patients and found almost identical levels in both biological fluids, which means that any elevation in the serum sulphate ions would likely be accompanied by similar changes in the synovial concentration of the ion. This finding suggested that the sulphate ions of GlcN sulphate may participate in the therapeutic activity of GlcN (Hoffer *et al.*, 2001). Nevertheless, when chondrocytes were incubated with GlcN-3-sulphate, no increase in the incorporation of ^{35}S-sulphate in GAG was observed (Qu *et al.*, 2006). It is important to mention that GlcN-3-sulphate is an ester form of glucosamine and is physically, chemically, and pharmacologically different from the commercially available salt; therefore, this observation is not an imitation of the real activity of GlcN sulphate salt in GAG synthesis. The role of the sulphate ion in the GlcN pharmacological effect still needs further investigation.

The effect of concurrent administration of chondroitin sulphate (CS)

The Glucosamine/chondroitin Arthritis Intervention Trial (GAIT), the largest randomized multi-center clinical study on GlcN in the United States, noted a trend of superior efficacy in pain relief in patients with moderate to severe OA who received the combination of GlcN HCl and CS over those who received each compound alone (Clegg *et al.*, 2006). The data prompted a pharmacokinetic study

to assess the effect of the combination on the pharmacokinetic parameters of each compound. Jackson *et al.* carried out the study on 29 subjects who were randomly assigned to receive either 1500 mg GlcN HCl (6 X 250 mg capsule), 1200 mg CS (6 X 200 mg capsule), or the combination. The results demonstrated significantly lower oral bioavailability for GlcN HCl when given in combination ($AUC_{0-\infty}$ was 2.38 ± 0.94 and 1.86 ± 0.89 μg/h/mL for GlcN HCl alone and in combination, respectively) (Figure 1.2.). The results were consistent with single and multiple dosing (Jackson *et al.*, 2010). More interestingly, the CS plasma level did not increase after oral dosing, either alone or in combination with GlcN, indicating that the superior efficacy in pain relief of the combination may not be due to the synergistic effect on the joint, but due to metabolic changes in the gut lining or in the liver that induced by CS and/or GlcN (Jackson *et al.*, 2010).

The influence of inflammation on the absorption of orally administered GlcN

Moderate to severe OA is characterized by joint inflammation. Local and systemic inflammation can highly affect drug and nutrient pharmacokinetics (Kulmatycki & Jamali, 2005; Peuhkuri *et al.*, 2010). Hence, it is expected that GlcN pharmacokinetics will be different in healthy individuals than it is with those who have an inflammation. Joint inflammation was induced in horses by an intra-articular injection of Escherichia coli (E. coli) lipopolysaccharide (LPS) in both radiocarpal joints. Twelve hours later, 20 mg/kg GlcN HCl was given by nasogastric intubation. Plasma and synovial fluid samples were obtained at different time intervals and the pharmacokinetic parameters were compared with

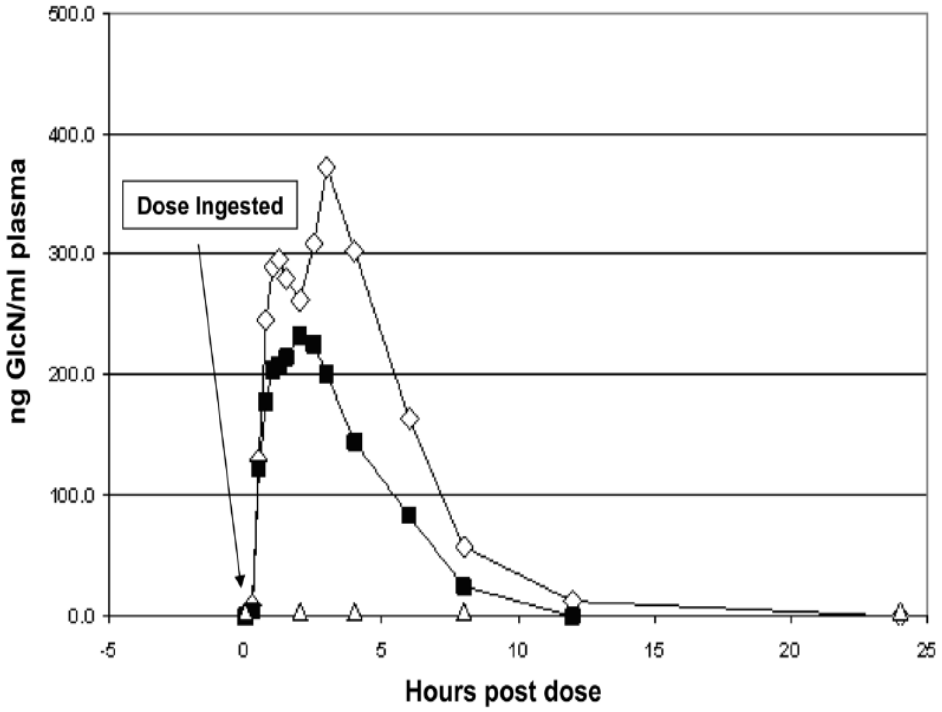


Figure 1.2. The effect of co-administration of CS on GlcN oral pharmacokinetics**,** showing the plasma concentration-time curve of GlcN in healthy human volunteers after the administration of GlcN HCl alone (◊) or in combination with CS (■). The basal level of GlcN was below detectable limits (Δ).

those obtained from the same horses before the induction of the joint inflammation (Meulyzer *et al.*, 2009). There was non-significant difference in GlcN plasma level at all the sampling time; however, GlcN concentration in the synovial fluid increased significantly ($p < 0.05$) from 92.7 ± 34.9 ng/mL in healthy joints to 422.3 ± 244.8 ng/mL in the inflamed joints (Figure 1.3) (Meulyzer *et al.*, 2009). The results indicated that joint inflammation did not affect the intestinal absorption of GlcN. However, it enhanced the compound distribution to the inflamed area (the joint), most probably by inducing extravasations through the synovial membrane.

1.1.2. Distribution

GlcN is highly distributed in the body. The volume of distribution of GlcN at a steady state (Vdss) is around 0.6 L/kg in dogs and horses, and 2.12 ± 1.08 L/kg in rats (Adebowale *et al.*, 2002; Aghazadeh-Habashi *et al.*, 2002b; Meulyzer *et al.*, 2008). In human, the reported Vd value after an i.v. dose of 800 mg GlcN sulphate is 0.07 L/kg (Setnikar *et al.*, 1986), which is a fairly small value, probably due to the low sensitivity of the method of analysis.

The distribution of GlcN in the rat organs was studied after an oral and i.v. administration of the radiolabeled compound. After i.v. administration, a rapid decrease in the total plasma radioactivity was observed in the first 30 min, followed by a peak at 2 h, and then a slow decline. After 10 min, the radioactivity was found in all organs including the cartilage, with the highest accumulation in

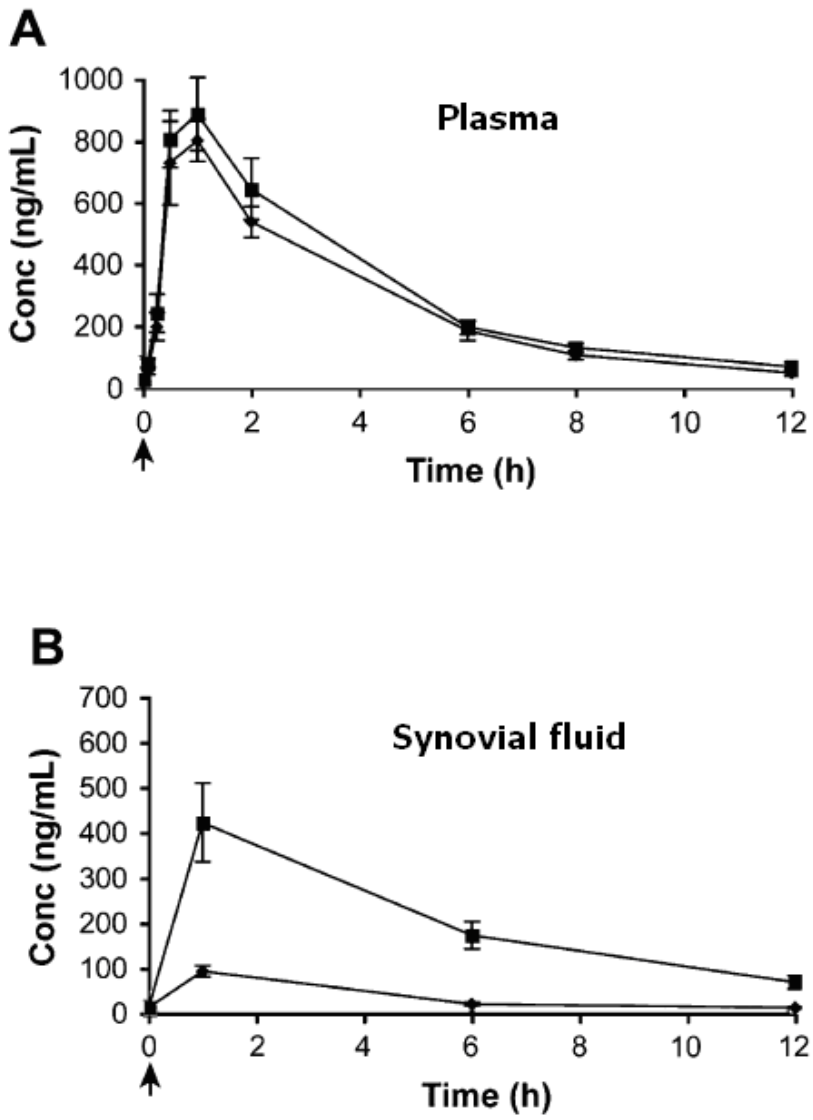


Figure 1.3. Effect of knee inflammation on GlcN plasma and synovial fluid levels in the horse. The figure shows GlcN average concentration in plasma (A) and synovial fluid (B) after nasogastric administration of 20 mg/Kg GlcN HCl to horse (n = 8) before induction of knee-inflammation (♦) and after induction of knee-inflammation (■).

The inflammation was induced by an intra-articular injection of LPS to both radiocarpal joints 12 h before dose administration. The figure shows higher levels in the synovial fluid of the inflamed joint.

the liver and kidneys. After 30 min, most of the plasma radioactivity was associated with plasma proteins. About 20% of the administered radioactivity was found in the liver 30 min post dose. Radioactivity could be detected in all organs, even 144 h after the dose was administered (Setnikar & Rovati, 2001).

A two-dimensional image of the whole body section of a rat was taken 15 min after i.v. administration of D-[1-^{14}C]GlcN HCl using Ambis 4000 detector (Figure 1.4). The image showed that GlcN is highly distributed in the body, mainly in the kidneys, liver, brain, skin, and cartilage, but the liver and kidneys have the highest levels (Giraud *et al.*, 2000). Analysis of the plasma samples obtained at different time intervals after the i.v. dose administration showed that the plasma radioactivity decreased rapidly to reach its minimum value after 15 min and then started to increase to reach its peak at 2 h post injection, followed by a very slow disappearance (Giraud *et al.*, 2000).

When radiolabeled GlcN sulphate was administered orally, low levels of radioactivity appeared rapidly in the plasma. The highest radioactivity was in the liver and kidneys. The radioactivity reached its maximum level after 2 h in the liver, after 4 h in the plasma and kidneys, and after 8 h in the femoral cartilage. In the intestinal content, the radioactivity decreased rapidly up to 1 h, and then reached its highest level after 2 h, followed by a slow decline (Setnikar & Rovati, 2001). This pattern of rapid disappearance and re-appearance of the radioactivity from the intestinal content may indicates uptake of GlcN by the intestinal tissues for some biotransformation processes. Early studies showed that the radioactivity

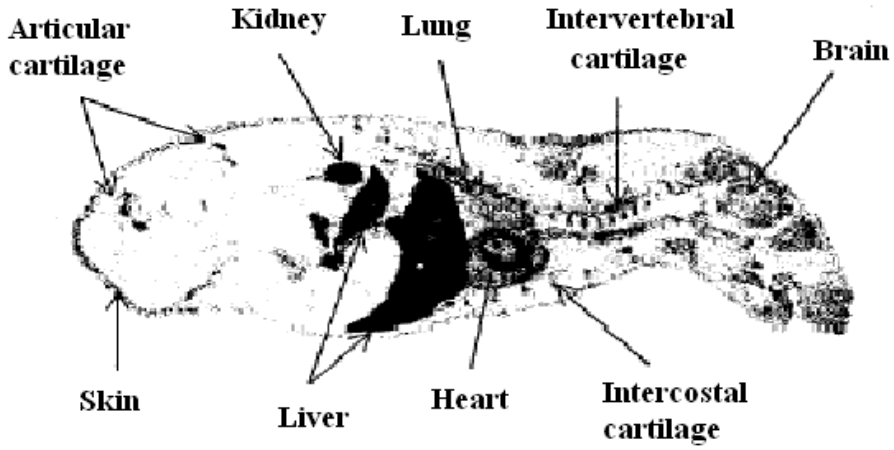


Figure 1.4. Two dimensional image of GlcN distribution in rat 15 min after i.v. dose of ^{14}C-GlcN HCl. The image shows high distribution in the liver and kidneys.

of i.p. administered ^{14}C-GlcN is detected in the intestinal brush border of the rat 90 min and in the intestinal secretion 150 min after dose. Analysis of the intestinal secretion revealed that GlcN is incorporated into glycoprotein, which is the main constituent of the mucus secretion (Forstner, 1970).

There is some reported data indicated that GlcN can pass easily through the blood brain barrier (BBB) and participate in the formation of brain glycoprotein and ganglioside (Popov, 1985). On the other hand; there is no available data about the ability of GlcN to cross the placental barrier, however, a study has conducted in 54 women using GlcN during pregnancy found no increase in the potential risk of malformation or abortion with the use of the compound. As 50 out of 54 gave a live birth, with no major malformation and no deviation from the normal average birth weight or the median gestational age (Sivojelezova *et al.*, 2007).

GlcN does not bind to plasma or synovial fluid protein in human, dog, rat and mice (Setnikar *et al.*, 1986; Persiani *et al.*, 2009b); hence, no drug-drug interaction due to protein displacement was reported with its treatment. However it is incorporated by the liver into plasma glycoprotein, mainly beta-globulin (Setnikar *et al.*, 1986) and α-acid glycoprotein (Aronson, 1982) which play an important role in drug binding.

The distribution of GlcN to the synovial fluid and cartilage tissue

As GlcN effect on the joints is expected, its distribution to the synovial fluid receives some attention. The synovial level of GlcN was measured in horses

after 1 and 12 h from the i.v. and nasogastric administration of 20 mg/kg GlcN HCl. The basal GlcN level in the synovial fluid was below detectable limits prior to dose administration. The levels elevated to reach 1.5-2.5 μg/mL (9-15 μM) around 4% of the serum level and 0.05-0.12 μg/mL (0.3-0.7 μM) around 9% of the serum level, after i.v. and oral dosing, respectively. The GlcN serum level was below detectable limits 6 h post dose, while it could be detected in the synovial fluid at 12 h post dose (Laverty *et al.*, 2005), indicating fast distribution and slow elimination of the compound from the site of action.

In another study, GlcN plasma and synovial fluid levels were measured in horses after i.v. and nasogastric administration of 20 mg/kg GlcN sulphate (Dona®, Rotta Pharmaceutics) and HCl salt. Synovial fluid was withdrawn from the radiocarpal joint of the animals before the dose and at 1, 6 and 12 h post dose. The basal level of GlcN in the synovial fluid before dose administration was not significantly different from the plasma basal level (range 0.01-0.25 μg/mL) (Meulyzer *et al.*, 2008). After oral administration, the synovial fluid level increased and reached a maximum value 1 h post dose. The Cmax in the synovial fluid of the sulphate salt was significantly higher ($p = 0.0004$) than that of the HCl salt (0.154 ± 0.032 μg/mL and 0.093 ± 0.035 μg/mL for the sulphate and HCl salts, respectively). These values represent 14.3% and 9.9% of the corresponding Cmax in plasma of each animal group (Meulyzer *et al.*, 2008). The significant difference in the synovial level of GlcN from its different salts remained at 6 h ($p = 0.02$) but not at 12 h post dose. This significant difference reflected the increase in the absolute oral bioavailability of the sulphate salt. Intravenous administration

did not show any difference between the two salts, which indicates a difference in the intestinal absorption between both GlcN salts (Meulyzer *et al.*, 2008).

Persiani *et al.* studied GlcN distribution to the synovial fluid in 12 OA patients (6 male and 6 female). Before administering GlcN, the median endogenous GlcN level in the synovial fluid was 0.036 μg/mL (range, 0.01-0.067 μg/mL). This level increased by 21.5 fold after two weeks of daily administration of 1500 mg crystalline GlcN sulphate to be 0.78 μg/mL (range, 0.58-3.25 μg/mL). Moreover, the experiment showed that the synovial concentration at 3 h post GlcN oral dose is 25% of the corresponding plasma level at that time point (Persiani *et al.*, 2007).

Evidence has shown that joint inflammation has a positive effect on GlcN distribution to the synovial fluid. The Cmax achieved in the inflamed joint of the horse after nasogastric administration of 20 mg/kg GlcN was 0.42 ± 0.24 μg/ mL. This value was almost four times higher than that obtained after administration of the same dose to the same horses before induction of joint inflammation (0.09 ± 0.03 μg/mL). The increase in the synovial level did not reflect an increase of GlcN in plasma, indicating that joint inflammation only altered the compound distribution but did not affect its oral absorption (Figure 1.3). It is worth mentioning that the synovial fluid white blood cell count (WBC) and the total protein (TP) significantly increased after an inflammation was induced in the horse joint. Administering GlcN did not significantly reduce those inflammatory parameters, in spite of its relatively high synovial fluid level (Meulyzer *et al.*, 2009).

The distribution of GlcN to the cartilage was also studied. Three hours after oral administration of 98 mg/kg GlcN sulphate (Donna®, Rotta Pharmaceuticals) to rabbits, GlcN level increased in the cartilage (pooled sample from the knee, hip and shoulder) from 0.461 ± 0.090 μg/g in controls to 1.040 ± 0.190 μg/g. At the same time, a strong correlation between GlcN concentration in rabbits plasma and cartilage (R^2 = 0.971) was observed (Pastorini *et al.*, 2011).

1.1.3. Elimination

GlcN is eliminated rapidly from the plasma. The terminal half life of the unchanged compound in animal and human plasma ranges from 1.5 to 4 h (Adebowale *et al.*, 2002; Aghazadeh-Habashi *et al.*, 2002b; Du *et al.*, 2004; Zhang *et al.*, 2006b; Meulyzer *et al.*, 2008; Jackson *et al.*, 2010). There is no difference in the half life of GlcN HCl and sulphate salts (Meulyzer *et al.*, 2008). Persiani *et al.* reported that GlcN terminal half life in healthy human is 15 h, however, this number is an estimation based on the time to reach a steady state which according to the study achieved by the third day of dose administration. The study mentioned that the half life cannot be determined from the terminal phase as it did not achieved under their experimental conditions, and the plasma concentration of GlcN remained above the baseline level even after 48 h from the oral administration of the compound (Persiani *et al.*, 2005).

GlcN is rapidly incorporated by the liver into plasma protein; the elimination half life from plasma protein is 70 h (Setnikar *et al.*, 1993). The total body clearance (CL) after the i.v. administration of GlcN is 39.25 ± 1.87 L/h and

9.73 ± 4.93 L/h in horse and dog, respectively (Adebowale *et al.*, 2002; Du *et al.*, 2004), and 2.61 ± 0.8 L/ h/kg in rat (Aghazadeh-Habashi *et al.*, 2002b). A significantly lower CL/F value was reported in horses for GlcN sulphate compared to GlcN HCl (2.99 ± 0.77 L/h/kg and 5.10 ± 1.54 L/h/kg for sulphate and HCl salts, respectively) (Meulyzer *et al.*, 2008).

Urinary excretion

Kidney excretion is the main mechanism for eliminating GlcN after i.v. and i.m. doses. The urinary excretion of GlcN was measured using an ion exchange chromatography method after the i.v. administration of 800 mg GlcN sulphate to 6 healthy volunteers. A very fast renal elimination of the compound was observed, in which 31.7 ± 5.1% of the administered dose was recovered unchanged in the urine 2 h post dose administration and reached 38% in 24 h. (Setnikar *et al.*, 1986). Using the radiolabeled compound, the total radio-activity recovered in the urine 24 h post dose administration was 26%, 37%, and 10% for i.v., i.m., and oral doses respectively. The reported human urinary excretion of cold GlcN after an oral dose is less than 1% in 3 h (Biggee *et al.*, 2006) and 1.81 ± 0.99 % in 24 h (Guan *et al.*, 2011). In another study, the total amount excreted in human urine after 13 h of the administration of 1500 mg GlcN was corrected to the reported oral bioavailability of the compound (26%) and found to be representing 3.16 ± 0.18% and 2.43 ± 0.6% of the orally absorbed GlcN HCl and sulphate forms, respectively (Aghazadeh-Habashi & Jamali, 2011).

Hepatic metabolism

GlcN is not subjected to metabolism by liver cytochrome P450; however, GlcN is subjected to hepatic biotransformation. *In vitro* and *in vivo* experiments on the rat demonstrated a rapid uptake of exogenous ^{14}C-GlcN by the liver and incorporation into plasma glycoprotein, mainly beta-globulin and α-acid glycoprotein (Akamatsu *et al.*, 1976; Aronson, 1982; Setnikar *et al.*, 1993; Giraud *et al.*, 2000).

GlcN does not have an induction or inhibition effect on the human liver cytochrome P450, particularly, CYP1A2, CYP2E1, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 (Persiani *et al.*, 2009a).

Excretion of GlcN metabolite in the expired air

The metabolism and excretion of GlcN as CO_2 in the expired air has been studied extensively using the radiolabeled compound. In rats, and after the i.v. administration of ^{14}C-crystalline GlcN sulphate, 16% of the dose was excreted as $^{14}CO_2$ in the first 6 h, and reach 50% in 144 h post dose. After oral administration, 61% of the administered dose was excreted as $^{14}CO_2$ in the first 6 h. The number reached 81% in 144 h (Setnikar *et al.*, 1984). This metabolic behavior is different from glucose, in which the same percentage of radioactivity is excreted in the expired air after the oral and i.v. administration of the radiolabeled sugar (Setnikar & Rovati, 2001) (Table 1.2). The excessive production of $^{14}CO_2$ after the oral administration of GlcN was attributed to a possible hepatic first pass effect that converts GlcN or its metabolites to CO_2 and urea (Setnikar *et al.*, 1984).

The difference in the metabolic fate between i.v. and oral GlcN was first observed in 1968 upon studying the distribution of radioactivity of free and bound [1-^{14}C]GlcN in rats (Robinson, 1968). Interestingly, the same percentage of radioactivity was excreted in the expired air after the oral administration of labeled GlcN and labeled glycoprotein (Table 1.2).

Robinson thought that intestinal flora was behind the higher levels of radioactivity excreted in the expired air when the compound was administered orally. Thus, he carried out an experiment to test his hypothesis. He treated one group of rats with 0.5% neomycin in the drinking water for either 48 or 96 hours before oral administration of [1-^{14}C]GlcN and compared the metabolic fate of the labeled compound with the results obtained from normal rats. However, the metabolic fate of oral GlcN was exactly the same in antibiotic-treated rats as it was in normal rats. Robinson concluded that GlcN is not subjected to degradation by intestinal flora but is either metabolized by the gastrointestinal tract (GIT) to produce CO_2, or converted by the GIT to another compound that can be further metabolized to CO_2 (Robinson, 1968).

N-acetyl[1-^{14}C]GlcN shows a slight different metabolic pattern from GlcN, where almost the same percentage is excreted as CO_2 after oral administration, a higher percentage is excreted in the urine, and a lower percentage is retained in plasma. No radioactivity could be detected in the feces and intestinal tract 24 h after oral administration (Table 1.2) (Robinson, 1968).

Table 1.2. Difference in the metabolic fate of i.v. and oral doses of radiolabeled GlcN, glucose, GlcNAc, and glycoprotein

Compound	**Dose**	**Time**	**$^{14}CO_2$**	**Urine**	**Feces & intestine**	**Plasma**	**Ref.**
^{14}C-GlcN	i.v.	24	20%	26%	-	13%	(Robinson, 1968)
		144	50% (16% in the first 6h)	38%	2 %		(Setnikar *et al.*, 1984)
	Oral	24	50%	3%	10%	3%	(Robinson, 1968)
		144	81 % (61% in the first 6h)	6 %	5 %		(Setnikar *et al.*, 1984)
^{14}C-Glucose	i.v.	144	65 % (50% in the first 6h)	5 %	-		(Setnikar & Rovati, 2001)
	Oral	144	65 % (49% in the first 6h)	4 %	4 %		(Setnikar & Rovati, 2001)
^{14}C-labeled glycoprotein	i.v.	24	21%	2.3%		34%	(Robinson, 1968)
	Oral	24	50%	3%	10%	4%	(Robinson, 1968)
N-Acetyl[1-^{14}C]GlcN	i.v.	24	12%	51%	12%	0.7%	(Robinson, 1968)
	Oral		47%	7.5%	_	-	(Robinson, 1968)

Deamination of GlcN

The possibility of deamination of GlcN and GlcNAc by the animal tissues to form fructose-6-P, which can further utilized as an energy source was confirmed earlier (Comb & Roseman, 1958). In order to determine the significance of this metabolic pathway, food deprived rats administered i.p. doses of radiolabeled GlcN, GlcNAc or glucose and the comparative rate of oxidation, incorporation into liver macromolecules or urinary excretion was determined for each compound. The results showed that glucose and GlcNAc are more subjected to direct oxidation to provide energy than GlcN. On the other hand, GlcN showed higher tendency to be incorporated into liver macromolecules and sialic acid. (Kohn *et al.*, 1962).

Mammalian GlcN-6-P deaminase (GNPDA), the enzyme responsible for converting GlcN-6-P to fructose-6-P, has been cloned and localized (Wolosker *et al.*, 1998). Northern blot and Western blot analyses showed that this enzyme is localized with high density in the kidneys and small intestine, and with lower levels in the spleen, testes, ovary, brain, lungs and heart. Very small levels were found in the skeletal muscles and liver. Moreover, an immunohistochemical examination revealed that GNPDA is localized with high levels in the apical portion of the small intestine epithelial cells and with very small levels in the lamina propria and underlying muscles. In the kidney, the enzyme is mainly localized in the apical portion of the epithelium of the proximal convoluted tubules. The restricted distribution of GNPDA in cells with a high metabolic rate reflects its role in providing enough energy for those cells by converting GlcN-6-

P generated from a macromolecular breakdown to fructose-6-P that can be utilized in the glycolytic pathway (Wolosker *et al.,* 1998). This fact raises the possibility that orally administered GlcN is utilized by the intestinal tissue for energy production, especially at low luminal glucose levels.

The hexosamine biosynthetic pathway (HBP)

To date, the only well-known metabolic pathway for GlcN is the hexosamine biosynthetic pathway (HBP). Inside the cells, glucose is rapidly phosphorylated to glucose-6-P, which is then converted to fructose-6-P and enters into the glycolysis pathway to generate energy and CO_2. Glucose-6-P can also biotransformed to glucose-1-P, which enters the glycogenesis pathway to produce glycogen. A small portion of fructose-6-P (around 3%) enters into the HBP, in which fructose-6-P is converted to GlcN-6-P by the action of glutamine:fructose-6-phosphate amidotransferase (GFAT).

It is believed that exogenous GlcN directly undergoes phosphorylation inside the cells and is converted to GlcN-6-P (Figure 1.5). This compound is then acetylated to GlcNAc-6-P by glucosamine-phosphate-N-acetyltransferase and converted to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) by UDP-N-acetylglucosamine pyrophosphorylase. UDP-GlcNAc can undergo conversion to UDP-N-acetylgalactosamine (UDP-GalNAc) by UDP-N-acetylglucosamine-4-epimerase (Anderson *et al.,* 2005; Buse, 2006).

UDP-GlcNAc and other nucleotide hexosamines provide glycosidic precursors to form glycoproteins, proteoglycans, and glycolipids. Moreover,

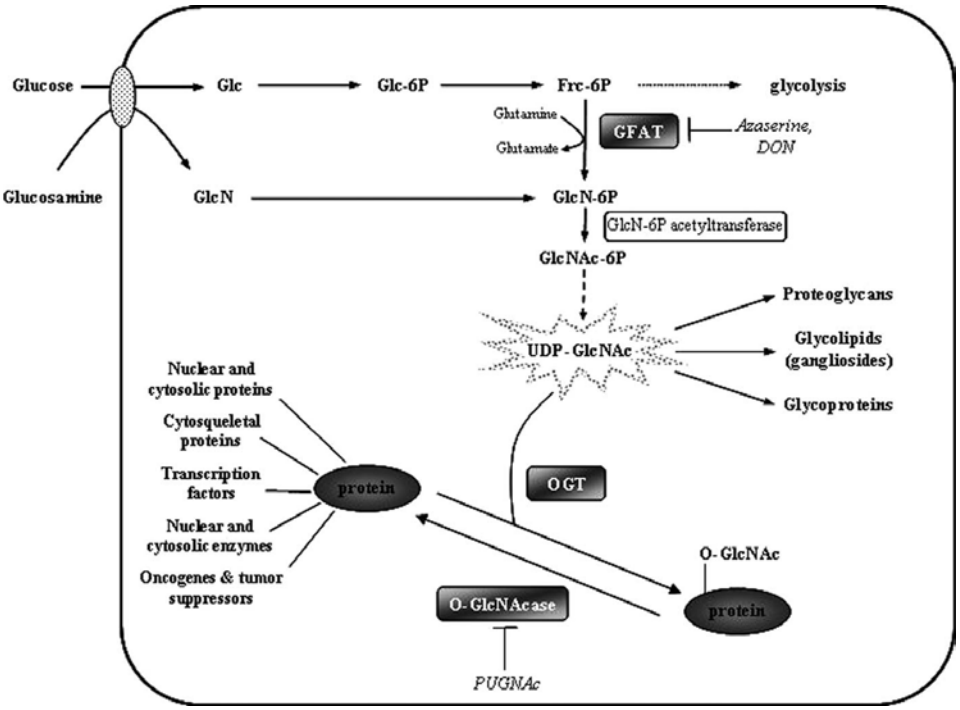


Figure 1.5. A schematic diagram of the hexosamine biosynthetic pathway (HBP).

Glc, glucose; Glc-6P, glucose-6-phosphate; Frc-6P, fructose-6-phosphate; GlcN, glucosamine; GlcN-6P, glucosamine-6-phosphate; GlcNAc-6P, N-acetylglucosamine-6-phosphate; UDP-GlcNAc, Uridine diphosphate-N-acetylglucosamine; GFAT, glucosamine:fructose-6-phosphate aminotransferase; OGT, O-linked-N-acetylglucosamine transferase; O-GlcNAcase, β-N-actylglucosaminidase.

UDP-GlcNAc is a substrate for the cytosolic O-linked-N-acetylglucosamine transferase (OGT), which glycosylated nuclear and cytosolic proteins with a single GlcNAc moiety on serine and threonine residues. The process is known as O-GlcNAc modification or O-GlcNAcylation, which is highly reversible by the action of another enzyme, *B*-N-acetylglucosaminidase (O-GlcNAcase). A dynamic cycle of addition and removal of O-linked-N-acetylglucosamine (O-GlcNAc) plays an important role in regulating cell growth and division, gene expression, enzyme activity and the structural integrity of the cytoskeleton (Love & Hanover, 2005).

Normally not more than 3% of cellular glucose enters into the HBP. In hyperglycemia, this pathway is activated, leading to insulin resistance and diabetic complications (Buse, 2006), which raises a serious concern about the effect of GlcN on glucose metabolism and its safety in diabetic patients.

The effect of disease on GlcN elimination

GlcN elimination after i.v. administration is not altered in patients suffering from liver diseases, uncomplicated diabetes, coronary sclerosis, and multiple myeloma, while a pronounced decrease in the urinary excretion occurs with renal disease and carcinoma, especially carcinoma of the main hepatic duct (Weiden & Wood, 1958).

1.1.4. Glucosamine pharmacokinetics after increasing oral dose

Linear relationships between the labeled GlcN dose and the corresponding $AUC_{0\text{-}120}$ and Cmax were observed in rats after the administration of 100, 1000, and 2700 mg of labeled GlcN sulphate (Setnikar & Rovati, 2001). The Tmax of the appearance of GlcN in deproteinized plasma decreased from 4 h to 0.5 h with increasing the oral dose; however the opposite was observed in the Tmax in total plasma (4 h for 100 mg and 8 h for higher doses). The linearity of GlcN pharmacokinetics with increasing oral dose was confirmed by the total percentage of radioactivity excreted in rat urine, feces, and expired air (Setnikar & Rovati, 2001).

In human, the dose-AUC relationship was found to be linear when the oral dose increased from 750 to 1500 mg, and deviated from linearity with a higher dose (3000 mg). The normalized AUC value for the higher dose was lower than expected if the relationship was linear, which suggests a capacity-limited absorption mechanism for GlcN (Persiani *et al.*, 2005). However, close examination of their data, suggests a linear relationship between the dose and the AUC for 750, 1500, and 3000 mg doses that is preceded by a saturable phase. The line passing through the data crosses the AUC axis at a point substantially higher than the origin (Figure 1.6). An increase in the Tmax value was also observed with higher GlcN doses in human (Persiani *et al.*, 2005).

1.1.5. GlcN pharmacokinetics after a repeated oral dose

The steady state plasma concentration was achieved after two days of daily administration of 1500 mg GlcN sulphate to human, as calculated from the

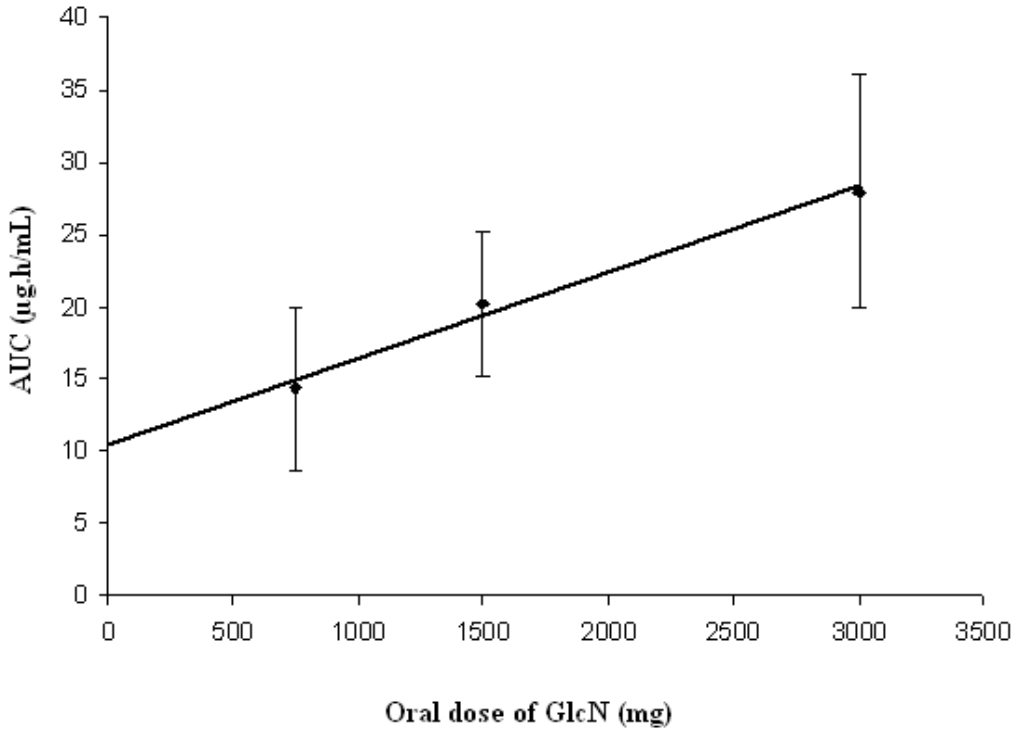


Figure 1.6. The relationship between the doses of orally administered of GlcN and the corresponding average $AUC_{0\text{-}48h}$ in human. The average AUC was obtained from the plasma concentration-time curves of GlcN in 12 healthy volunteers after administration of 750, 1500 and 3000 mg GlcN sulphate in an open-randomized-cross-over study.

Data used in the graph is obtained from *Osteoarthritis Cartilage* 13, Persiani S, Roda E, Rovati LC, Locatelli M, Giacovelli G & Roda A (2005) Glucosamine oral bioavailability and plasma pharmacokinetics after increasing doses of crystalline glucosamine sulfate in man, 1041-1049.

steady urinary excretion of GlcN (Setnikar & Rovati, 2001). In the rat, after the administration of 12.6 mg/kg labeled GlcN sulphate, the steady state was achieved in three days following administration, but most of the radioactivity was associated with plasma proteins. Only 10% of the radioactivity was found in deproteinized plasma. The average peak plasma protein concentration was 2.5 times the peak obtained after the first dose (Setnikar & Rovati, 2001). The urinary excretion accounted for 3.6 ± 0.9 in the first 24 h and increased to reach its maximum level of 8.2 ± 1.9 after the third day of administration. GlcN or its metabolites tends to accumulate in the body, where the radioactivity found in rat liver, kidneys and articular cartilage after six days of repeating the oral dose was three to five times greater than that found after a single oral dose (Setnikar & Rovati, 2001).

In dogs, a non-significant difference between single and multiple dosing pharmacokinetics was observed (Adebowale *et al.*, 2002). Recently, the pharmacokinetic parameters were determined in human after a single dose and a dose taken after three months of repeated administration of 1500 mg GlcN HCl. There was no significant change in the $AUC_{0-\infty}$; however, a significant decrease in the Cmax was observed with multiple administration (0.49 ± 0.16 μg/mL and 0.21 ± 0.093 μg/mL, for single and multiple dose, respectively) (Jackson *et al.*, 2010).

1.1.6. The mechanism of GlcN uptake by different tissues

Evidence showed that the tissue uptake and the intestinal absorption of GlcN are mediated by facilitated transporters. Tesoriere *et al.* studied GlcN and GlcNAc

transport through everted rat sacs using different concentrations of the radiolabeled compounds. The data generated from ^{14}C-GlcN indicated the presence of a saturable mechanism mediating its intestinal absorption, since the Lineweaver-Burk plot did not pass through the origin. Sodium deficiency had no effect on ^{14}C-GlcN transport. On the other hand, GlcNAc absorption was mediated by simple diffusion. Both GlcN and GlcNAc were absorbed in their intact form without metabolic changes (Tesoriere *et al.*, 1972).

The hepatic uptake of GlcN was studied extensively in rat hepatoma cells. The uptake followed Michaelis-Menten kinetics with a km of 20 mM, which indicates the contribution of a saturable transporting mechanism. The transport was facilitative since no accumulation against the concentration gradient was observed. At a higher GlcN concentration in the incubation medium, the uptake continued with a higher rate than expected after the saturation of the transporters, most probably by simple diffusion. The driving force for GlcN uptake by hepatocytes is its rapid phosphorylation inside the cells to GlcN-6-P (Plagemann & Erbe, 1973). Cytochalasin B (a potent inhibitor of glucose transporters) and glucose competitively inhibited GlcN uptake by the cultured cells (Ebstensen & Plagemann, 1972).

The same observation was found in the uptake of GlcN by rat brain synaptosomes, where a saturable transport mechanism was involved with km 2.5 ± 0.8 mM and Vmax 3.7 ± 1.2 nmol/mg/min. Glucose, mannose, 2-deoxy-D-glucose, 3-O-methyl-D-glucose, cytochalasin B, phloretin and phlorizin competitively inhibit GlcN uptake by synaptosomes. The uptake is slightly

inhibited by ammonia, manganese, and calcium. It is unaffected by magnesium, sodium, and lithium. Only phosphate ions showed a slight stimulatory effect on GlcN uptake (Tan *et al.*, 1977).

GlcN uptake by normal human chondrocytes and synovial fibroblast cell lines was also found to be mediated by a facilitative transport system. A considerably higher uptake of GlcN was observed in human chondrosarcoma (SW1353). The rapid phosphorylation of GlcN inside the cells led to the depletion of ATP pools. The study did not address the effect of glucose on the uptake of GlcN; however, it showed that GlcN reduced the glucose transporters' GLUT1 and 6 insertion on the chondrocytes' cell membrane, and hence inhibited glucose uptake in a non-competitive, dose-dependant way (Shikhman *et al.*, 2009). In contrast, a non-significant uptake of GlcNAc was observed on this cell line. Incubation of the cells with GlcNAc causes a mild but significant stimulation of glucose uptake (Shikhman *et al.*, 2009).

The involvement of GLUTs (particularly GLUT2) in GlcN transport was suggested when Uldry *et al.* expressed glucose transporters' GLUT 1, 2 and 4 in Xenopus oocytes and found that the three transporters were able to transport GlcN. Both GLUT1 and 4 shared an affinity to both glucose and GlcN, but the GLUT2 affinity to GlcN was almost 20 times higher than that for glucose (km = 0.8 ± 0.1 mM for GlcN and 17-20 mM for glucose) (Uldry *et al.*, 2002). Moreover, the uptake of GlcN into hepatocytes was found to be absolutely through GLUT2 transporters (Uldry *et al.*, 2002).

1.2. GlcN adverse effects and drug interaction

GlcN is a highly safe nutritional supplement. It is is well tolerated by animals, even with large oral doses ranging from 5-15 g/kg (Anderson *et al.*, 2005). The LD_{50} of GlcN after i.v. and i.p. administration is 1.7 g/kg and 5.2 g/kg, respectively, in the rat and 1.6 g/kg and 6.6 g/kg in the mice (Setnikar *et al.*, 1991).

The reported side effects are rare. Most are gastrointestinal, including abdominal pain, diarrhea, heartburn, and constipation. Nausea, vomiting, drowsiness, insomnia, and headaches are also reported (Kelly, 1998). Allergic reactions can occur in people allergic to shellfish and shellfish products. There are some reported cases of elevated blood cholesterol levels after the administration of GlcN; however, this effect was not proven clinically (Albert *et al.*, 2007). The combination of GlcN and CS may lead to a mild elevation of blood pressure, especially with sodium containing products, and worsen asthmatic conditions (Tallia & Cardone, 2002). GlcN is not recommended for diabetic patients because of the possibility of its interaction with glucose metabolism.

1.2.1. The effect of GlcN on glucose metabolism

The contribution of the HBP in the development of insulin resistance was proposed after the discovery that this resistance develops in isolated adipocytes only if glucose, insulin, and glutamine are present together in the cell medium (Traxinger & Marshall, 1989). This proposal has been supported by evidence that actinomycin D (a transcription inhibitor) can prevent development of insulin

resistance in isolated adipocytes but not in GlcN-treated isolated adipocytes. Since the only enzymatic step before GlcN enters the HBP is the amidation of fructose-6-P by GFAT, it was concluded that GFAT is the enzyme that has been inhibited by actinomycin D, resulting in the reduction in the development of insulin resistance (Marshall *et al.*, 1991).

Subsequent studies confirmed HBP's contribution to the development of insulin resistance; the over-expression of GFAT in skeletal muscle and adipose tissue of transgenic mice was associated with weight-dependent insulin resistance in randomly-fed mice (Hebert *et al.*, 1996; Cooksey & McClain, 2002), and the over-expression of OGT, the enzyme that utilizes UDP-GlcNAc to modify proteins by O-GlcNAcylation, induced type II diabetes in transgenic mice (McClain *et al.*, 2002). In humans, the GFAT activity was 46% higher in NIDDM skeletal muscle biopsies compared with normal subjects. However, there was a poor correlation between the increase in GFAT activity and the decrease in total body glucose uptake. This poor correlation indicted that the HBP is an important factor in the development of insulin resistance in humans, but other factors may be involved (Yki-Jarvinen *et al.*, 1996).

Knowing that GlcN is the initial component of the HBP, suggests that chronic administration of the compound to OA patients may induce or worsen diabetes. *In vivo* animal studies showed that GlcN infusion was able to induce severe skeletal muscle insulin resistance in normoglycemic rats but not in type II diabetic rats which were already insulin resistant (Rossetti *et al.*, 1995). The resistance was associated with a four to five fold elevation in the HPB end

product UDP-GlcNAc and UDP-GalNAc in rat muscles (Choi *et al.*, 2001). The mechanism by which GlcN induced insulin resistance is thought to be by inhibiting GLUT4 translocation to the cell membrane by reducing the insulin-stimulated insulin receptor substrate (IRS-1) tyrosine phosphorylation (Baron *et al.*, 1995; Patti *et al.*, 1999).

Moreover, some studies suggested that GlcN may induce diabetes by reducing insulin production and secretion by pancreatic β-cells. There are some reported data indicating that activating the HBP and /or exposure to high levels of GlcN can induce apoptosis and decrease the function of pancreatic beta cells (Shankar *et al.*, 1998; Anello *et al.*, 2004; D'Alessandris *et al.*, 2004; Lafontaine-Lacasse *et al.*, 2011).

Although most *in vitro* and *in vivo* animal studies highly supported the contribution of the HBP and GlcN administration in the development of insulin resistance, it is worth mentioning that the above studies used much higher concentrations (100-1000 times) than the maximum plasma levels achieved after oral administration of 1500 mg GlcN to human (Anderson *et al.*, 2005; Muniyappa, 2011; Simon *et al.*, 2011). Human studies, on the other hand, did not confirm the ability of GlcN to induce insulin resistance. There was no difference in insulin stimulated from skeletal muscle glucose uptake or in total body glucose uptake between subjects infused with 4 µmol (0.72 µg)/dL.min GlcN for 5 h (to simulate the HBP of glucose) and those given a placebo (Pouwels *et al.*, 2001). Improvement of insulin resistance in type II diabetic patients was not accompanied by a decrease in UDP-GlcNAc or UDP-GalNAc, which are the

stable metabolites of the HBP; in contrast, their tissue concentrations were significantly increased (Pouwels *et al.*, 2002).

In the same time, it is very unlikely that the recommended oral dose of GlcN (1.5 g/day) can induce insulin resistance; as the normal daily production of endogenous GlcN should be around 12 g after the administration of 200 to 350 g carbohydrates (2-5% enters the HBP) (Anderson *et al.*, 2005).

However, as OA patients are mostly elderly and obese (Felson *et al.*, 1987; Coggon *et al.*, 2001) who are either suffering from diabetes or at high risk of developing diabetes, the effect of orally administered GlcN on glucose metabolism received a lot of attention. As a result, many clinical trials were conducted on healthy, diabetic, and OA patients to evaluate the treatment's safety. Several reviews summarized the clinical outcome of these data and concluded that short-term use of GlcN does not induce or worsen insulin resistance (Anderson *et al.*, 2005; Stumpf & Lin, 2006), and that GlcN can be used safely by healthy humans for up to three years without significant adverse effects (Anderson *et al.*, 2005).

Data about the effect of long term administration (2-12 week) of GlcN in diabetic and pre-diabetic patients is limited. In the comprehensive review (Simon *et al.*, 2011) which summarized the clinical outcome on the effect of oral administration of GlcN on glucose metabolism up to December 2009, only six articles were found about studies conducted on diabetic or pr-diabetic individuals (Tapadinhas *et al.*, 1982; Scroggie *et al.*, 2003; Yu *et al.*, 2003; Muniyappa *et al.*, 2006; Albert *et al.*, 2007; Biggee *et al.*, 2007b). Only one of those studies

reported worsening glucose tolerance in some individuals (3 out of 16) with undiagnosed diabetes (Biggee *et al.*, 2007b). The small sample size and lack of randomized allocation highly weakened the outcome (Simon *et al.*, 2011). On the other hand, short- and long- term administration of GlcN to healthy subjects (12 clinical trials) did not demonstrate any significant adverse effects on glucose metabolism (Simon *et al.*, 2011). To date, there is no final conclusion about GlcN safety in diabetic individuals. Some authors advise that a non-significant reduction in glucose tolerance should be considered, and that more long-term studies are required to evaluate GlcN effect on diabetic and pre-diabetic patients before reaching a final conclusion (Dostrovsky *et al.*, 2011).

1.2.2. GlcN-drug interaction

Only few reports about GlcN drug interaction are available. A strong interaction was reported with warfarin, in which an increase in the international normalized ratio (INR) was observed after concurrent administration with either GlcN or GlcN/CS combination, which increases the risk of bleeding (Knudsen & Sokol, 2008). Some *in vitro* studies reported that GlcN may induce resistance toward some anticancer drugs, mainly doxorubicin, etoposide, and teniposide, but no clinical studies support these observations (Yun *et al.*, 1995).

1.3. GlcN and arthritis

1.3.1. Glycoproteins, glycolipids, and proteoglycans

Glycoproteins

Glycoprotein is a protein in which an oligosaccharide moiety is covalently attached to a polypeptide chain. Glycoprotein is usually classified into O-linked glycans (in which GalNAc is attached to the O-terminus of either threonine or serine) and N-linked glycans (in which GlcNAc is attached to the N-terminus of asparagine). Adding amino sugars to the protein moiety occurs in the endoplasmic reticulum and Golgi apparatus of the cells (Schachter, 1986). The formed glycoprotein then migrates to the cell surface where it either remains attached to the cell membrane, or may be excreted out of the cells. P-glycoproteins, α-acid glycoproteins, α and β immunoglobulins are examples of glycoproteins in the body that contain GlcNAc. The mucus secretion of the digestive and respiratory tracts is mainly glycoproteins of high sugar content which gives them their viscous properties (Berninsone, 2006).

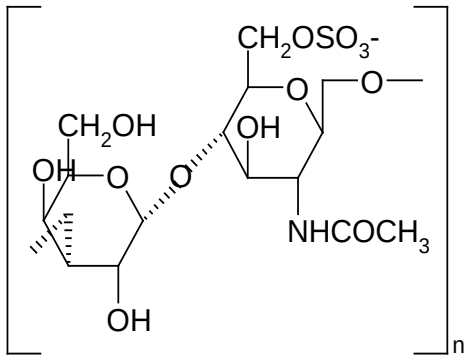
Glycolipids

Glycolipids are lipids that are covalently bonded to monosaccharides or polysaccharides. They are normally found at the outer surface of the cell membrane and associated with phospholipids. They have an important role in cell recognition and cell-cell communication. They also provide energy to cells. Sphingolipids and gangliosides are examples of glycolipids that contain GlcNAc or GalNAc (Maccioni *et al.*, 2002). The terminal sugar of the polysaccharide chain of glycolipids and glycoprotein at the erythrocytes cell membrane are essential for blood-group typing (Mehta, 1980).

Proteoglycans

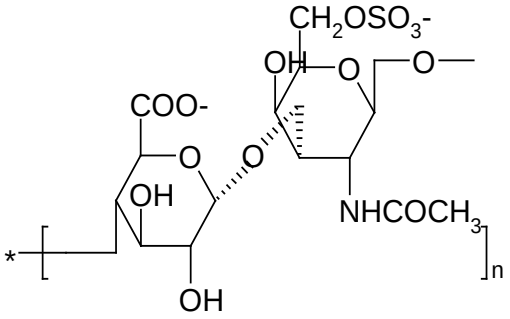
Proteoglycans are heavily glycosylated glycoproteins, composed of one or more GAGs covalently attached to a protein core. GAGs are mucopolysaccharides that consist of a long chain of repeating disaccharide units. The repeating unit is usually amino sugars, either GlcNAc or GalNAc that are attached to hexose or hexuronic acids (Figure 1.6) (Esko *et al.*, 2009). CS, keratan sulphate, dermatan sulphate, hyaluronic acid (HA), heparin, and heparin sulphate are all GAGs that present in the extracellular matrix of the cells and connective tissues. GlcN-6-P is considered the main precursor of all proteoglycans since, GalNAc is formed endogenously from GlcNAc by isomerization (Kelly, 1998).

Connective tissues are mainly composed of collagen and proteoglycans. The articular cartilage is a type of connective tissue that covers the ends of the bones and facilitates smooth gliding of bones at the articular joints. It functions as weight bearing, wear-resistant, and low friction material through its specific mechanical properties that allow it to resist compression and shear forces. Chondrocytes of the articular cartilage synthesize and excrete the matrix components (collagen II and proteoglycans) (Kuettner *et al.*, 1982). The balance between proteoglycans and collagen II components of the extracellular matrix of the articular cartilage gives the cartilage its mechanical properties (Asanbaeva *et al.*, 2007). Aggrecan, the major proteoglycan in the articular cartilage, is composed of CS and keratan sulphate attached to a protein core (Figure 1.8).



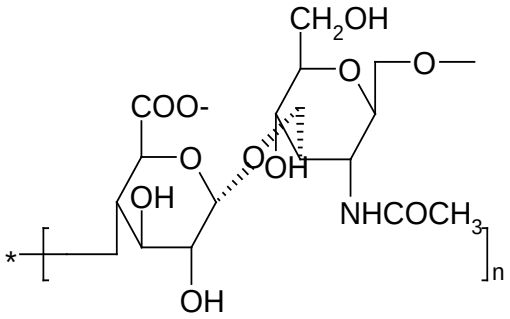
Keratan sulfate

D-Galactose N-acetyl-D-**glucosamine-**6-sulfate



Chondroitin 6-sulfate

D-Glucuronic acid N-acetyl-D-**galactosamine**-6-sulfate



Hyaluronate

D-Glucuronic acid N-acetyl-D-**glucosamine**

Figure 1.7. Chemical structure of the main glycosaminoglycans (GAGs) in the articular cartilage.

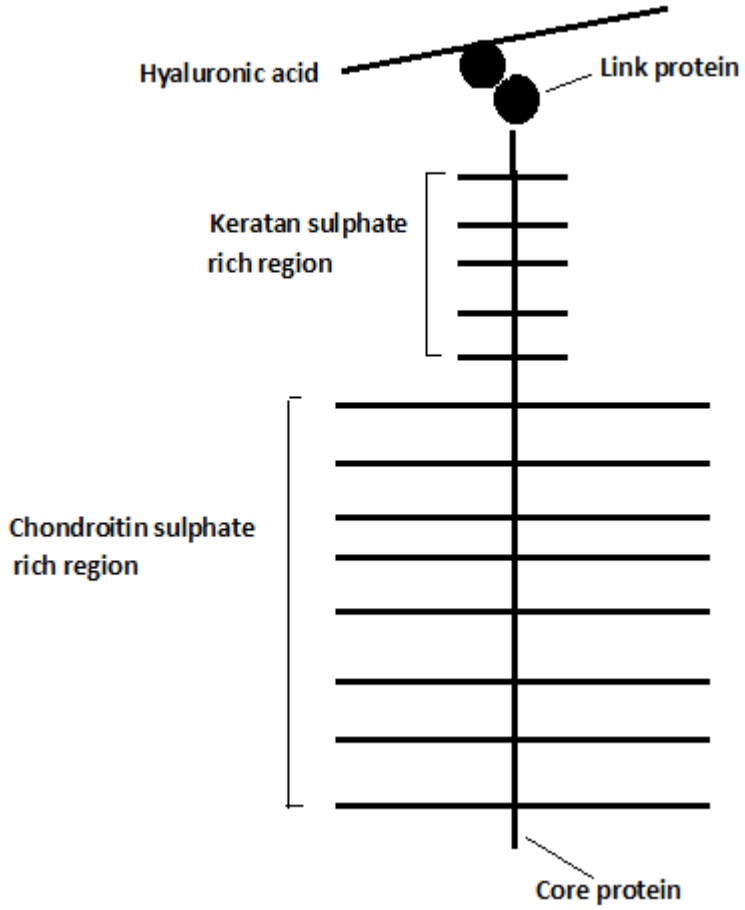


Figure 1.8. Aggrecan structure, showing the distribution of keratan sulphate and chondroitin sulphate around a protein core to form a feather like shape which bind to hyaluronic acid to form large aggregates.

This proteoglycan has a high affinity for HA, which is synthesized and secreted by the synovial membrane (Knox *et al.,* 1988) to form large aggregates. The sulphate ions provide fixed negative charges to the tissue and allow it to swell by attracting water and resisting compression. The collagen network resists the GAGs excessive swelling and gives the tissue its tensile stiffness, and strength. Any disruption of this balance can lead to loss of the articular cartilage function and integrity (Asanbaeva *et al.*, 2007)

1.3.2. Osteoarthritis

OA is a chronic degenerative disease of the joints characterized by excessive degradation of the extracellular matrix of the articular cartilage, and synovial inflammation and sclerosis of the subchondral bone (Felson *et al.*, 2000; Dieppe *et al.*, 2002). OA is considered the main cause of morbidity and disability among the elderly in Canada and the United States. Around three million (one of 10) Canadians are diagnosed with OA. The elderly are the most susceptible, with almost 30% of males and 40% of females over 70 diagnosed with a degree of OA (Kopec *et al.*, 2007).

Normally, cartilage tissue maintains its integrity and functionality by a balanced cytokine-mediated anabolic and catabolic process (the building of new components and degradation of old components). In OA, the balance is shifted toward catabolism, with an over-production of catabolic enzymes as aggrecanase and matrix metalloproteinase (MMPs). This results in cartilage degradation and releasing of higher levels of catabolic products into the synovial fluid. The process stimulates an over-expression of inflammatory cytokines (interleukin-1-

beta (IL-1β) and tumor necrosis factor-alpha (TNF-α)) by the synovial membrane and initiates an inflammatory reaction (Kim *et al.*, 2011). When the joint capsules are inflamed, the joints swell and stiffen, causing pain and stimulating further production of the catabolic enzymes and more degradation of the articular cartilage. In severe cases, joint mobility is lost, and complete joint replacement is required. The exact cause is unknown, but there are some known contributing factors. Trauma, excessive exercise, anatomic abnormalities, genetic factors, and metabolic disorders are some contributing factors (Felson *et al.*, 2000). There is also a strong association between body mass index and the incidence of knee OA (Jiang *et al.*, 2011).

OA is mostly seen in weight-bearing joints, specifically the hip and knee, but it can affect any other joint. The disease is usually asymmetric, which means the swelling and stiffness are usually observed in one knee or hip joint. The disease progression is slow and highly variable among patients; some patients diagnosed with OA stay in the mild stage for decades, while others deteriorate (Spector *et al.*, 1992; Dieppe *et al.*, 1997). Complications from OA are rare; however, disability and loss of independence can lead to loss of job loss and psychological problems.

To date there is no cure for OA; the treatment is based on alleviating the symptoms. Managing OA requires a combination of non-pharmacological measures (education, mild exercise, and weight reduction) and pharmacological therapy. Acetaminophen is usually the first line analgesic medications in mild non-inflamed cases. Recommendations for treating inflamed joints include topical

or oral NSAIDs, COX-2 inhibitors, and the intra-articular injection of corticosteroids. Opioid analgesics can be used to alleviate severe pain. The intra-articular injection of HA is also used to enhance cartilage regeneration; however it is not recommended by the American Academy of Orthopedic Surgeons (AAOS). Nutritional supplements as GlcN; CS; dimethyl sulfoxide (DMSO); MSM; Vitamin C, D and E; avocado soybean unsaponifiables; and S-adenosylmethionine are frequently used by OA patients, although their therapeutic benefit is not well established (Zhang *et al.*, 2010).

1.3.3. Rheumatoid arthritis

RA is a chronic, inflammatory, autoimmune disease of the synovial joints characterized by the progressive erosion of the articular cartilage and the underlying bone. The disease affects one to two percent of the population worldwide, leading to irreversible joint deformity, destruction, and loss of joint mobility (Firestein, 2005). RA is a disease of middle age (it usually starts at age 40-60), and women are two to three times more susceptible than men (Gabriel, 2001).

RA is an auto-immune disease in which the body's immune system attacks the synovial membrane of the synovial joint, leading to severe inflammation. What triggers the disturbance in the immune system is unknown; however, multiple genetic, environmental, and dietary factors may contribute to its etiology (Tobon *et al.*, 2010).

The exact pathogenesis is not well understood. It is believed that the disease starts when CD4+ T lymphocytes are activated by arthritogenic antigens in the synovial membrane, and stimulate monocytes, macrophages, and synovial fibroblasts to secrete MMPs enzymes and pro-inflammatory cytokines (IL-1, IL-6, and TNF-α). MMPs initiate the degradation of the cartilage and bone, while the pro-inflammatory cytokines cause inflammation of the synovial membrane (Feldmann & Maini, 1999; Smith & Haynes, 2002).

RA is mainly seen in the hand (metacarpophalangeal, proximal, and interphalangeal joints), wrist, and knee joints, but it can affect any articular joints. The disease is characterized by swelling and tenderness of the joint with severe morning stiffness that can last for one hour. It is symmetric (identical swelling and stiffness is observed in both hands and knees at the same time). As the disease progresses, joint deformity and loss of mobility usually occur. Fever, fatigue, malaise, weight loss, skin nodules (hard tissue lumps), and visual disturbance are symptoms associated with rheumatoid arthritis (Grassi *et al.*, 1998). The disease itself is not life threatening; however it induces systemic inflammation that trigger numerous complications, some of which are life-threatening. Lung fibrosis, renal amyloidosis, and cardiovascular complications are the most common fatal complications associated with RA (Husby, 1985; Roschmann & Rothenberg, 1987; Kaplan, 2006).

There is no cure for RA. The aim of the treatment is to reduce joint pain and prevent joint damage and loss of function. According to the American College of Rheumatology (ACR) guidelines (2002), after the diagnosis is

confirmed by a rheumatologist, treatment should start with patient education, and physical and occupational therapy. Pharmacological treatment is dependent on the disease severity, but usually starts with non-steroidal anti-inflammatory drugs (NSAIDs) to reduce pain and swelling. Gastro protective drugs are recommended to prevent NSAID-associated GIT side effects. COX-2 selective inhibitors are preferable than NSAID for treating inflammation because of the lack of GIT side effect. Intra-articular corticosteroids and/or low-dose systemic steroids are frequently used. The use of disease-modifying anti-rheumatic drugs (DMARDs) is now recommended even in early stages. Hydroxychloroquine (HCQ), sulfasalazine (SSZ), methotrexate (MTX) and leflunomide are common non-biological DMARDs used to treat RA. Infliximab, etanercept, and adalimumab are examples of anti-tumor necrotic factor alpha (anti-TNFα) medications, known as biological DMARDs (Newsome, 2002).

According to the 2008 ACR guidelines for non-biological and biological DMARD-use in RA, methotrexate or leflunomide are used for RA patients in early stages. The combination of methotrexate and hydroxychloroquine is required in moderate to high disease activity. The triple combination of methotrexate, hydroxychloroquine, and sulfasalazine is indicated with patients with moderate to high disease activity with poor prognosis. The use of anti-TNFα should be limited to those who did not respond to non-biological DMARDs (Saag *et al.*, 2008).

There are no statistics on using GlcN to treat RA. In adjuvant arthritis (AA), a widely used RA model, GlcN has shown to be effective in preventing the

disease (Hua *et al.*, 2005). Due to its safety record, GlcN should be considered as an alternative to other anti-inflammatory drugs. Unfortunately, data are not available on how effective the compound is on RA.

1.3.4. The GlcN debate

GlcN has been used therapeutically for arthritis since the 1960s. In spite of its popularity and how well it sells, its clinical effectiveness is still questionable (Block *et al.*, 2010; Aghazadeh-Habashi & Jamali, 2011). The ability of GlcN to improve OA symptoms and modify the disease progression has been tested on OA animal models. In most of these studies, OA was induced into the animal by anterior cruciate ligament transaction (ACLT) which highly mimic human OA (Tiraloche *et al.*, 2005; Wang *et al.*, 2007; Chen *et al.*, 2010; Naito *et al.*, 2010). In one study, oral administration of 100 mg of GlcN HCl/day for 8 weeks to rabbit model of OA (n = 16) starting from week 3 after surgery, lead to partial disease modifying, site specific effect. The loss of proteoglycans and the rate of disease progression were significantly lower in the lateral tibial (but not femoral) plateau cartilage of GlcN treated group compared to placebo treated group (Tiraloche *et al.*, 2005). In another study on rabbit OA model, oral administration of 100 mg GlcN HCl/day from day 1 after ACLT induction up to 8 weeks was able to decrease the rate of subchondral bone turnover, however, it did not cause significant changes in subchondral bone structure or mineralization (Wang *et al.*, 2007). In rat OA model, oral administration of 1000 mg/kg/day GlcN HCl from day 0-56 after ACLT caused a substantial decrease in collagen type II degradation (Naito *et al.*, 2010). Intra-articular injection of 0.5 ml of 2.5 mM GlcN to rabbit

knees at week 5 and 6 from induction of OA by vitamin A injection suppressed the development of the disease (d'Abusco *et al.*, 2007). Histological examination of the cartilage of GlcN treated group showed more homogenous chondrocytes cellularity and less fissures and fragmentation than the untreated group (d'Abusco *et al.*, 2007).

In a randomized, double blind, positive controlled study on OA dogs, oral administration of GlcN HCl and CS twice daily lead to significant ($p < 0.001$) decrease in pain score, weight-bearing and the disease severity by day 70. Nevertheless; the combination was less effective than the tested positive control (carprofen), as the symptoms improvement appears faster with carprofen administration (McCarthy *et al.*, 2007)

Although, animal studies suggest that GlcN has a potential for disease modifying and pain reliving activity. Most of the human clinical studies suggest that GlcN is not better than a placebo in improving joint function or decreasing OA pain (Block *et al.*, 2010; Wandel *et al.*, 2010; Markenson, 2011; Stuber *et al.*, 2011). One of the most famous clinical studies that aimed to determine the beneficial efficacy of GlcN HCl, CS, and the two in combination in improving OA symptoms was the Glucosamine/Chondroitin Arthritis Intervention Study (GAIT) (Clegg *et al.*, 2006). The wide-randomized, double-blind study was carried out on 1583 subjects from 16 clinical centers. All of the subjects had different degrees of knee OA. They were divided randomly into five groups. The patients in each group received either a placebo, 500 mg GlcN HCl three times daily, 400 mg CS three times daily, 500 mg GlcN HCl + 400 mg CS three times

daily, or 200 mg celecoxib daily. The results showed that celecoxib, which is an approved medication for OA treatment, has a 10% higher response rate (20% reduction in knee pain from baseline at week 24) than a placebo with $p < 0.05$, while this percentage was not significant with GlcN HCl or CS alone. However, a trend toward pain improvement in patients of moderate to severe knee OA was observed in patients who received the GlcN HCl and CS combination. Interestingly, the study reported a 60.1% response rate from the placebo (Clegg *et al.*, 2006).

The results triggered a two-year follow-up randomized study with radiographic criteria Kellgren/Lawrence [k/L] grade 2 or 3 and at least 2 mm joint space width (JSW) on 572 patients with moderate to severe knee OA. In this study, GlcN HCl alone was more effective in decreasing JSW loss than the combination (GlcN HCl + CS), although the difference didn't reach a statistically significant level (Sawitzke *et al.*, 2010). Patients with moderate OA were more responsive to GlcN HCl treatment than those with severe cases. Celecoxib did not show significant benefit in decreasing JSW loss (Sawitzke *et al.*, 2010). Possible reasons for the observed non-significant outcome, as explained by the authors, included the small sample size, the difficulty in measuring JSW in OA patients, and the unexpected small loss in JSW (Sawitzke *et al.*, 2010).

In 2009, The National Institute of Health Research (NIHR) carried out a systemic review to assess the clinical and cost effectiveness of GlcN in knee OA. The results demonstrated evidence on GlcN sulphate efficacy in decreasing joint pain and joint space loss, and improving joint function. However, the study did

not get to a final decision about GlcN cost effectiveness and recommended further researches on GlcN sulphate (Black *et al.*, 2009).

The problem with accurately measuring the OA progression as a factor in the non-significant clinical results of GlcN was addressed by Miller *et al*. in their comprehensive review about GlcN and CS (Miller & Clegg, 2011). Usually, OA progression is estimated from questionnaires about pain severity, functional activity and stiffness according to Western Ontario and McMaster Universities Osteoarthritis index (WOMAC), or Lequesne index. The test is highly subject to patient opinion, hence, associated with high variability. The progression can also be estimated by JSW measurements that require a full extension of the knee, which is not easy for OA patients (Miller & Clegg, 2011). A poor correlation between self-assessed OA symptoms and the radiographic joint changes was reported. Some patients who have radiographic knee OA are asymptomatic. In a seven-year follow-up study, 25% of the patients who reported pain improvement did not have any measurable radiographic changes, which highlight a possibility of false reporting of improvement due to patients adapting to the pain (Johnson *et al.*, 2007). The inability to accurately measure the disease progression in humans can highly affect a decision about GlcN efficacy and lead to the observed controversy.

GlcN is approved as a prescribed drug for treating OA in Europe. In Canada and the USA, the Food and Drug Administration (FDA) considers it to be a dietary supplement. Food supplements are not subject to clear regulations for their quality control and content uniformity as prescribed medicine. Adebowale *et*

al. studied the deviation of the actual content of GlcN and chondroitin sulphate in fourteen GlcN-containing products and eleven CS preparations from the claimed amount. A great variability was observed and the deviation percent reaches 115% in some cases. The highest deviation was found in some cheap products where the actual content was not more than 10% of the claimed amount (Adebowale *et al.*, 2000) Russell *et al.* did another study on fourteen commercially available human oral preparations of GlcN and obtained the same result with a variability range from 41-108% (Russell *et al.*, 2002). The results raise a serious question about the reliability of the systemic reviews that pooled data used different commercial products of GlcN.

Moreover, animal studies that show promising effects of GlcN used doses much higher than those used in the human clinical studies (range 100-1000 mg/kg in animals compared to 20 mg/kg in human) (Aghazadeh-Habashi & Jamali, 2011). Similarly, *in vitro* studies on articular tissues and cell lines, which suggested therapeutic benefits for GlcN in OA, used supra-physiological concentrations of GlcN (50-5000 μM) (Block *et al.*, 2010). The reported average maximum plasma concentration in humans after oral administration of the conventional dose (1500 mg) is only 10 μM (range 2.7-17 μM), indicating a possibility of patient under-dosing as a cause for the lack of significant clinical effect of GlcN on OA patients (Aghazadeh-Habashi & Jamali, 2011).

OA patients are usually elderly and obese (Hochberg *et al.*, 1995; Russell *et al.*, 2002; Sowers & Karvonen-Gutierrez, 2010), with or without other complicated diseases. To date, no study has addressed the effect of underlying

diseases and medications on the pharmacokinetics and pharmacodynamics of GlcN, which indeed can influence its therapeutic outcome.

1.3.5. How GlcN can improve osteoarthritis

Little information is available about the mechanism by which GlcN improves OA symptoms. For a long time, it was assumed that GlcN, by its downstream contribution in the HBP, stimulates GAGs production in chondrocytes, which helps regenerate the articular cartilage and slow down OA progression. This assumption was supported by *in vitro* studies that showed that GlcN can increase proteoglycan and HA production by human chondrocytes and synovial cells (Dahl & Husby, 1985; Bassleer *et al.*, 1998; Uitterlinden *et al.*, 2008; Igarashi *et al.*, 2011).

Although the former assumption is completely true when talking about endogenously formed GlcN, exogenous GlcN is not easily incorporated into GAGs. In a study on Swarm rat chondrosarcoma cells, the ratio of incorporation of exogenous to endogenous GlcN in UDP-GlcNAc was 1:375, indicating preferential incorporation of the endogenously formed GlcN (Sweeney *et al.*, 1993). In human chondrocytes, only 1.6% of galactosamine (GalN) and CS was derived from exogenous GlcN when the cells incubated with 12 μM ^{3}H-GlcN, this percentage increased with increasing exogenous GlcN concentration to reach 30% at 1 mM (Mroz & Silbert, 2004).

The metabolic role of GlcN and GlcNAc in GAGs and HA production by human chondrocytes was investigated using the radiolabeled compounds

(Shikhman *et al.*, 2009). The rapid uptake of GlcN by chondrocytes was observed, however, GlcN uptake causes a concentration-dependent inhibition of glucose uptake. Rapid phosphorylation of GlcN inside the cells led to the depletion of the cellular ATP pool and the inhibition of GLUTs insertion on the cell membrane and hence, inhibition of glucose uptake. It was expected that GlcN-6-P would enter into the HBP and stimulate the formation of GAGs and HA, but, the synthetic process was significantly reduced. In contrast, GlcNAc uptake by human chondrocytes was insignificant, nevertheless, it stimulates glucose uptake and the synthesis of GAGs and HA (Shikhman *et al.*, 2009).

GlcN has two opposing effects on the cartilage. On isolated human OA chondrocytes, GlcN at a concentration range of 1-150 μM caused a dose-dependent increase in aggrecan mRNA expression and aggrecan production (Dodge & Jimenez, 2003). In another study on human osteoarthritic cartilage explants, GlcN significantly inhibits aggrecan and collagen type II mRNA at 5 mM concentration, while this inhibition was not significant at 500 μM (Uitterlinden *et al.*, 2006). The above results show that GlcN is able to increase aggrecan production at concentrations up to 150 μM. Above this level it starts to possess inhibitory activity. It worth to mention that after the oral administration of GlcN sulphate, the maximum reported level found in the synovial fluid was 0.7 μM (Laverty *et al.*, 2005), which is lower than the concentration that can induce a harmful effect. However, it is not clear if this low concentration is capable to induce beneficial effect.

Nowadays, most of the studies on GlcN indicate anti-inflammatory activity of the compound. GlcN and CS and their combination at a concentration of 5 μg/mL and 20 μg/mL (27.9-112 μM) were able to decrease inducible nitric oxide (iNOS) and COX-2 mRNA expression in bovine articular cartilage explants treated with IL-1. These changes are associated with elevated nitric oxide (NO) and prostaglandin E2 (PGE_2) that play important roles in the inflammatory pathogenesis of OA (Chan *et al.*, 2005b). GlcN sulphate at a high concentration (1-5 mg/mL, 5.6-27.9 mM) was able to decrease MMP-2 and 9 by inhibiting the expression of urokinase-plasminogen activator (u-PA) and plasminogen activator inhibitor-1 (PAI-1) in the synovial fluid (Chu *et al.*, 2006). Moreover, GlcN sulphate at concentrations of 10-500 μg/mL (0.5-2.7 mM) inhibits the activity of the inflammatory mediators TNF-α, IL-1β and PGE_2 in osteoblast-like MG-63 cells, and promotes cell differentiation (Kim *et al.*, 2007). The anti-inflammatory activity of GlcN can explain its superior activity in moderate to severe OA, which are characterized by joint inflammation, and makes it more suitable for treating RA. However, the concentrations used in the previous experiments are still considered higher than the reported levels in the synovial fluid after the oral administration of GlcN. The exact mechanism of GlcN in OA treatment remains unclear and further studies are recommended.

1.4. The study rationale and objectives

Patient under-dosing is proposed as one of the reasons for the inconsistent clinical outcome and the insignificance results observed with GlcN in OA patients (Aghazadeh-Habashi & Jamali, 2011). Increasing the administered oral dose is expected to inhance the therapeutic benefit of GlcN. As the recommended oral dose of GlcN is already considered excessively large (1500 mg/day) and inconvenient due to its high frequency (3 X 500 mg caplets or 3 X 2 X 250 mg tablets), increasing either the daily dose or intake frequency seem impractical. A new formulation with improved bioavailabity is a good alternative. However, before designing such a formulation, it is necessary to have a good understanding of GlcN pharmacokinetics and the mechanisms of oral absorption.

GlcN is known to have a low oral bioavailability. Our previous lab work on the rat indicated that the gut, not the liver is behind the low oral bioavailability of GlcN, since i.p. administration of the compound led to 100% absolute bioavailability, while the absolute bioavailability was only 19% after oral administration (Aghazadeh-Habashi *et al.*, 2002b).

The mechanism by which the gut decreases GlcN oral bioavailability is unknown. Early studies on the radiolabeled GlcN intestinal uptake suggested the presence of facilitated transporters that mediate the process (Tesoriere *et al.*, 1972). The transporter-mediated uptake of GlcN was also observed in the brain and hepatic tissues (Plagemann & Erbe, 1973; Tan *et al.*, 1977). Some studies suggested that glucose facilitative transporters GLUTs are involved in GlcN uptake by the cells (Uldry *et al.*, 2002). The involvement of transporters in GlcN

intestinal absorption points to the possibility of a capacity-limited process behind the observed low oral bioavailability of GlcN.

Moreover, *in vitro* and *in vivo* studies on the rat indicated that exogenous GlcN can be utilized by the intestinal tissue for glycoprotein formation, which is the main constituent of the intestinal mucus secretion (Forstner, 1970). At the same time, some bacterial species (e.g. E coli and lactic acid bacteria) are able to deaminate GlcN-6-P and convert it to fructose-6-P by GNPDA for energy and carbon supply (Koser *et al.*, 1961; Oliva *et al.*, 1995). In the intestine, bacteria are abundantly present in the distal lumen (particularly ileum and colon). This fact prompted us to question the contribution of intestinal microflora in decreasing the oral bioavailability of GlcN.

Furthermore, little information is available about the effect of glucose on GlcN intestinal absorption. Biggee *et al.* followed the increase of plasma GlcN in 16 OA patients for 3 h after the oral administration of 1500 mg GlcN sulphate with and without 75 g glucose (during glucose tolerance test) and noticed a delay in the plasma appearance of GlcN accompanied by a non-significant increase in its plasma level (Biggee *et al.*, 2007a), The study attributed the delayed absorption of GlcN to the competition with glucose on the intestinal transporters. The study also suggested that glucose is competing with GlcN on hepatic glucose transporters and decreasing GlcN hepatic uptake, which resulted in the observed increase in the plasma level (Biggee *et al.*, 2007a). Actually, the competitive inhibition of GlcN uptake and transport by glucose and cytochalasin B (GLUT2 inhibitor) was reported earlier in studies conducted on GlcN uptake by rate

hepatoma cells and brain synaptosomes (Ebstensen & Plagemann, 1972; Tan *et al.*, 1977).

Expression of GLUT 1, 2, and 4 in Xenopus oocytes showed that the three transporters are able to transport glucose and GlcN, but only GLUT2 has higher affinity to GlcN (Uldry *et al.*, 2002). In enterocytes, glucose is absorbed from the intestinal lumen by sodium/glucose co-transporters SGLT1 (located at the brush border membrane) and then delivered to the bloodstream by GLUT2, which is located mainly at the basolateral membrane in the cells. However, at a high luminal glucose level, GLUT2 migrates to the brush border and participates in absorbing luminal glucose (Drozdowski & Thomson, 2006). If GLUT2 is transporting GlcN, then glucose level should play a role in stimulating or inhibiting GlcN oral bioavailability. Thus far, no study has been conducted to determine the exact role of GLUT2 in transporting GlcN through the gut.

1.4.1. The primary aim of the thesis

The primary aim of this work was to shed light onto GlcN absorption kinetics and the different factors that contribute to the low oral bioavailability of GlcN, using the rat as an animal model.

1.4.2. General hypothesis

Two factors contribute to the low gut availability of GlcN: the presence of capacity-limited transporter that mediates GlcN intestinal absorption, and the biotransformation or degradation of GlcN by the intesinal tissues and microflora.

1.4.3. Objectives

Objective 1. To develop a sensitive anaytical method for GlcN in biological fluids.

Objective 2. To confirm the the site of the first-pass loss of GlcN.

Hypothesis: Hepatic metabolism or bitransformation of GlcN contribute in decreasing GlcN oral bioavailability.

Specific objective:

To determine the hepatic availability of GlcN in the rat after the i.p. administration of small doses (10 and 50 mg/kg).

Objective 3. To investigate GlcN linear absorption kinetics.

Hypothesis: GlcN intestinal absorption is non-linear due to the presence of capacity-limited transporters, which highly limit its oral bioavailability.

Specific objectives:

a. To study GlcN pharmacokinetics in the rat after increasing the oral dose.
b. To study of GlcN absorption kinetics on the everted rat gut segments incubated with different concentrations of GlcN.

Objective 4. To determine intestinal tissue's contribution in decreasing the oral bioavailability of GlcN.

Hypothesis: GlcN is biotransformed or degraded by the intestinal tissue, which decreases its oral bioavailability.

Specific objectives:

a. To examine GlcN recovery after incubation with everted rat gut segments.
b. To determine the main site of absorption of GlcN.

Objective 5. To investigate intestinal microfloral contribution in decreasing GlcN oral bioavailability.

Hypothesis: Intestinal microflora metabolize GlcN and hence decreses its oral bioavailability.

Specific objectives:

a. To calculate the recovery of GlcN from its incubation with rat feces.

b. To compare the oral pharmacokinetics of GlcN in control and antibiotic-treated rats.

Objective 6. To study the role of glucose and glucose transporter (GLUT2) in GlcN intestinal absorption.

Hypothesis: Glucose and GlcN compete on the same intestinal transporter, hence, the glucose leuminal level can affect the gut availability of GlcN.

Specific objectives:

a. to examine the contribution of GLUT2 in GlcN intestinal absorption using everted gut sacs.

b. to determine the effect of high and low glucose levels on GlcN transport through everted gut sacs.

Objective 7. To inspect the effect of low fat food on the oral bioavailability of GlcN.

Hypothesis: Co-administration of GlcN with food can decrease its oral bioavailability.

Objective 8. To determine the effect of verapamil on GlcN oral bioavailability.

Hypothesis: Concurrent administration of other medications with GlcN may alter its oral bioavailability and, hence, its therapuetic outcome.

Specific objectives:

To compare the oral pharmacokinetics of GlcN taken alone or in combination with verapmil.

CHAPTER 2[1]

Improved sensitive high performance liquid chromatography assay for GlcN in human and rat biological samples with fluorescence detection

2.1. Introduction

GlcN (2-amino-2-deoxy-D-glucose) is an amino sugar that lacks a chromophore in its structure, hence it is undetectable in the ultraviolet (UV)-visible ranges. Early studies on GlcN pharmacokinetics have been carried out using the radiolabeled compound which could not distinguish between the parent compound and its biosynthetic or degradation product (Setnikar *et al.*, 1986). The first reported high performance liquid chromatography (HPLC) methods for determining GlcN in plasma were based on the derivatization of GlcN with phenylisothiocyanate (Liang *et al.*, 1999) or 1-naphthyisothicyanate (Aghazadeh-Habashi *et al.*, 2002a) to form a compound that can be detected in the UV region. Although those methods were considered sensitive, their lower limit of quantification (LLOQ) was not enough to study GlcN pharmacokinetics in animal models receiving low oral doses of GlcN, or in humans (LLOQ = 1.25 µg/mL) while the average Cmax in human plasma after 1500 mg dose is 1.8 µg/mL (Aghazadeh-Habashi & Jamali, 2011).

More recently, sensitive liquid chromatography methods have been reported that involve detection of the intact or derivatized GlcN product by

[1] *A version of this chapter has been published. Ibrahim & Jamali. 2010. J Pharm Pharm Sci. 13 (2):128-35.*

electrospray ionization (ESI) with mass spectroscopy (MS) (Table 2.1). Suitable instruments for these assays, however, are not commonly available.

In 2006, Zhang *et al.* reported an HPLC method with florescence detection for determining GlcN in human plasma after derivatization with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) (Zhang *et al.*, 2006b). The derivatization yields two anomer peaks for GlcN (stereoisomers of cyclic sugars that differ in their configuration at the anomeric carbon). This method was later modified by the addition of vertilmicin sulphate as an internal standard (Huang *et al.*, 2006b). The LLOQ of this method was 0.1 µg/mL, which is suitable for studying GlcN pharmacokinetics in human and rat biological fluids. Our attempts to utilize this method in our lab failed because of broad interfering peaks and the elevation of the HPLC pump pressure following repeated injections. The goal of this work was to improve and optimize the reported method for determination of glucosamine in the rat and human biological fluids.

Table 2.1. Different reported analytical methods of GlcN in animal and human plasma.

Technique	Plasma	Derivatizing agent	Linear range (μg/mL)	Ref.
HPLC-UV	Beagle dogs	Phenylisothiocyanate	1.25-20	(Liang *et al.*, 1999)
HPLC-UV	rat	1-naphthylisothiocyanate	1.25-400	(Aghazadeh-Habashi *et al.*, 2002a)
LC-ESI-MS	human	Phenylisothiocyanate	0.1-20	(Huang *et al.*, 2006a)
HPLC-ESI-MS/MS	human		0.01-1	(Roda *et al.*, 2006)
HPLC-Fluorescence	human	FMOC-Cl	0.01-10	(Zhang *et al.*, 2006b)
LC-ESI/MS/MS	horse		0.01-1	(Beaudry & Vachon, 2008)
LC-MS/MS	human		0.004-4	(Zhong *et al.*, 2007)
HPLC-Florescence	rat	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate	0.1-30	(Wang *et al.*, 2008)
HPLC-Electrochemical	human		0.01-2	(Pashkova *et al.*, 2009)

2.2. Experimental

2.2.1. Material and reagents

GlcN HCl, mannosamine HCl**,** amantadine HCl (1-aminoadamantane HCl, ADAM), and FMOC-Cl were purchased from Sigma-Aldrich Canada, LTd**.** (Oakville, ON, Canada). HPLC grade acetonitrile and water were obtained from Caledon Laboratories Ltd, (ON, Canada).

2.2.2. Solutions and standards

GlcN stock solution was prepared by dissolving 60.15 mg GlcN HCl in 100 ml water to yield 0.5 mg/mL GlcN free base. The stock solution was then kept in the fridge at 4ºC. At the analysis day the stock solution was further diluted with water to obtain a standard solution 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, and 20 µg/mL. Mannosamine HCl was used as an internal standard (IS) for which a stock solution was prepared by dissolving the appropriate amount in water to obtain 10 µg/mL. The derivatizing reagent solution (8 mM) was prepared freshly by dissolving 10.345 mg FMOC-Cl in 5 mL acetonitrile. A borate buffer 0.2 M was prepared by dissolving 6.18 g boric acid in 425 mL water followed by a pH adjustment to 8.5 by 10 M NaOH. A 300 mM solution of amantadine HCl (ADAM) was prepared by dissolving 281 mg in 5 mL 1:1 (v/v) acetonitrile-water.

2.2.3. Sample preparation and derivatization

Aliquots of 0.1 mL of human plasma were spiked with 50 µL of 10 µg/mL IS. Plasma proteins were precipitated by the addition of 200 µL acetonitrile

followed by 1 min vortex mixing and centrifugation for 3 min at 10 000g. An aliquot of 100 μL of the supernatant was then transferred to a clean dry test tube, and 50 μL of borate buffer 0.2 M was added, followed by of 50 μL of freshly prepared FMOC-Cl. This was followed by 1 min vortex-mixing and incubation in a water bath at 30^{o}C for 30 min. After incubation, 50 μL of ADAM was added to the test tubes to react with the excess derivatizing agent. The samples were then diluted with 1 mL acetonitrile-water (1:1) and 5 μL was injected into the HPLC system.

2.2.4. Chromatographic conditions

GlcN analysis was carried out using a Shimadzu prominence HPLC system (Mandel Scientific, Guelph, ON, Canada) consisting of a DGU-20A5 degasser, a LC-20AT pump, a SIL-20A autosampler, a CTO-20AC column oven, a RF-10AxL fluorescence detector and a CBM-20A communication bus module. The integration was performed using Shimadzu Class VP 7.4 software. Chromatographic separation was achieved on Phenomenex C18 (100 mm X 4.6 mm, id 3 μm) reverse phase column, guarded with a Phenomenex Security Guard Cartridge C_{18} (4 mm x 3 mm) column, both purchased from Phenomenex (Torrance, CA, United States). The mobile phase consisted of 0.1% glacial acetic acid in HPLC-grade water (solvent A) and acetonitrile (solvent B). A gradient elution was programmed as follows: 0-13 min, 23% solvent B; 13-15 min, solvent B increased gradually to 90%; 15-23 min, 90% solvent B; 23-25 min, solvent B is decreased gradually to 23%; 25-35, 23% solvent B. The flow rate was 1mL/min

and the column oven temperature was set at 40^{o}C. The detection was carried out at an excitation wavelength of 263 nm and an emission wavelength of 315 nm. The sample run time was 35 min. The peak area was used in all calculations.

2.2.5. Validation

Calibration samples were prepared by spiking aliquots of 0.1 mL plasma with GlcN to yield standard samples in the range of 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, and 20 μg/mL. The standard curves were constructed by plotting a GlcN: IS peak ratio versus the added concentration of GlcN using a least squares fitting procedure. Three calibration curves were constructed on the same day to determine intra-day variability. The method was repeated on three different days to determine the inter-day variability. The accuracy was determined from the % error.

% error = (average observed concentration – added concentration) x100/ add concentration.

The coefficient of the variation (CV%) was used to estimate the assay precision.

*CV% = (Standard deviation/ mean observed concentration)*100.*

2.2.6. Recovery

Solutions of 0.05, 0.5, 5, and 20 μg/mL of GlcN were prepared in plasma and in water and analyzed as mentioned before, each in triplicate. The percentage recovery of GlcN from plasma was estimated from *% recovery = GlcN peak area*

*in plasma sample/GlcN peak area in water sample)*100.* The recovery of mannosamine from plasma was also determined using the same approach.

2.2.7. Stability

Short term stability: The stability of the samples during analysis was tested using four standard samples of GlcN in plasma (0.05, 0.5, 5, and 20 µg/mL) Samples were prepared and derivative as mentioned before. The samples were analyzed at 0, 4, 8, and 24 h after derivatization. The % accuracy and CV% were calculated.

Freeze and thaw stability: Four standard samples of GlcN in plasma (0.05, 0.5, 5 and 20 ųg/mL) were prepared and kept in the freezer at -20^{o}C for 24 h. Samples were then removed from the freezer and allowed to thaw at room temperature. The samples were then refrozen for another 24 h. The method was repeated two more times. Aliquots of the standard samples were derivatized and analyzed after each freeze-thaw cycle, and the % accuracy and CV% were determined.

2.2.8. Application

The method was used to detect GlcN in human plasma separated from blood collected from the arms vein of a male adult at 0, 1 and 6 h after a single 3X500 mg oral dose of GlcN sulphate (Webber Naturals, Coquitlam, BC, Canada, Lot # 567521), and in plasma of 5 male Sprague Dawley rats separated from the jugular veins blood collected through surgically inserted catheters at 0, 0.25, 0.5,

0.45, 1, 2.5, 3, 4, 6 and 8 h after single oral doses of 200 mg/kg GlcN HCl. We also collected the total urine and feces output of the rats during 0-6 h post-dose.

2.3. Results

The HPLC chromatogram of blank human plasma (Figure 2.1a) shows a clean period between 7-18 min, in which almost no peaks of endogenous compounds were observed. Both GlcN and mannosamine (IS) appeared as equal size resolved anomer peaks. The retention times were 10.6 and 13.1 min for mannosamine α- and β-anomers, and 11.7 and 14.6 min for GlcN α- and β-anomers (Figure 2.1b &.c). There is no observed interference between GlcN and the IS peaks. However, there is interference between the GlcN first peak and an endogenous peak. Our trials to resolve this interference resulted in broad peaks and significant elongation of the elution time. Since the ratio of the anomer peaks of GlcN and mannosamine were identical, we preferred to use the second peak of each compound for our quantification analysis.

The assay was found to be linear over the examined range of 0.05-20 μg/mL in human plasma with a typical regression equation of $y = 0.307x + 0.003$ and a correlation coefficient of 0.999. We set the lowest limit of quantification at 50 ng/mL. The inter- and intra-day variations were less than 20% for the 50 ng/mL samples and less than 10% for other concentrations. The accuracy ranged from -1.72 to 2.46 (Table 2.2).

The percent recovery of GlcN from plasma was determined to be 119 ± 9.1%, 100 ± 4.1%, 97.2 ± 2.1% and 98.2 ± 1.8% for 0.05, 0.5, 5 and 20 μg/mL,

respectively. For mannosamine, the percent recovery from plasma was 95.9 ± 2. Derivatized GlcN was found stable during the analysis in all of the plasma samples. It was also stable in plasma after three freeze and thaw cycles (Table 2.3).

In human plasma, the baseline GlcN was below the detection limit. A single 1500 mg oral dose of GlcN yielded plasma concentrations of 184 ng/mL at 1 h post dose (Figure 2.1c), and 42 ng/mL at 6 h post-dose. In rat plasma endogenous GlcN was highly variable (range 0-0.25 µg/mL) (Figure 2.2), however, the method was suitable for detecting GlcN in rat plasma up to 8 h after the oral administration of 200 mg/kg of GlcN (Figure 2.4), which showed a concentration range from 0.1 to 7.43 µg/mL.

Applying the method to undiluted rat urine resulted in a crowded chromatogram. Ten times dilution was essential for detecting GlcN in urine (Figure 2.3a). A measurable concentration of GlcN was detectable in blank rat urine samples which, when corrected for the dilution factor, represents about 3 µg/mL. We did not carry out inter-day validation tests for GlcN in urine. However, the calibration curves using 10 times dilution of spiked rat urine samples was linear over the range (1-20 µg/mL), which represents 10-200 µg/mL when corrected for dilution. The intra-day CV% was less than 10% for all the tested concentrations (Table 2.4).

The method was applicable for the analysis of rat feces. The chromatograms generated using 10 or 100 times diluted fecal homogenate were identical to those observed when the compound was tested in water.

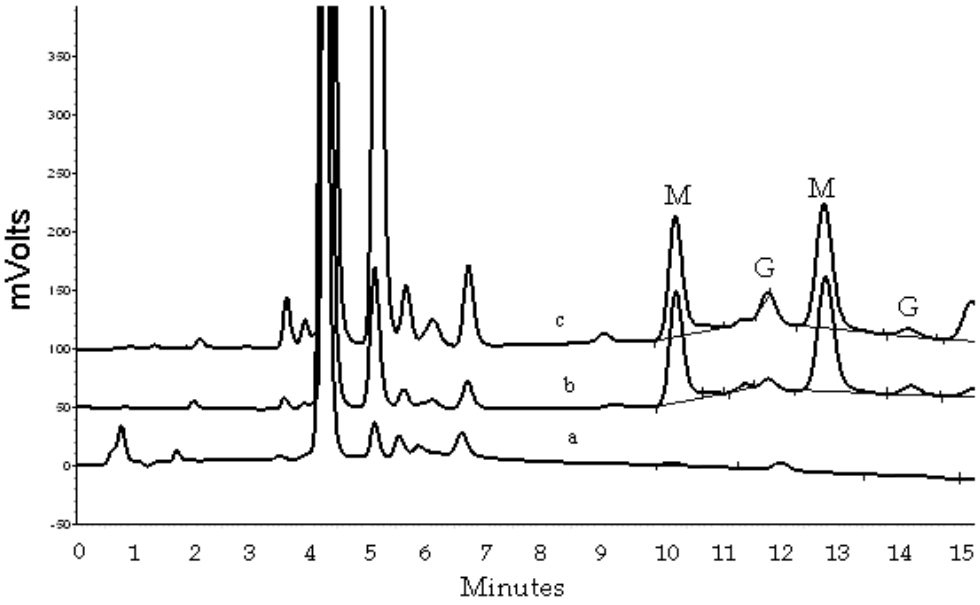


Figure 2.1. HPLC chromatogram of blank human plasma (a), blank human plasma spiked with 250 ng/mL GlcN (b), and plasma obtained from a healthy volunteer 1 h after the oral administration of 3X500 mg GlcN sulphate showing 184 ng/ml GlcN (c); G, GlcN; M, mannosamine.

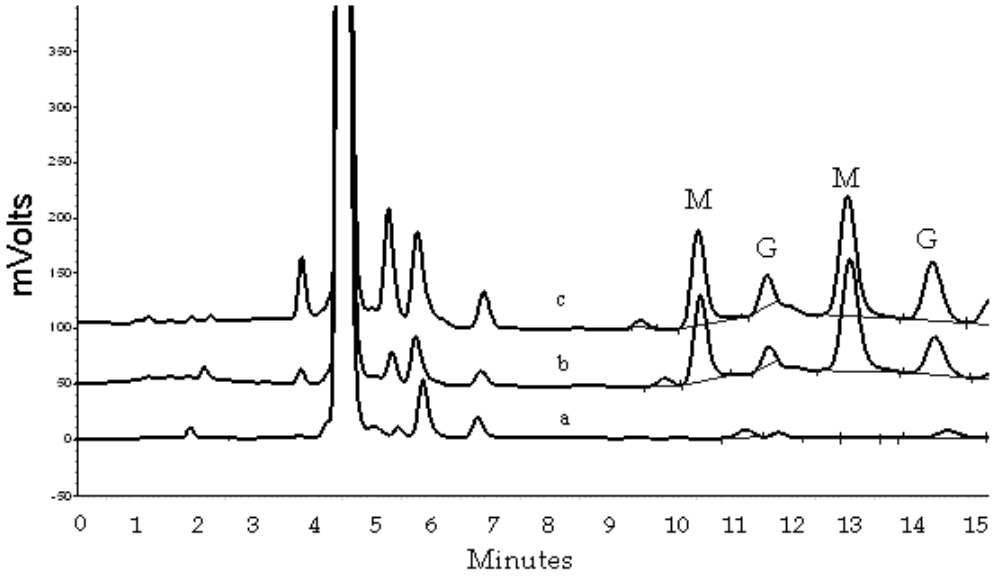


Figure 2.2. HPLC chromatogram of blank rat plasma (a), blank rat plasma spiked with 1ug/mL GlcN (b), and plasma obtained from a rat 2 h after the oral administration of 200 mg GlcN (c); G, GlcN; M, mannosamine.

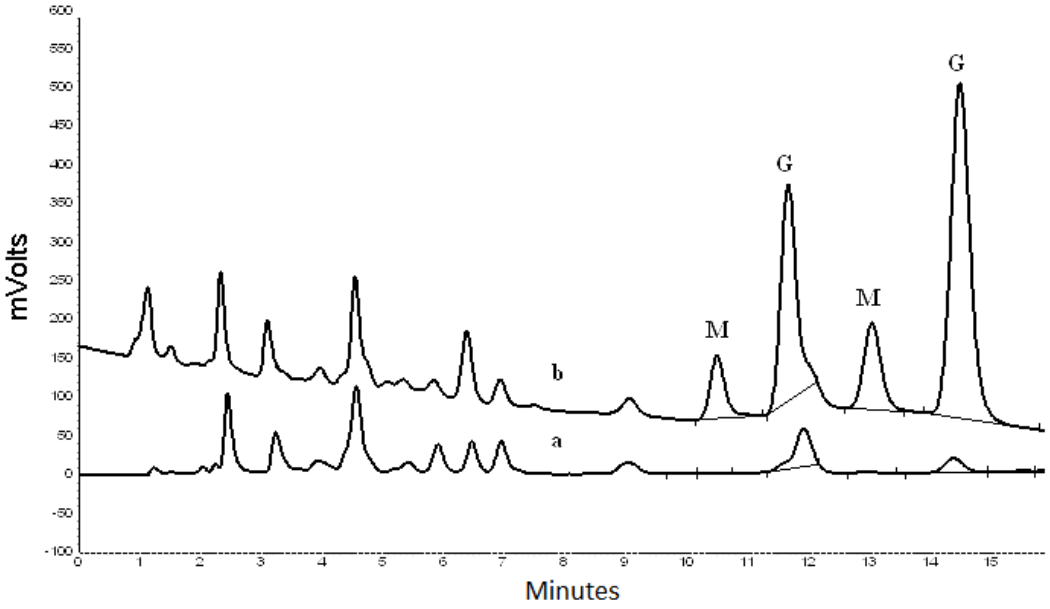


Figure 2.3. HPLC chromatogram of ten times diluted blank rat urine (a), and ten times diluted rat urine collected over 6 h after the oral administration of 200 mg/kg GlcN (b). G, GlcN; M, mannosamine.

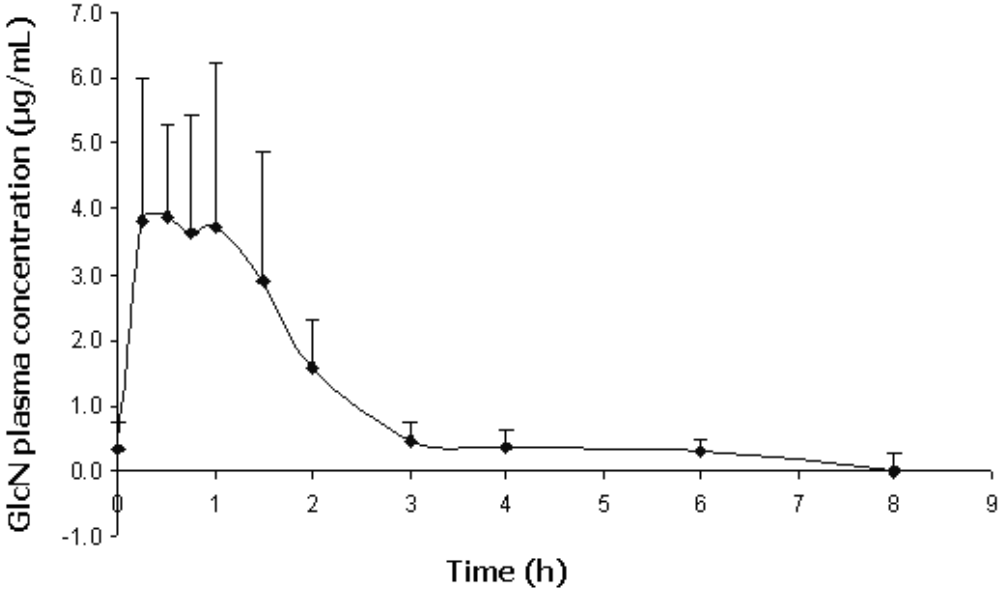


Figure 2.4. Average plasma concentration of GlcN in rats (n = 5) after the oral administration of 200 mg/kg dose.

Table 2.2. Precision and Accuracy of GlcN assay in human plasma.

Added Concentration (µg/mL)	Mean observed concentration (µg/mL)	CV%	% error
Intra-day			
0.05	0.051	2.49	1.21
0.5	0.491	4.87	-1.71
5	4.919	4.12	-1.62
20	20.491	2.33	2.46
inter-day			
0.05	0.049	4.86	-1.72
0.5	0.500	1.63	-0.03
5	5.007	1.70	0.14
20	20.30	1.24	1.49

Table 2.3. Stability of derivatized GlcN in human plasma samples

Added Concentration (µg/mL)	Mean observed concentration (µg/mL)	CV%	% error
During analysis (24h)			
0.05	0.058	11.23	12.7
0.5	0.482	1.41	0.5
5	4.913	1.54	-1.7
20	20.693	2.63	-1.3
Freeze-thaw stability (3 cycles at -20^{o}C)			
0.05	0.051	9.10	10.78
0.5	0.520	6.55	-3.08
5	4.904	1.21	-1.32
20	20.161	0.25	0.28

Table 2.4. Precision and accuracy of GlcN assay in rat urine

Added Concentration (µg/mL)	Mean observed concentration (µg/mL)	CV%	% error
Intra-day			
1	0.96	5.16	-3.73
2.5	2.60	3.83	4.08
5	4.78	5.37	-4.38
10	10.65	8.73	6.52
20	19.50	3.85	-2.49

2.4. Discussion

In our attempts to conduct a detailed pharmacokinetic study on GlcN, we used a previously reported method that involved formation of an FMOC-GlcN derivative (Zhang *et al.*, 2006b). Application of this method obtained a crowded chromatogram, in which GlcN peaks were trapped between two huge peaks of the endogenous compounds. Upon repeated injection, the large peaks gradually overlapped GlcN peaks. This was accompanied by an elevation in the HPLC pump pressure, which indicated the presence of contaminants. Moreover, the recovery of GlcN from plasma samples was only 38%. Attempts to improve the recovery by increasing the amount of the derivatizing agent resulted in more interfering peaks.

FMOC-Cl was first used as a derivatizing agent for amino acids in 1983. The advantage of this compound was that it had the ability to react rapidly with primary and secondary amines in alkaline media to form a highly fluorescent derivative (Einarsson *et al.*, 1983). This method was successfully used to analyze amino acids in protein (Vogt *et al.*, 1987; Golaz *et al.*, 1996). It was also utilized to analyze catecholamines in biological fluids (Descombes & Haerdi, 1992). In all the reported methods for analyzing amino compounds using FMOC-Cl, the aqueous part of the mobile phase was an acetate buffer, not pure HPLC water. Acidifying the mobile phase makes the acidic analytes (amino acids) less polar so they can retain on the reversed phase column for a longer time. Adding 0.1% acetic acid to the HPLC water mobile phase resulted in a very clean, reproducible chromatogram (Figure 2.1a). At the same time, we decreased the percentage of

acetonitrile in the first 13 min from 30% to 23% to allow a maximum separation of the peaks.

Huang *et al.* reported that FMOC-CL precipitated when acetonitrile concentration dropped below 30% (Huang *et al.*, 2006b). To avoid precipitation we injected aliquots of only 5 μL of samples after dilution with 1:1 acetonitrile-water. Under our conditions, most of the plasma interfering peaks eluted after the appearance of mannosamine HCl that we used as the internal standard. Mannosamine has a chemical structure and physical properties similar to glucosamine except in the amino group at the position 2 (Figure 2.5A). This group is equatorial in glucosamine and axial in mannosamine. This small difference in the three-dimensional orientation allows them to elute at different times (11.7 and 14.6 min for GlcN α- and β-anomers, and 10.6 and 13.1 min for mannosamine α- and β-anomers). The reaction with FMOC-Cl adds a large molecule to the amino group at position 2, and creates a steric hindrance that prevents any possible inter-conversion between GlcN and mannosamine (Figure 2.5B).

For the amino acid analysis, the excess derivatizing agent has been generally removed by extraction with pentane or by reaction with ADAM to form a hydrophobic complex that elutes towards the end of the run time (Gustavsson & Betnér, 1990). Since FMOC-Cl and FMOC-GlcN peaks are eluted at different times, Zhang *et al.* and Huang *et al.* did not need to apply the sample cleaning step (Huang *et al.*, 2006b; Zhang *et al.*, 2006b). However, under our experimental

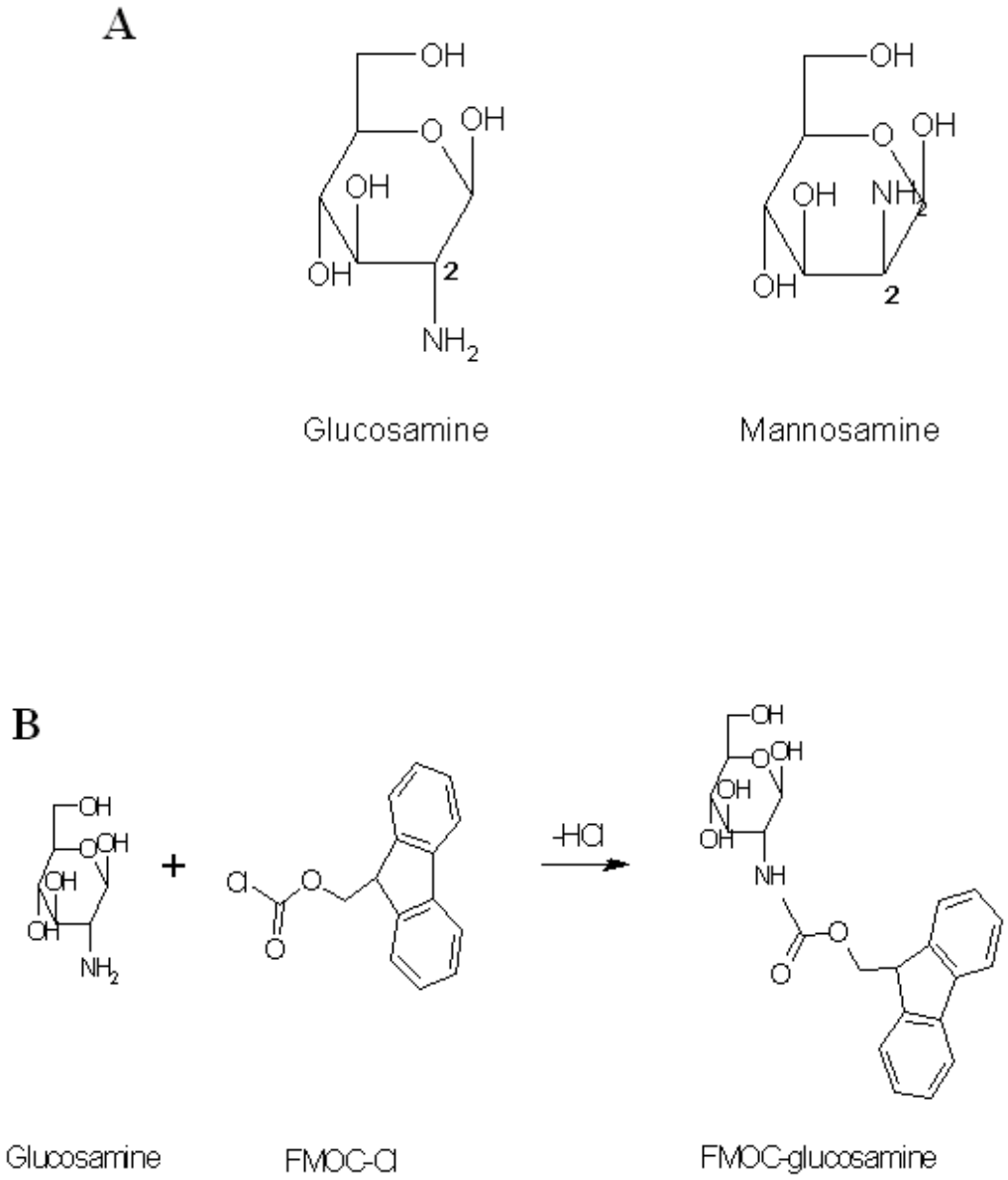


Figure 2.5. The chemical structure of GlcN and mannosamine, showing the difference in the space orientation of the amino group at position 2 (A), and the formation of FMOC-GlcN (B).

condition, we noticed a small peak that interfered with that of GlcN. This interfering peak was eliminated when we added ADAM. In addition, the sample cleaning rendered the baseline more stable even after doubling the amount of FMOC-Cl (from 4 mM to 8 mM), which enables us to achieve a lowest limit of quantification at 50 ng/mL and a percent recovery of 98% and 96% for GlcN and mannosamine, respectively.

The method is both accurate and reproducible. It is also more sensitive than the originally reported method (Zhang *et al.*, 2006b), and provides a more facile procedure than others (Roda *et al.*, 2006; Zhong *et al.*, 2007; Beaudry & Vachon, 2008). The application of the assay in pharmacokinetic studies of GlcN in the rat allowed the analysis of plasma concentrations up to 8 h post-dose. In our previous report we had analyzed the pharmacokinetics of GlcN only up to 4 h due to the lack of sensitivity (Aghazadeh-Habashi *et al.*, 2002b). The previously reported method (Aghazadeh-Habashi *et al.*, 2002a) did not detect baseline GlcN concentrations in the rat due to the inherent lack of sensitivity. In the present study, on the other hand, we did observe baseline GlcN levels but were generally low, but as high as 250 ng/mL in a single rat. This may raise questions about the reliability of the post 4 h concentration. We did not correct for the baseline values as we were not certain of the consistency of the endogenous baseline levels during the 8 h experiment. This is inevitable with sensitive assays developed for the determining endogenous compounds. For GlcN, the post 4 h concentrations are rather low; hence, their contribution to the overall area under the curve should be

negligible. However, they may introduce error in the calculation of the compound's terminal half-life.

The assay is also suitable for measuring GlcN in human plasma. We detected no baseline GlcN in the pre-dose human sample. Jackson *et al.* and Zhong *et al.* also did not detect baseline GlcN concentrations in their subjects (Zhong *et al.*, 2007; Jackson *et al.*, 2010). Roda *et al*., on the other hand, have reported a mean of 64 ± 47 ng/mL endogenous GlcN (Roda *et al.*, 2006). It is worth mentioning that the reported overall GlcN concentrations of Roda *et al*. are substantially greater that those reported by Jackson *et al.*, and Zhong *et al.* Nevertheless, similar to what we have observed in the rat, the possibility of endogenous GlcN must also be considered for interpreting human plasma samples containing low concentrations of the compound.

We did not completely validate the GlcN assay in urine. Nevertheless, this is the first reported HPLC method for determining unchanged GlcN in urine samples. The total GlcN excreted in human urine in 3 h has previously been determined by ion exchange chromatography with amperometric detection to be less than 1% of the administered dose (Biggee *et al.*, 2006). Our results suggest 1.2% cumulative urinary excretion in the rat over a period of 6 h after oral dosing. In this context, it is important to consider the relative bioavailability of oral GlcN doses (Aghazadeh-Habashi *et al.*, 2002b). The GlcN concentration in human plasma appears to be dependent on the administered product. The reported values following 1500 mg of GlcN HCl (Jackson *et al.*, 2010) or sulphate (Persiani *et al.*, 2005) are approximately three-fold different from each other. We found 184 and

42 ng/mL of GlcN in the plasma of a human after a single 1500 mg oral dose of the sulphate salt. The 6 h sample was below the set sensitivity of the assay, i.e., 50 ng/mL. However, we injected only 5 μL of the final solution to prevent pump pressure build-up. An increased volume of injection of only those samples with lower concentration, however, is not expected to cause pressure build-up but will improve the assay sensitivity. Our observed concentrations in human plasma samples are in agreement with those reported by (Jackson *et al.*, 2010). Nevertheless, the method offers sensitivity within the reported concentration ranges.

CHAPTER 3[2]

Factors affecting the intestinal absorption and oral bioavailability of GlcN

3.1. Introduction

Pharmacokinetic studies confirmed the low oral bioavailability of GlcN in human and in different animal species (Setnikar *et al.*, 1993; Adebowale *et al.*, 2002; Aghazadeh-Habashi *et al.*, 2002b; Du *et al.*, 2004). Setnikar *et al.* attributed the low oral bioavailability to the hepatic first pass metabolism. However, our lab's previous work on rats showed that GlcN is completely absorbed after i.p. administration, while only 19% reaches the systemic circulation when the compound is administered orally. The results pointed to the gut as the main cause of the observed low bioavailability (Aghazadeh-Habashi *et al.*, 2002b).

The mechanism by which the gut can lower the oral bioavailability of GlcN is unknown. Early studies on the intestinal absorption of radiolabeled GlcN suggest that its influx into the intestinal cells is mediated by facilitative transporters (Tesoriere *et al.*, 1972). Using Xenopus oocytes, the transporters involved have been revealed to be those that facilitate glucose absorption (GLUT1, 2, and 4), with GLUT2 demonstrating a 20-fold greater affinity for GlcN than that for glucose, albeit with a lower transporting capacity (km and Vmax values were 0.8 ± 0.1 mM and 3610 ± 520 pmol/oocyte/h, respectively for GlcN and 17-20 mM and 12,000 pmol/oocyte/h, respectively for glucose,) (Uldry *et al.*, 2002). The involvement of transporters in GlcN absorption raises the

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possibility of a capacity-limited intestinal absorption for the compound. This probability was addressed by Persiani *et al.,* who noticed a deviation from the linearity between the oral doses of crystalline GlcN sulphate and the corresponding AUC (Persiani *et al.*, 2005). A close examination of their data, however, revealed that a linear relationship exists between the dose and the AUC for 750, 1500, and 3000 mg doses, which raises questions about the involvement of capacity limited transporters in the observed low oral bioavailability of GlcN.

There is a possibility that the intestinal tissues biotransform GlcN and incorporate it into glycoproteins, which are the main constituent of the intestinal mucous secretion (Kohn *et al.*, 1962; Forstner, 1970). Intestinal tissues may also use GlcN as a source of energy through deamination and conversion to fructose-6-P, which then utilized by the glycolytic pathway. Moreover, intestinal bacteria may utilize GlcN as a source of carbon (Koser *et al.*, 1961).

In this work we used rats as an animal model as we tried to shed light on how capacity-limited transporters, intestinal tissue, and microflora contribute to decreasing the oral bioavailability of GlcN.

3.2. Materials and methods

3.2.1. Material

D**(+)**-GlcN HCl, galactosamine HCl, D-mannosamine HCl, neomycin trisulphate salt hydrate, tetracycline HCl, bacitracin, amantadine HCl (1-aminoadamantane HCl), FMOC-Cl (9-fluorenylmethoxycarbonyl chloride), cytochalasin B, quercetin dihydrate, and verapamil HCl were purchased from

Sigma-Aldrich Canada, LTD**,** (Oakville, ON, Canada). Cyclosporine A (Sandimmune® 50 mg/mL ampoules) was obtained from the Sandoz Pharmaceuticals Corporation (East Hanover, NJ, United States), HPLC grade acetonitrile and water were purchased from Caledon Laboratory Ltd (Georgetown, ON, Canada). All other chemicals and solvents were commercial products of analytical or HPLC grades.

3.2.2. Animals

The study protocol was approved by the University of Alberta Animal Care Committee. Adult male Sprague Dawley rats were used. Animals were housed in a temperature-controlled room at 21 ± 1ºC with 12 h of light per day and fed standard rodent chew food of 23% crude protein, 4.5% crude fat, 6% crude fibre, 8% ash, and 2.5% minerals.

3.2.3. Rat preparation and drug administration

Rats (250-300g) were cannulated under anaesthesia by inserting a silastic catheter into their right jugular vein (0.58 mm i.d. x 0.965 mm o.d.; Clay Adams, Parsippany, NJ, USA). They were allowed to recover overnight. Food was withdrawn from the animals 12 h before GlcN was administered. The next morning each rat was transferred to a separate metabolic cage for pharmacokinetic studies. GlcN solutions were prepared by dissolving equivalent amounts of GlcN HCl in water for oral dosing and in saline for parental administration.

3.2.4. Confirmation of the site of first-pass effect

Three groups (n = 3-5 rats, weight 250-300 g) of rats were dosed intravenously (i.v.) or intraperitoneally (i.p.). One group (n = 5) was dosed intravenously with 10 mg/kg of GlcN through the catheter followed by 0.5 ml of saline for rinsing. The other two groups received either 10 (n = 3) or 50 (n = 5) mg/kg of GlcN i.p. dose. Blood samples were withdrawn at 0, 0.083, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, and 6 h after dose into heparinised tubes. Plasma was separated immediately by centrifugation, and stored at -20°C until analysis. Urine was collected at 3, 6, 9 and 24 h post i.v. dose and kept frozen at -20 °C for analysis.

3.2.5. The pharmacokinetics of GlcN after increasing the oral dose

For studying GlcN absorption kinetics, rats were divided into three groups (n = 5-6 rats). Each group was administered, 200, 400 or 600 mg/kg GlcN by oral gavages. Serial blood samples were collected just before the oral dose and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 6 h after administration. Samples were collected into heparinised tubes, and plasma separated immediately by centrifugation, and stored at -20°C until analysis. Total rat urine and feces were collected for 6 h post-GlcN administration.

3.2.6. Everted gut sacs preparation

Adult male Sprague Dawley rats (weight, 350-400 g) were deprived of food with free access to water 12 h before the experiment. Rats were anesthetised with halothane and the intestine was exposed by abdominal midline incision. Four

10 cm sequential segments were removed 10 cm after the ligament of Treitz (jejunum) and immersed immediately into ice cold bicarbonate buffer (118 mM NaCl, 4.75 mM KCl, 2.50 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM MgSO4, 25 mM $NaHCO_3$, and 11 mM dextrose, pH 7.4). The segments were cleaned and everted using a glass rod followed by one end legation with a silk thread as previously described (Wilson & Wiseman, 1954). A silastic catheter (0.58 mm i.d. x 0.965 mm o.d.) was inserted into the other end and tied.

3.2.7. GlcN movement across the rat gut

Everted segments were prepared as before and filled with 2 ml of fresh oxygenated Kerbs-Henseleit bicarbonate buffer and placed in 40 ml of different concentrations of GlcN HCl (20, 40, 80, and 160 μg/ml) in a Kerbs-Henseleit bicarbonate buffer continuously aerated with oxygen (95%) and carbon dioxide (5%) in a perfusion apparatus at 37°C. Samples (0.5 ml) were withdrawn from the mucosal and serosal fluid at 0, 15, 30, 45, and 60 min and kept frozen at -20°C to be analyzed for GlcN. The collected volume was replaced with an equal volume of the buffer.

3.2.8. GlcN recovery from incubation with the rat everted gut segments

After 60 min of incubating the everted gut segments with 80 μg/ml GlcN as mentioned above, the segments were emptied, washed with saline, and weighed before freezing at -20°C. The total volume of the serosal fluid was determined and

samples were obtained from both serosal and mucosal fluid and kept frozen at -20°C to be used in studying GlcN recovery. The mass balance of what was lost from the mucosal fluid and gained to the serosal fluid was calculated. The total recovery was calculated from the total amount of GlcN in the serosal fluid, mucosal fluid, and gut sac tissue divided by the total amount added to the mucosal fluid at the start of the experiment.

3.2.9. Site specific absorption of GlcN

For determining GlcN transport through different parts of the intestine, 7 cm segments representing the duodenum (just below the ligament of Treitz), jejunum (20 cm away from the ligament of Treitz), ileum (above the cecum) and colon (below the cecum), were obtained from rat (n = 4, weight, 300-350 g). The segments were cleaned and everted as described under everted gut sacs preparation section. The everted segments were then incubated in 40 mL of 20 μg/mL of GlcN in a Kerbs-Henseleit bicarbonate buffer continuously aerated with oxygen (95%) and carbon dioxide (5%) in a perfusion apparatus as above at 37°C. Samples (0.5 ml) were withdrawn from the mucosal and serosal fluid at 0, 15, 30, 45, and 60 min and kept frozen at -20°C to be analyzed for GlcN. The collected volumes were immediately replaced with an equal volume of the buffer.

3.2.10. The influence of glucose, glucose transporter (GLUT2) and sodium dependent transporters on the intestinal absorption of GlcN

Four sequential jejunum segments were excised from each rat (n = 5-6, weight, 300-350 g) and prepared as previously mentioned and filled with 2 mL of the Krebs-Henseleit buffer. Two segments of each rat were incubated in 40 mL of the buffer, continuously aerated with oxygen (95%) and carbon dioxide (5%) in a perfusion apparatus as above at 37°C. The incubation medium contains 80 μg/mL GlcN HCl in presence of 0 or 100 mM of glucose, or glucose transporter GLUT2 inhibitors, cytochalasin B (0.1 mM) or quercetin dihydrate (0.1 mM). The other two segments (positive control segments) were incubated in Krebs-Henseleit buffer containing 80 μg/mL GlcN HCl.

To investigate the involvement of sodium-dependent transporters, the segments were incubated in a sodium-free medium in which sodium chloride of the Krebs-Henseleit bicarbonate buffer was replaced by choline chloride as previously described (Ganapathy & Radhakrishnan, 1980; Russell *et al.*, 1988). Samples (0.5 mL) from the serosal fluid were collected at 0, 15, 30, 45, and 60 min and kept at -20^{o}C for analysis. The samples were replaced immediately with the blank buffer. The rate and extent of the GlcN transport to the serosal side was then determined.

3.2.11. Involvement of the intestinal microflora in the low oral bioavailability of GlcN

Eighteen male Sprague Dawley rats were assigned to two groups (n = 9/group). One group was orally administered a combination of 100 mg/kg neomycin trisulphate, 50 mg/kg tetracycline HCl, and 50 mg/kg bacitracin twice

daily for two days, while the other group received saline using the same regimen. On the second day both groups were cannulated in the jugular vein as described above. They were given the last treatment dose and withdrawn from food for 12 h. When 200 mg/kg GlcN was administered orally, serial blood samples were collected into heparinised tubes just before and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 9, and 24 h post dose and kept frozen at -20°C for analysis. The total urine (0-9 h and 9-24 h) and feces outputs (0-24 h) were also collected and kept frozen at -20°C until analyzed for GlcN.

For *in vitro* determination of GlcN metabolism by intestinal bacteria, aqueous solutions equivalent to 1.25, 2.5, 5 and 10 mg of GlcN were added to 1 g feces and left overnight. The fecal suspensions were then analyzed for GlcN recovery.

3.2.12. The effect of food on the oral bioavailability of GlcN

To study the effect of food on GlcN bioavailability, a cross-over study was conducted in which rats were assigned randomly into two groups, fed and fasted (n = 5/group, weight 250-300 g). The fed group had free access to food and water prior to and during the experiment, whereas the fasted group was deprived of food 12 h prior and during the experiment with free access to water throughout. Both groups received 200 mg/kg GlcN by oral gavage, and serial blood samples were collected at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, and 6 h post administration. After a one-week washout period, the rats were re-cannulated in their left jugular vein and switched from one group to another.

3.2.13. The effect of verapamil on the oral bioavailability of GlcN

Ten male Sprague Dawley rats were assigned to two groups (n = 5/group, weight 250-300 g). The rats were cannulated in their right jugular vein as described above and deprived of food 12 h prior to the experiment with a free access to water. In the experiment day the rats were transferred into metabolic cages and one group (n = 5) was given an oral dose of verapamil HCl equivalent to 25 mg/kg verapamil. Two hours later each rat received 200 mg/kg GlcN. Serial blood samples were collected just before the oral dose and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, and 6 h after administration. Plasma samples were transferred to heparinised tubes and the plasma was separated immediately by centrifugation and stored at -20°C until analysis. Urine was collected over 9 h after dose. The pharmacokinetic parameters obtained from each group were then compared.

To investigate if P-glycoprotein transporters are involved in GlcN intestinal absorption another rat group (n = 4) was given an oral dose of P-glycoprotein inhibitor (cyclosporine A) 30 mg/kg, 2 h before the oral administration of 200 mg/kg GlcN. Plasma and urine samples were collected as mentioned before, and the pharmacokinetic parameters were calculated.

3.2.14. Analysis of the samples

The rat plasma and urine samples were analyzed by the HPLC method described in Chapter 2. Briefly, 100 μL of rat plasma or ten-times diluted urine samples were spiked with 50 μL of 10 μg/ml mannosamine as an internal standard. Plasma proteins were precipitated by adding 200 μL acetonitrile

followed by 1 min of vortex-mixing and 3 min centrifugation at 10,000 rpm. An aliquot of 100 μL of the supernatant was transferred into a test tube and 50 μL of borate buffer was added followed by 50 μL of freshly prepared FMOC-Cl. This was followed by 1 min of vortex-mixing and incubation in a water bath at 30^{o}C for 30 min. After incubation, 50 μL of amantadine HCl was added and samples were diluted with 1 ml of acetonitrile-water (1:1). Five μL were then injected into the HPLC system (Shimadzu prominence, Mandel Scientific, Guelph, ON, Canada). The chromatographic separation was achieved on a Phenomenex C18 (100 mm X 4.6 mm, id 3 μm) reversed phase column, using 0.1% acetic acid/acetonitrile gradient mobile phase at a 1 ml/min flow rate with a column oven temperature of 40^{o}C. Detection was carried out at an excitation and emission wavelength of 256 nm and 315 nm, respectively. The method was validated over the range of 0.05-20 μg/ml with CV < 15%.

Fecal pellets were placed in a 50 ml centrifuge tube, softened by adding 25 mL of deionized water and vortex mixed for 10 min before being centrifuged at 3000 rpm for 8 min; 1 mL of the fecal suspension was then transferred to a 1.5 microtube and centrifuged at 10,000 rpm for 5 min. The supernatant was immediately analyzed as described above for GlcN. The intestinal segments were allowed to thaw at room temperature before being transferred to a 50 mL centrifuge tube. The segments were homogenized in water (3 mL/ g wet tissue) using a Brinkmann Homogenizer (Kinematica AG, Littaulucerne, Switzerland) and the supernatant was then analyzed as previously described.

The everted sac fluid samples were analyzed by modification of HPLC-UV method reported previously by our lab (Aghazadeh-Habashi *et al.,* 2002a). In summary, 50 μL of the internal standard (galactosamine HCl) and 400 μL of acetonitrile were added to 200 μL of the samples in 2 ml centrifuge tubes and then vortex mixed and centrifuged before being passed through ion exchange columns (Extract Clean SPE SAX, Altech Association, Inc., 2051 Waukegan Rd., Deerfield, IL 60015) and evaporated. An aliquot of a derivatizing agent (200 μL of 88 mg 1-naphthylisocyothiate in 1ml of methanol, acetonitrile, triethylamine; 1:1:0.3) was added to the test tubes and left at room temperature for 20 min. The reaction was then stopped by adding 400 μL of 1.5% of acetic acid. The excess reagent was extracted by adding 1 ml chloroform. Subsequently, 100 μL of the upper aqueous layer was injected onto a reversed-phase isocratic HPLC system as previously reported. The method was linear over the range of 1.25-400 μg/ml, with the CV < 10 %.

3.2.15. Pharmacokinetic analysis

The data were analyzed using the non-compartmental method. The AUC was calculated using the log-linear trapezoidal rule. The elimination rate constant (λz) was calculated from the linear regression of the terminal phase and the $t_{½}$ from $0.693/\lambda z$. In general, plasma samples collected at or beyond 6 h have a GlcN concentration in the basal range; i.e., the AUC_{0-6} was approximately equal to the $AUC_{0-\infty}$. The total body clearance (CL) was calculated from dose/AUC_{0-6}. The renal clearance (CL_r) was calculated from the total amount excreted in the urine in

24 h after i.v. administration/AUC_{0-6}. Cmax and Tmax were the experimentally observed peak concentration and its time of attainment, respectively. The absolute bioavailability (F) following oral and i.p. doses was calculated from the equation

$$F = \frac{AUCpo\ or\ ip}{AUCiv} X \frac{DOSEiv}{DOSEpo\ or\ ip}$$

3.2.16. Statistics

Values were expressed as mean ± standard deviation. The statistical significance between two groups was examined using the two-tailed student's t-test. The difference between more than two means was examined using a one-way ANOVA followed by Tukey's multiple comparison tests. The significance level was set at $p < 0.05$.

3.3. Results

3.3.1. Confirmation of the site of first-pass effect

Following bolus i.v. doses, the GlcN plasma concentration declined rapidly in a multi-compartmental fashion and almost reached the basal levels in 2 h. GlcN is still detectable in plasma after 2 h, but with a low concentration and pronounced fluctuation (Figure 3.1). In spite of its rapid disappearance from plasma, GlcN is continually excreted in rat urine up to 24 h post i.v. administration (Figure 3.2). The cumulative amount excreted in urine represents 35.34 ± 12.24% of the administered dose. Determining the terminal half-life of the compound using the terminal three points of the plasma concentration-time curve was not feasible due to the observed fluctuation. Using the urinary excretion plot, GlcN t½ was 4.96 ± 3.45 h (Table 3.1).

After the i.p. doses, GlcN absorbed rapidly to reach its peak plasma level in less than 15 min, followed by a rapid decline. The decline pattern after a 10 mg/kg i.p. dose was almost identical to that observed after a 10 mg/kg i.v. dose (Figure 3.3). Increasing the i.p. dose from 10 to 50 mg/kg resulted in a proportional increase in the AUC_{0-6}. Calculating the hepatic availability revealed a complete absorption of the compound from its i.p. doses, as it was almost 100 % for both doses (Table 3.2). Following the oral doses, the F ranged from 0.05 to 0.06 (Table 3.2 and 3.3).

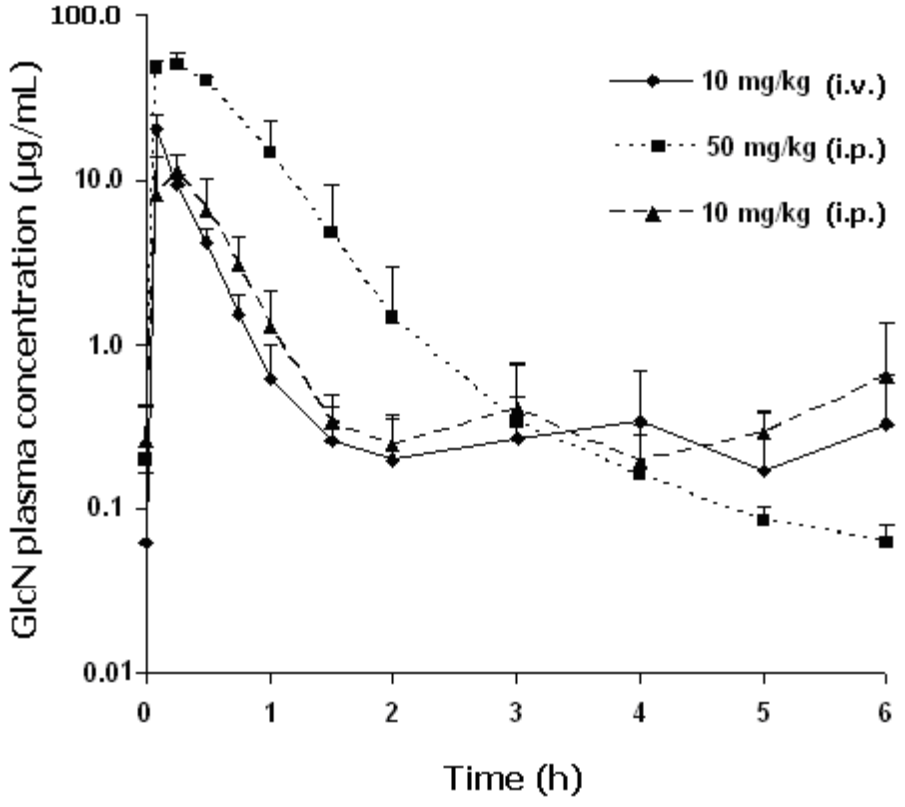

Figure 3.1. The mean plasma concentration- time curve of GlcN in male Sprague Dawley rats, after administration of 10 mg/kg (i.v.) dose, and 10 and 50 mg/kg (i.p.) doses. Except for 10 mg/kg (i.p.) (n = 3) all groups included five rats. The data represent the means ± SD.

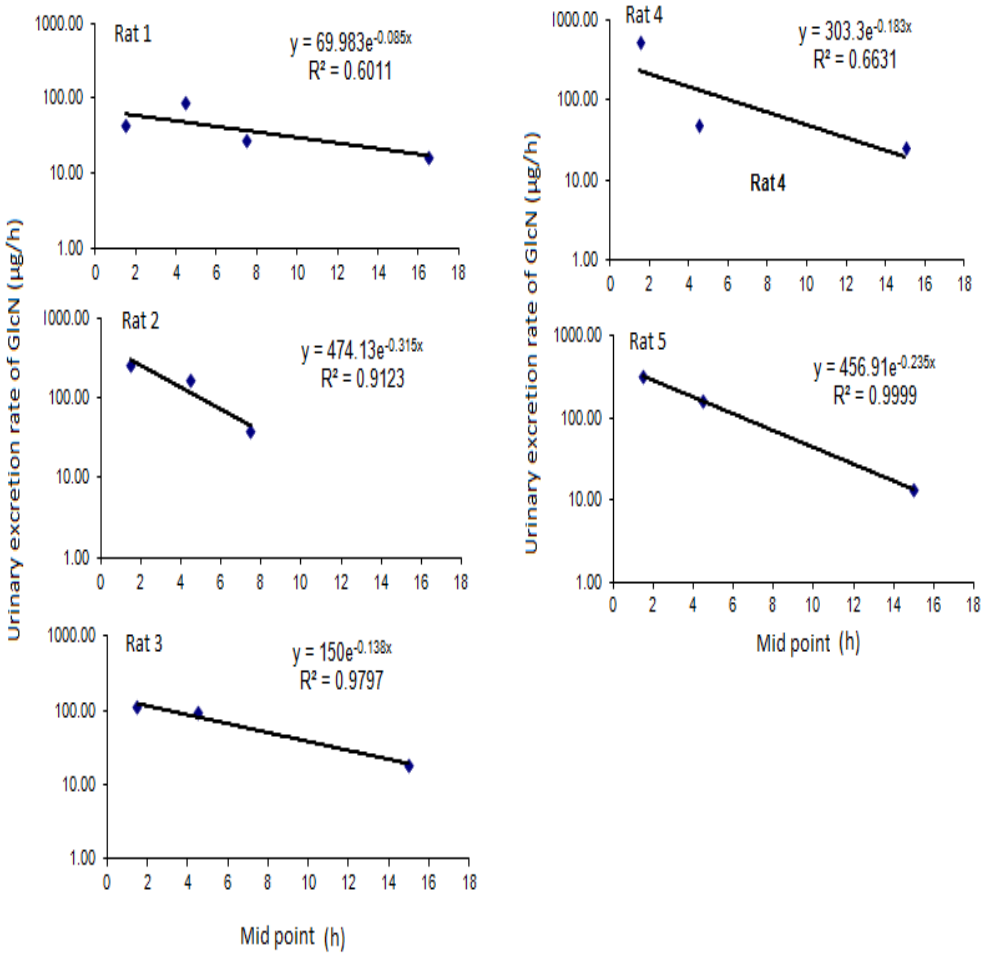

Figure 3.2. The urinary excretion rate plot of GlcN after i.v. administration of 10 mg/kg to male Sprague Dawley rats (n = 5). The urine was collected at time intervals up to 24 h post dose administration.

Table 3.1. GlcN pharmacokinetic parameters in the rat (n = 5) calculated from the urinary excretion rate plot after i.v. administration of 10 mg/kg GlcN. The data represent the means ± SD.

Parameter	**i.v., 10 mg/kg**
n	5
K, h^{-1}	0.19 ± 0.10
Kr, h^{-1}	0.07 ± 0.04
t½, h	4.96 ± 3.45
Fr	0.35 ± 0.06
% of dose excreted in urine in 24h	35.34 ± 12.24

n, number of rats; k, elimination rate constant; kr, renal elimination rate constant; t½, elimination half life; fr, fraction of dose eliminated in urine.

Table 3.2. GlcN pharmacokinetic parameters in the rat (mean ± SD) after different routes of administration

Parameter	**10 mg/kg (i.v.)**	**10 mg/kg (i.p.)**	**50 mg/kg (i.p.)**	**200 mg/kg (oral)**
n	5	3	5	5
C_o, μg/mL	0.07 ± 0.1	0.26 ± 0.17	0.2 ± 0.22	0.33 ± 0.42
T_{max}, h		0.19 ± 0.10	0.22 ± 0.07	0.80 ± 0.48
C_{max}, μg/mL		12.37 ± 1.54	51.38 ± 7.07	5.27 ± 2.36
Vc, mL/kg	478.3 ± 91.97			
AUC_{0-6}, μg.h/mL	7.81 ± 1.38	7.86 ± 1.70	38.24 ± 2.0	9.03 ± 2.63
CL, mL/min/kg	21.97 ± 3.9			
CL_r, mL/min/kg	7.63 ± 2.9			
t½, h	na	na	3.04 ± 1.55	2.85 ± 1.2
F		1.00 ± 0.22	0.98 ± 0.05	0.06 ± 0.02*

n, number of rats; T_{max}, time to reach maximum plasma concentration; C_o, plasma concentration at time zero; C_{max}, maximum plasma concentration; Vc, volume of distribution; AUC; area under plasma concentration-time curve; CL, total body clearance; CL_r, renal clearance; t½, elimination half life; na, not applicable**;** *, significant difference from i.v. ($p < 0.05$)

3.3.2. The pharmacokinetics of GlcN after increasing the oral dose

GlcN appears rapidly in plasma after it is orally administered. The AUC values proportionally increased with the dose elevation within the examined range of 200-600 mg/kg (Table 3.3 and Figure 3.3). A delay in the Tmax was observed with a 600 mg/kg dose. Substantial fluctuation was observed in the individual rat AUC profile compared to the i.v. curves (Figure 3.4). The terminal half-life of the 200 mg/kg dose is not significantly different from the value obtained from the urinary excretion data after the i.v. dose, while a significant decrease in the value of t½ was observed with the 400 and 600 mg/kg doses ($p < 0.05$, Figure, 3.5, Table 3.2)

A significant linear relation was found between the administered GlcN doses and the corresponding AUC_{0-6}, despite a great variability in AUC values, especially at the highest oral dose ($r = 0.892$; $p < 0.05$, Fig 3.6). A linear relationship was also observed between the Cmax and the examined dose range ($p < 0.05$).

Calculating the absolute oral bioavailability of the three oral doses showed that an average of 6% (range 2-10%) of the administered dose was able to reach the systemic circulation.

The percentage urinary excretion of GlcN was constant over the examined dosage range (Table 3.3). Approximately 1% of the administered oral dose was found in urine which, correcting for F, amounts to 10-50% of the amount absorbed.

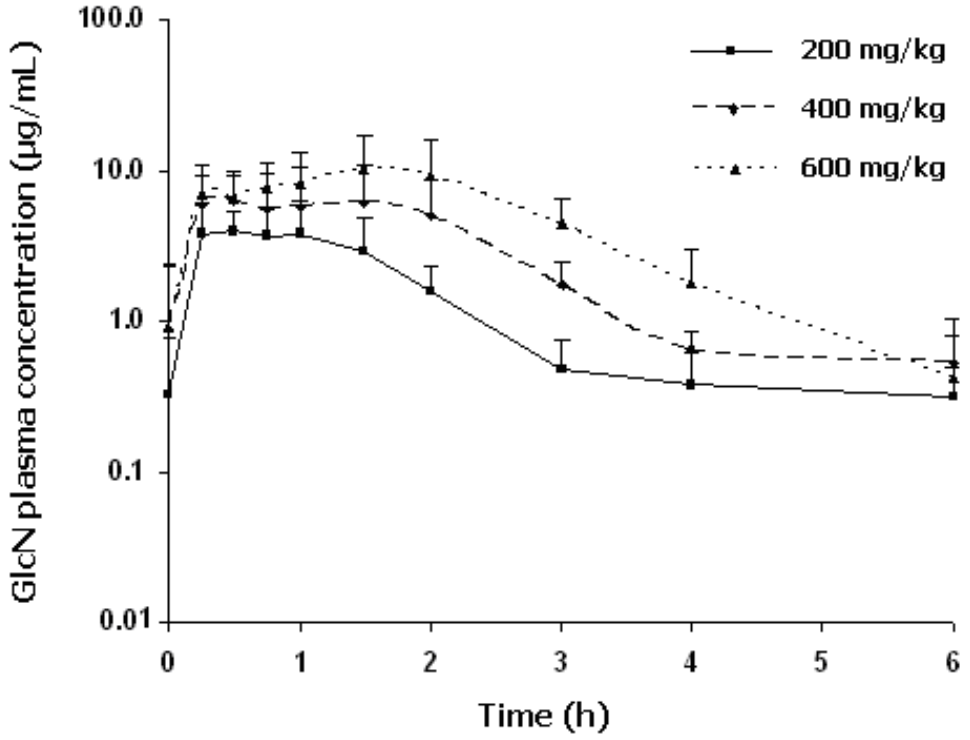

Figure 3.3. The mean plasma concentration-time curve of GlcN after the oral administration of 200, 400, and 600 mg/kg doses to male Sprague Dawley rat (n = 5-6). The data represent the means ± SD

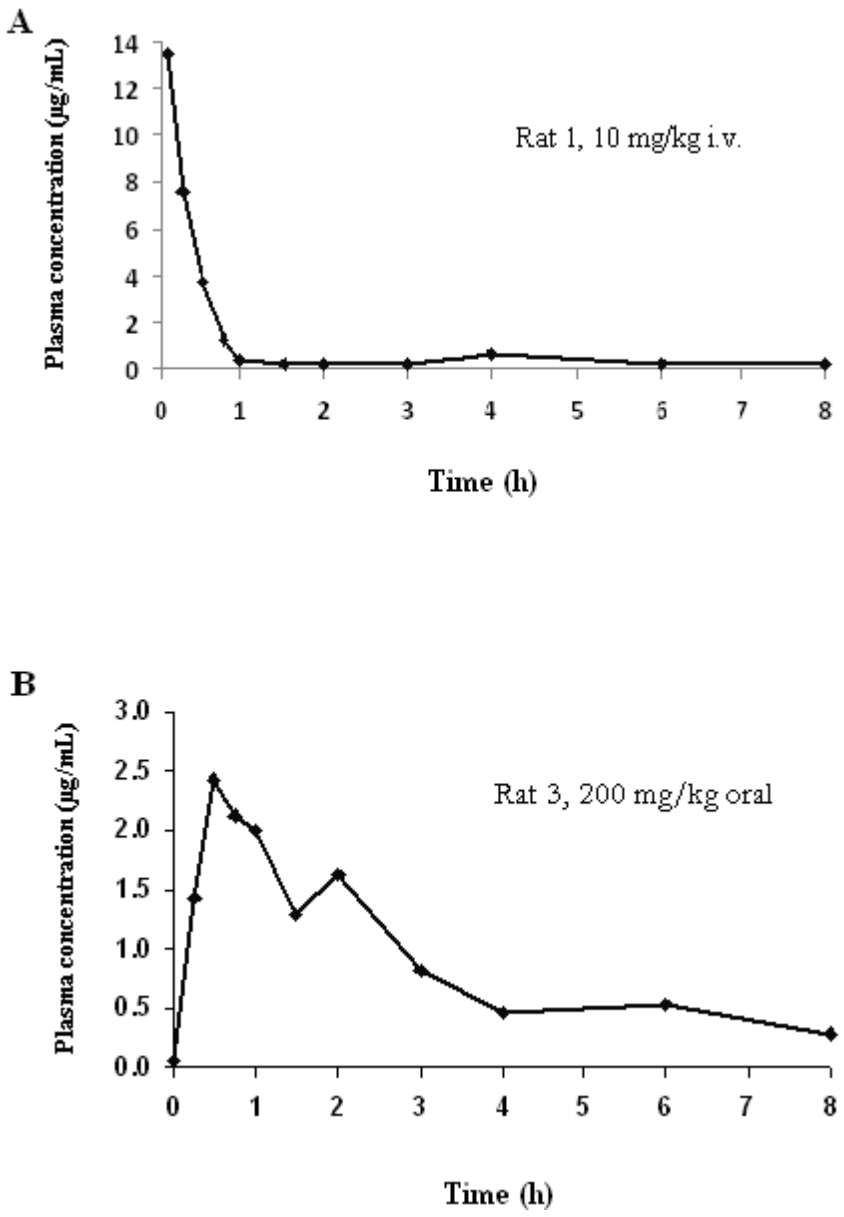

Figure 3.4. The plasma concentration-time profiles in typical individual rats after i.v. administration of 10 mg/kg GlcN (A) and oral administration of 200 mg/kg GlcN (B).

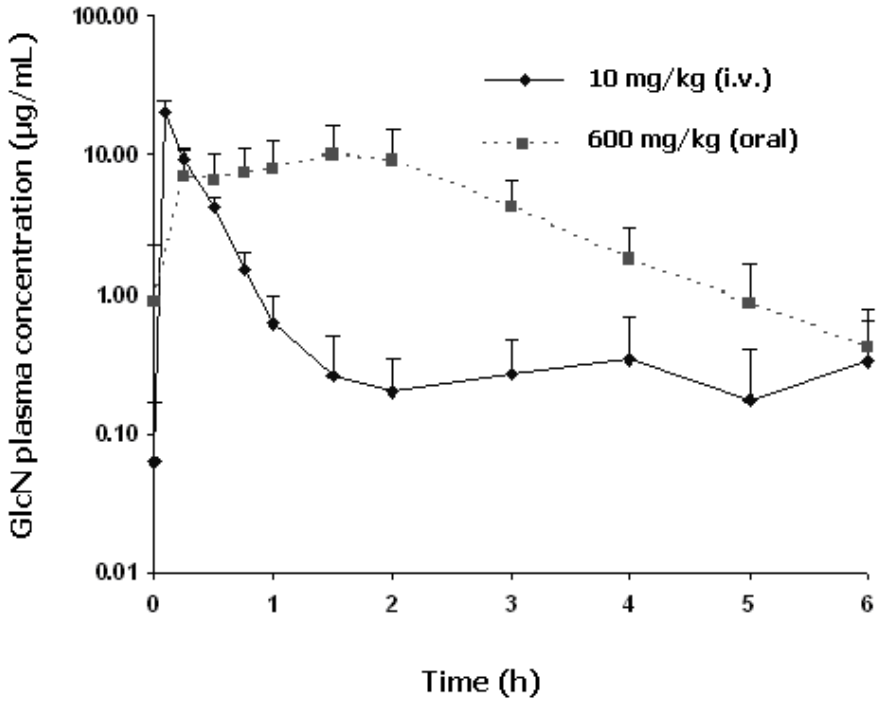

Figure 3.5. The mean plasma concentration vs. time plots of GlcN after an i.v. dose of 10 mg/kg (n = 5) and an oral dose of 600 mg/kg (n = 6) in male Sprague Dawley rat. The data represent the means ± SD.

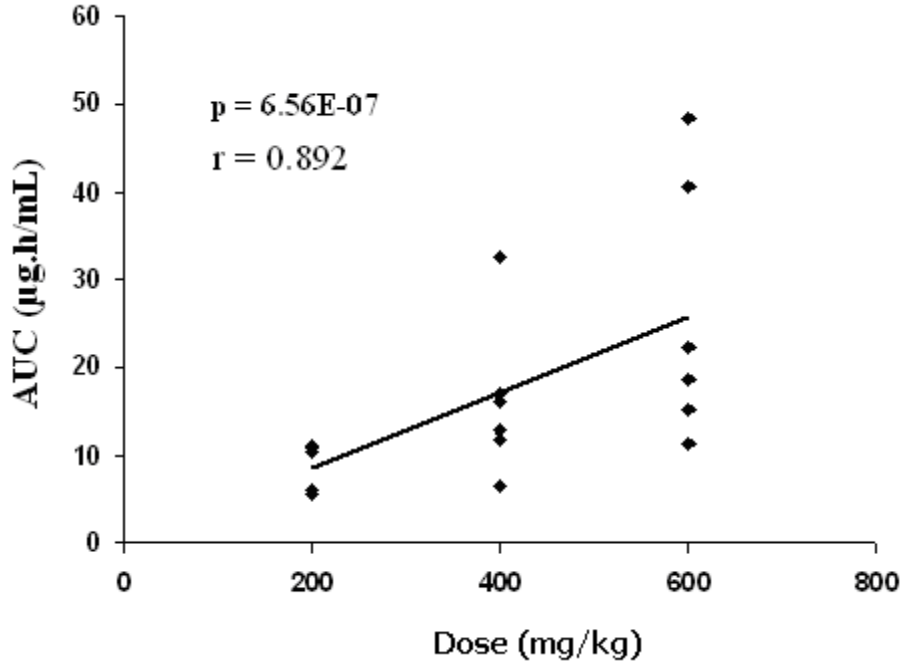

Figure 3.6. The linear regression plot of GlcN oral doses (200, 400 and 600 mg/kg) and the corresponding $AUC_{0\text{-}6}$ in the individual male Sprague Dawley rats. The plot shows a significant linear relationship ($p < 0.05$).

Table 3.3. GlcN pharmacokinetic parameters in the rat (mean ± SD) following oral administration of 200, 400 and 600 mg/kg doses

Parameter	**200 mg/kg**	**400 mg/kg**	**600 mg/kg**
n	5	6	6
C_o, μg/mL	0.33 ± 0.42	0.39 ± 0.68	0.9 ± 1.36
C_{max}, μg/mL	5.27 ± 2.36	8.38 ± 4.39	11.35 ± 6.75
T_{max}, h	0.80 ± 0.48	0.79 ± 0.62	1.13 ± 0.67
AUC_{0-6}, μg.h/mL	9.03 ± 2.63	16.12 ± 8.88	26.32 ± 14.82
K_{last}, h^{-1}	0.29 ±0.13	0.61 ± 0.32*	0.74 ± 0.26*
t½, h	2.84 ± 1.21	1.36 ± 0.51*	1.04 ± 0.40*
% of dose in urine (0-6 h)	1.2 ± 0.5	1.13 ± 0.2	1.13 ± 0.4
F	0.06 ± 0.02	0.05 ± 0.03	0.06 ± 0.03
CL/F, mL/min	158.63 ± 72.64	154.67 ± 80.38	147.10 ± 68.02

n, number of rats; T_{max}, time to reach maximum plasma concentration; C_o, plasma concentration at time zero; C_{max}, maximum plasma concentration; AUC_{0-6}; area under plasma concentration-time curve; K_{last}, elimination rate constant; t½, elimination half life; CL, total body clearance; F; absolute oral bioavailability; *, significant difference from 200 mg/kg ($p < 0.05$).

3.3.3. GlcN movement across the rat gut

GlcN passes through the everted rat gut. A proportional increase in the cumulative amount in the serosal fluid is observed with an increasing GlcN concentration in the incubation medium (Figure 3.7A). After 60 min, the concentration in the serosal fluid represents almost 10% of the initial concentration of the compound in the incubation media within the examined range (Figure 3.7B). A significant linear relationship is found between the accumulation rate of GlcN to the serosal fluid and its concentration in the incubation medium ($r = 0.84$; $p < 0.05$; Figure 3.8A), indicating linear absorption kinetics.

The passage of GlcN through the gut membrane appeared to be site-specific, as a significantly higher permeability was noticed form the duodenum in comparison to the other intestinal parts ($p < 0.05$, using ANOVA followed by Tukey's multiple comparison test) (Figure 3.9).

A substantial loss of GlcN was observed when the compound was incubated with the everted rat segments. After the incubation of 80 μg/ml of GlcN HCl with the everted rat segment for 60 min, an average of 14.52 ± 6% could not be accounted for in the mass-balance determination (Figure 3.10).

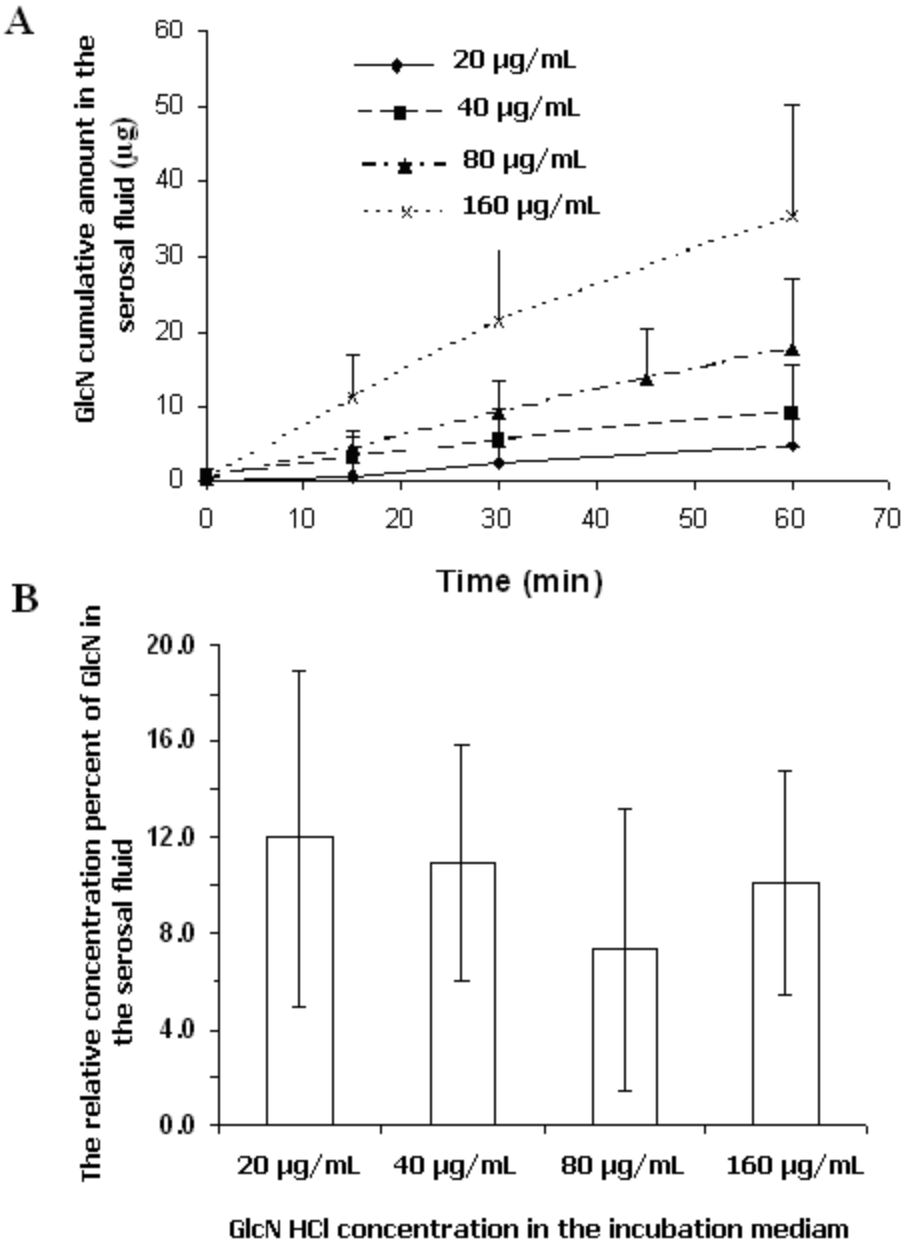

Figure 3.7. GlcN transport through the everted rat segments. Showing the average increase in the cumulative amount of GlcN in the serosal fluid over 60 min of incubation of the everted rat gut (n = 5 rats, 4 segments/rat) with 20, 40, 80 and 160 µg/mL GlcN HCl (A), and the average relative concentration percent of GlcN in the serosal fluid to the mucosal fluid after 60 min incubation (B). The data represent the mean ± SD.

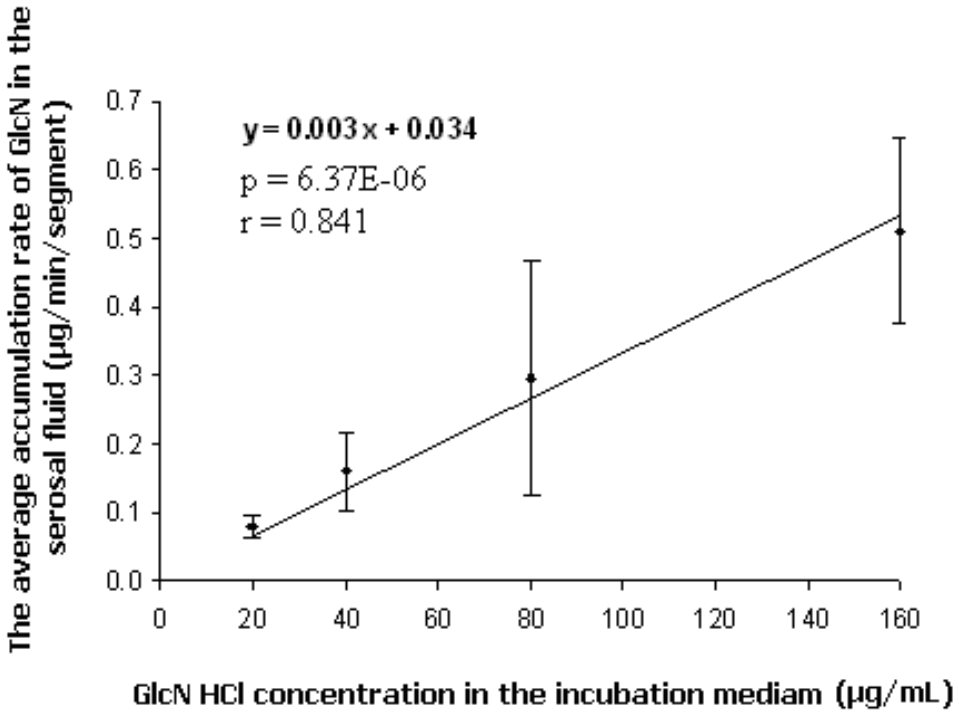

Figure 3.8. The linear relationship between the average accumulation rate of GlcN in the serosal fluid of the everted rat gut segments and GlcN HCl concentration in the incubation medium. The data represent the mean ± SD (n = 5 rats per group, 4 segments/rat)

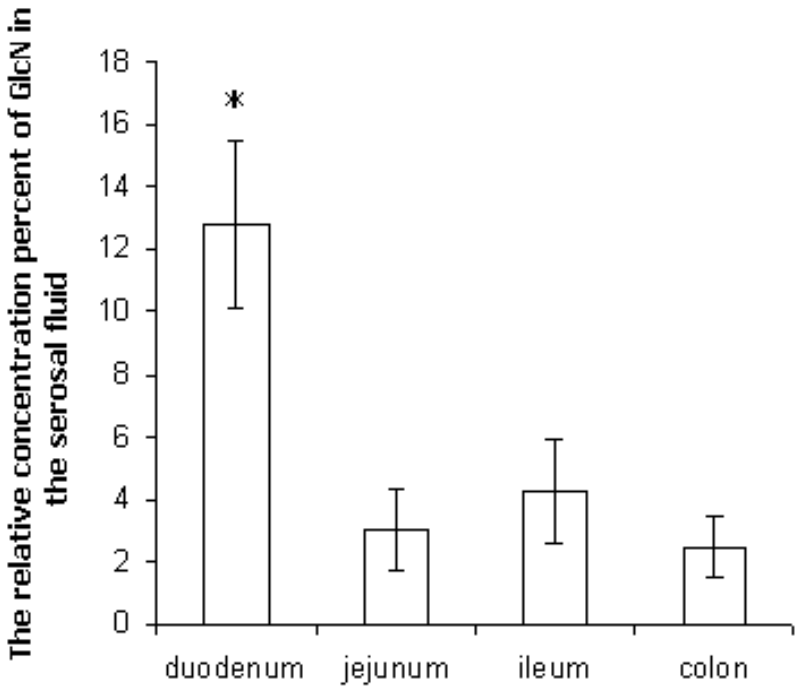

Figure 3.9. The permeability of GlcN through the different intestinal parts of the rat. The graph represents the average relative concentration of GlcN in the serosal fluid of the everted gut segments of the rat (n = 4). The segments excited from the rat intestine representing the duodenum (just below the ligament of Treitz), the jejunum (20 cm away from the ligament of Treitz), the ileum (above the cecum), and the colon (below the cecum). The segments were everted and filled with Krebs-Henseleit buffer and incubated with 20 µg/mL GlcN for 60 min in a perfusion apparatus at 37^{o}C and continuously aerated with O_2/CO_2 (95%:5%). *Statistically significant difference of the duodenum from the other parts ($p < 0.05$) using ANOVA followed by Tukey's multiple comparison test.

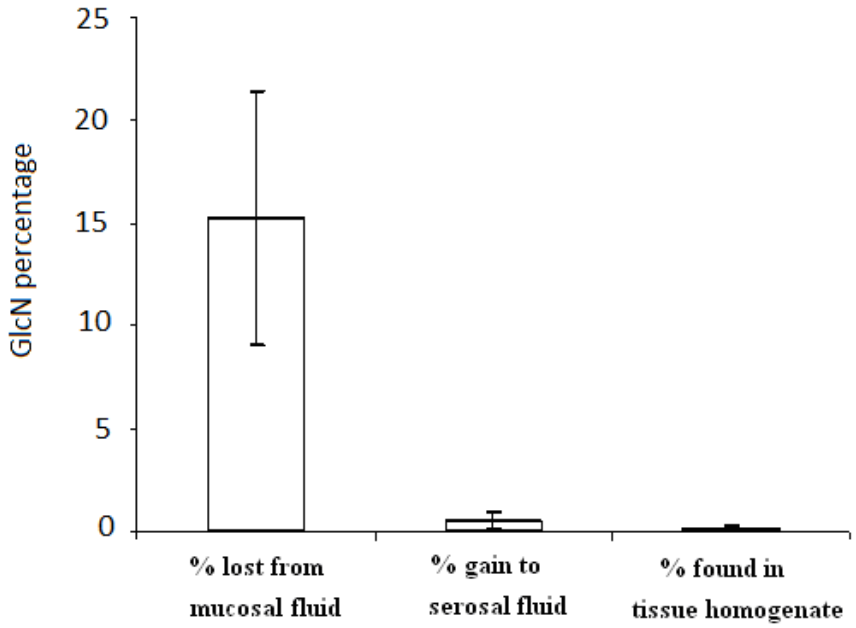

Figure 3.10. GlcN recovery after incubation with the everted rat segments. Segments from the rat jejunum (n = 5 rats, 2 segments/rat) were excited, everted, and filled with Krebs-Henseleit buffer, then incubated with 80 µg/mL GlcN HCl in Krebs-Henseleit buffer for 60 min in a perfusion apparatus at 37°C and continuously aerated with O_2/CO_2 (95%:5%). The serosal fluid, mucosal fluid, and the segment tissues were analyzed at the end of the incubation period to determine the total recovery of GlcN.

3.3.4. The influence of glucose and glucose transporter (GLUT2) and sodium dependent transporters on the intestinal absorption of GlcN

The incubation of the everted rat segments with 80 μg/mL GlcN for 60 minutes in the presence of 0.1 mM cytochalasin B (GLUT2 inhibitor) decreased the rate and extent of accumulation of GlcN in the serosal fluid; however, this decrease was not statistically significant. On the other hand, adding 0.1 mM quercetin dihydrate (a potent specific GLUT2 inhibitor) to the incubation media significantly decreased the accumulation rate ($p < 0.05$), from 0.29 ± 0.13 μg/min/segment for GlcN alone to 0.12 ± 0.04 μg/min/segment for GlcN in the presence of the GLUT2 inhibitor, which is almost half the former value. Nevertheless, inhibiting GLUT2 did not completely block GlcN movement from the mucosal to the serosal fluid (Figure 3.11A & B). There is no apparent potential interaction or competition between glucose and GlcN on the intestinal absorption, as a non-significant difference in the rate and extent of accumulation of GlcN in the serosal fluid was observed when the segments were incubated with 0, 10, and 100 mM glucose (Figure 3.12).

Moreover, non-significant difference in the rate and extent of accumulation was observed by incubating the segments with GlcN in a sodium-free Krebs-Henseleit buffer in which sodium chloride was replaced with an equivalent amount of choline chloride (Figure 3.13).

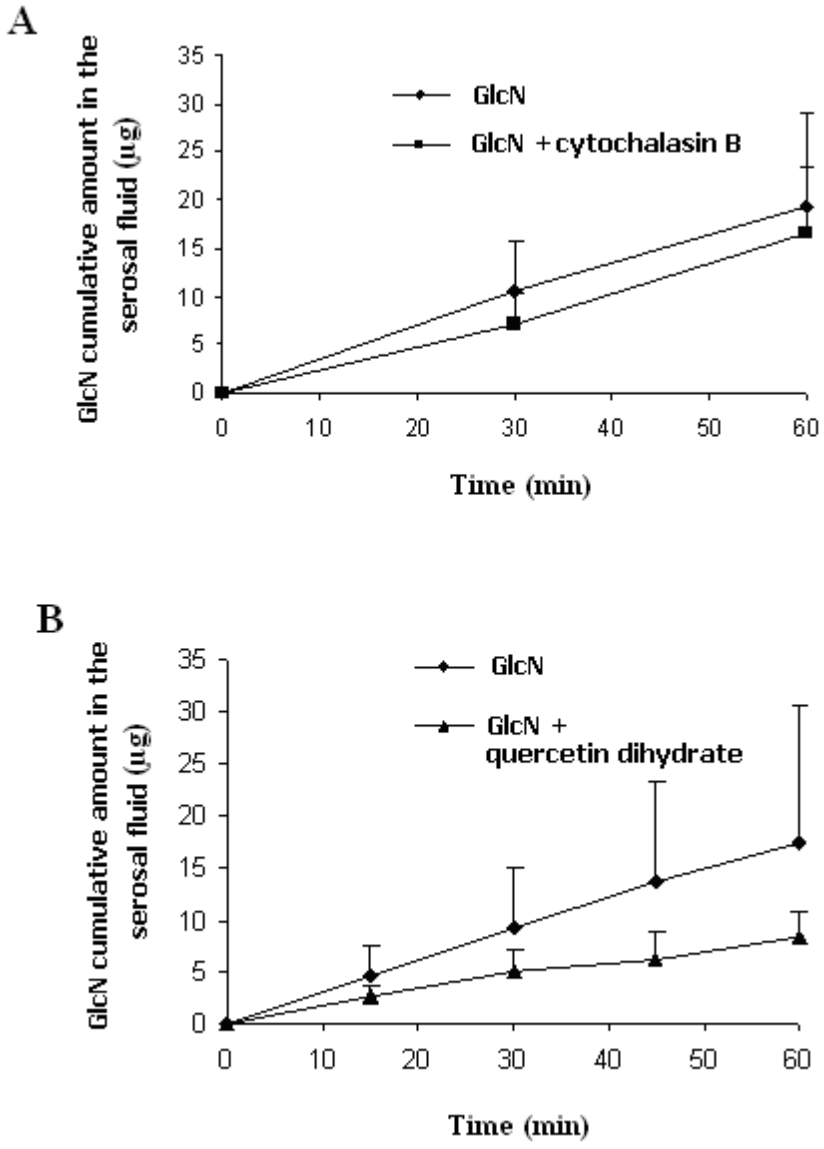

Figure 3.11. The effect of glucose transporter (GLUT2) inhibitors on GlcN transport through the everted rat gut. Segments from the rat jejunum (n = 5-6 rats, 4 segments/rat) were excited, everted, and filled with Krebs-Henseleit buffer. The segments were then incubated with 80 µg/mL GlcN HCl in Krebs-Henseleit buffer for 60 min in a perfusion apparatus at 37°C, continuously aerated with O_2/CO_2 (95%:5%) with or without GLUT2 inhibitors (0.1 mM cytochalasin B (A) or 0.1 of mM quercetin dihydrate (B)). The data represent the means ± SD. The effect of quercetin on the rate and extent of GlcN transport was significant at $p < 0.05$.

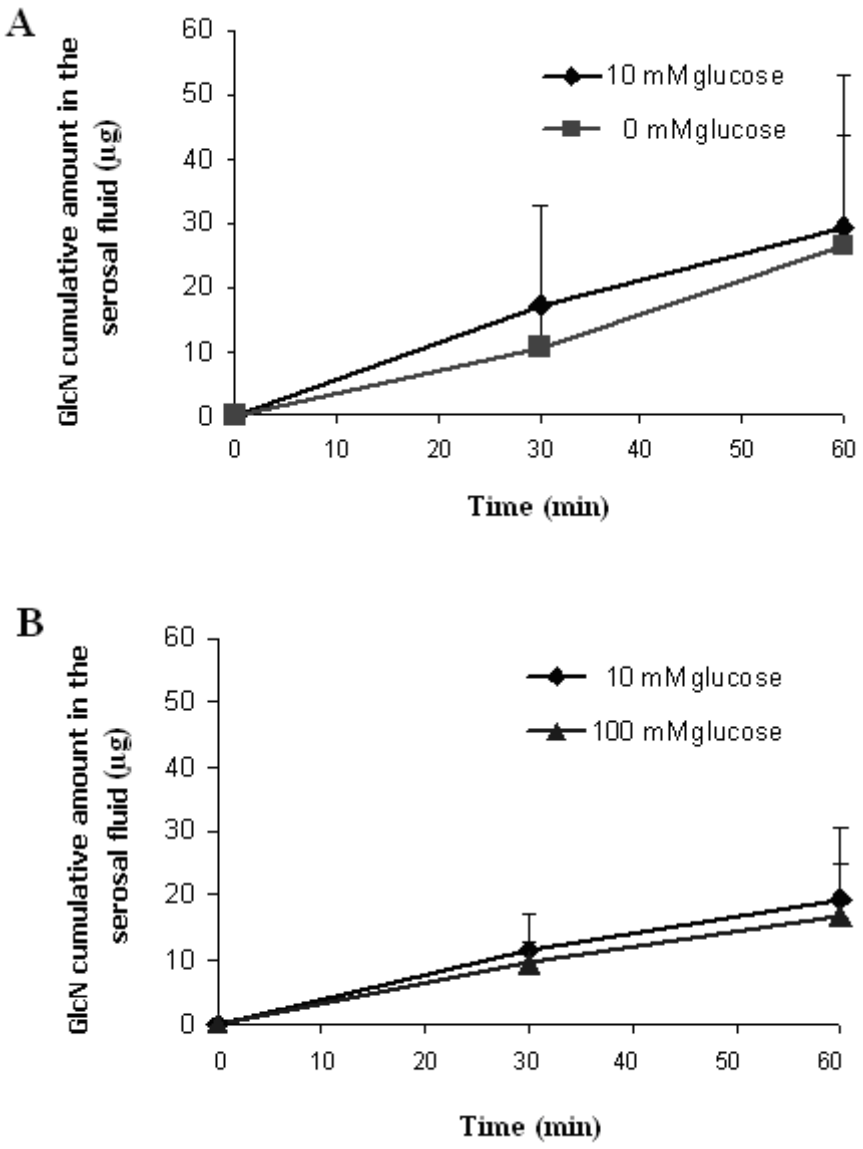

Figure 3.12. The effect of glucose concentration on GlcN transport through the everted rat gut. Segments from the rat jejunum (n = 5-6 rats, 4 segments/rat) were excited, everted, and filled with Krebs-Henseleit buffer. The segments were then incubated with 80 µg/mL GlcN HCl in Krebs-Henseleit buffer for 60 min in a perfusion apparatus at 37°C, continuously aerated with O_2/CO_2 (95%:5%) in the absence of glucose (A) or the presence of a high (100 mM) glucose level (B). Segments incubated in 10 mM of glucose were used as a positive control. The data represent the mean ± SD.

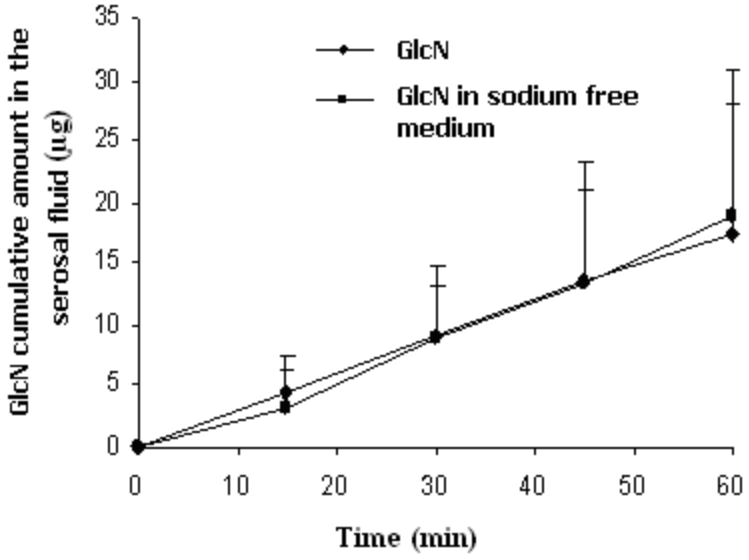

Figure 3.13. The effect of sodium on GlcN movement through the everted rat gut. Segments from the rat jejunum (n = 5 rats, 4 segments/rat) were excited, everted, and filled with Krebs-Henseleit buffer. The segments were then incubated with 80 µg/mL GlcN HCl in either normal Krebs-Henseleit buffer or in sodium free Krebs-Henseleit buffer (sodium chloride was replaced by an equivalent amount of choline chloride) for 60 min in a perfusion apparatus at 37^{o}C, continuously aerated with O_2/CO_2 (95%:5%). The data represent the mean ± SD.

3.3.5. The involvement of the intestinal microflora in the low oral bioavailability of GlcN

The plasma concentration time-curve of the orally administered GlcN in control and antibiotic-treated rats is shown in Figure 3.14. Treating the rat with antibiotics results in almost doubling in the mean AUC_{0-9} and Cmax values (Figure 3.14, 3.15 and Table 3.4). However these changes were found statistically insignificant ($p > 0.05$) due, likely, to the observed variability in the calculated values. A non-significant difference between the two groups was observed in the total AUC_{0-9} and in the terminal AUC_{3-9} (Figure 3.15). The same observation was noticed in the percent of the oral dose excreted unchanged in the urine over the two collection periods 0-9 h and 9-24 h as compared to the controls (Fig 3.16A and Table 3.4).

In the meantime, a pronounced and significant increase in the percent of oral dose eliminated unchanged in the feces of the treated group was observed (Figure 3.16B). To account for the variability, the AUC_{0-9} values were plotted vs. the percentage of administered does found in the feces of the antibiotic treated rats. The result suggested a negative relationship that did not amount to a significant correlation ($r = -0.654$, $p = 0.056$; Figure 3.17). However, using Cook's distance for detecting outliers ($D_i > 1$), we could identify one outlier with only 4.48% of dose found in 24 feces. By excluding this specific value a very strong relation was obtained with p-value of 0.013.

Moreover, incubating 10 mg of GlcN with 1 gm of the rat feces for 24 h, results in a 35% loss of the compound. This percentage increased with decreasing the amount added to the feces to reach 95% loss with 1.25 mg GlcN (Figure 3.18).

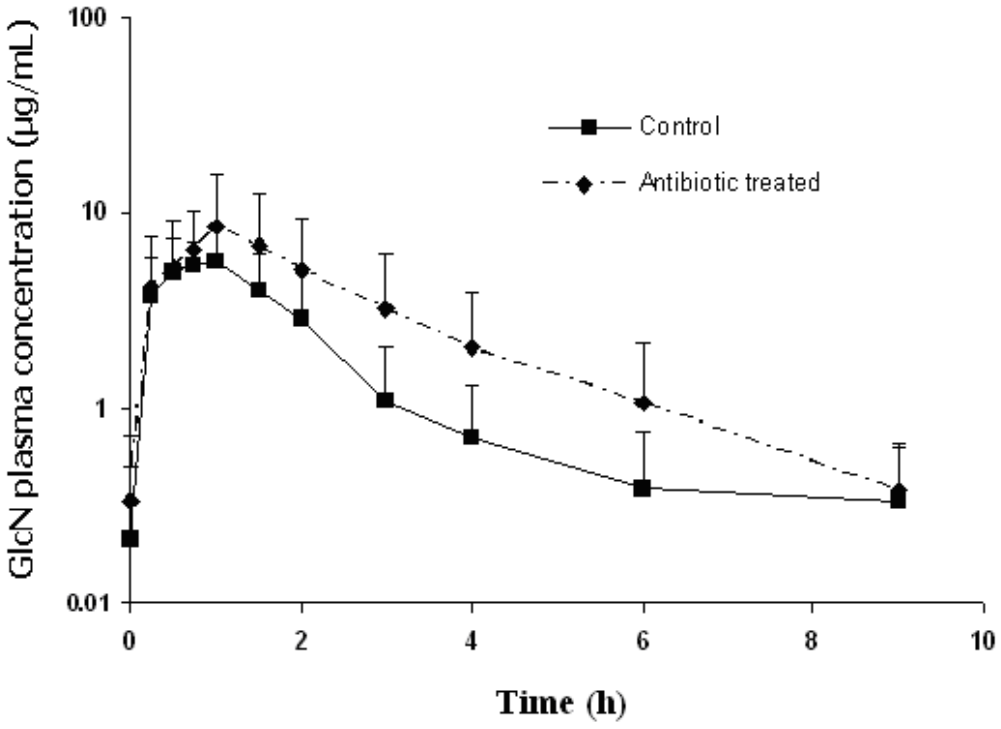

Figure 3.14. The mean plasma concentration-time curve of GlcN in control and antibiotic-treated rats (n = 9/group). The antibiotic treated group was orally administered a combination of 100 mg/kg neomycin trisulphate, 50 mg/kg tetracycline HCl, and 50 mg/kg bacitracin twice daily for two days. The control group received saline by the same regimen. In the third day both groups were given 200 mg/kg GlcN orally. The data represent the means ± SD.

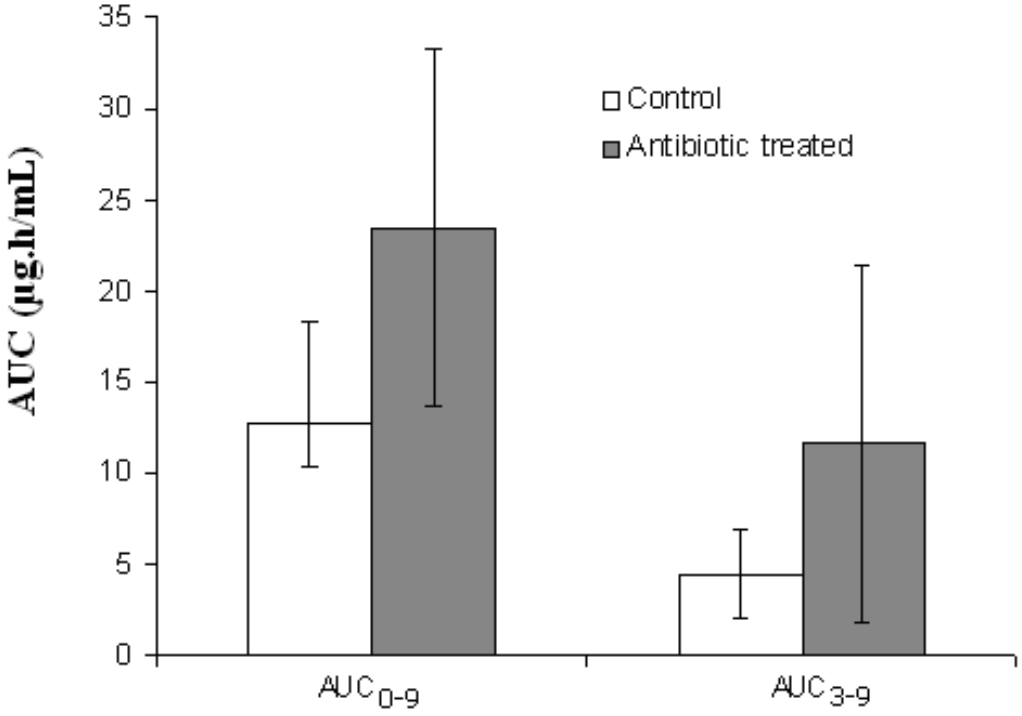

Figure 3.15. The mean AUC of GlcN in control and antibiotic-treated rats (n = 9/group) over two time periods: 0 to 9 h and 3 to 9 h. The antibiotic treated group was orally administered a combination of 100 mg/kg neomycin trisulphate, 50 mg/kg tetracycline HCl, and 50 mg/kg bacitracin twice daily for two days. The control group received saline using the same regimen. In the third day both groups were given 200 mg/kg GlcN orally. The data represent the means ± SD.

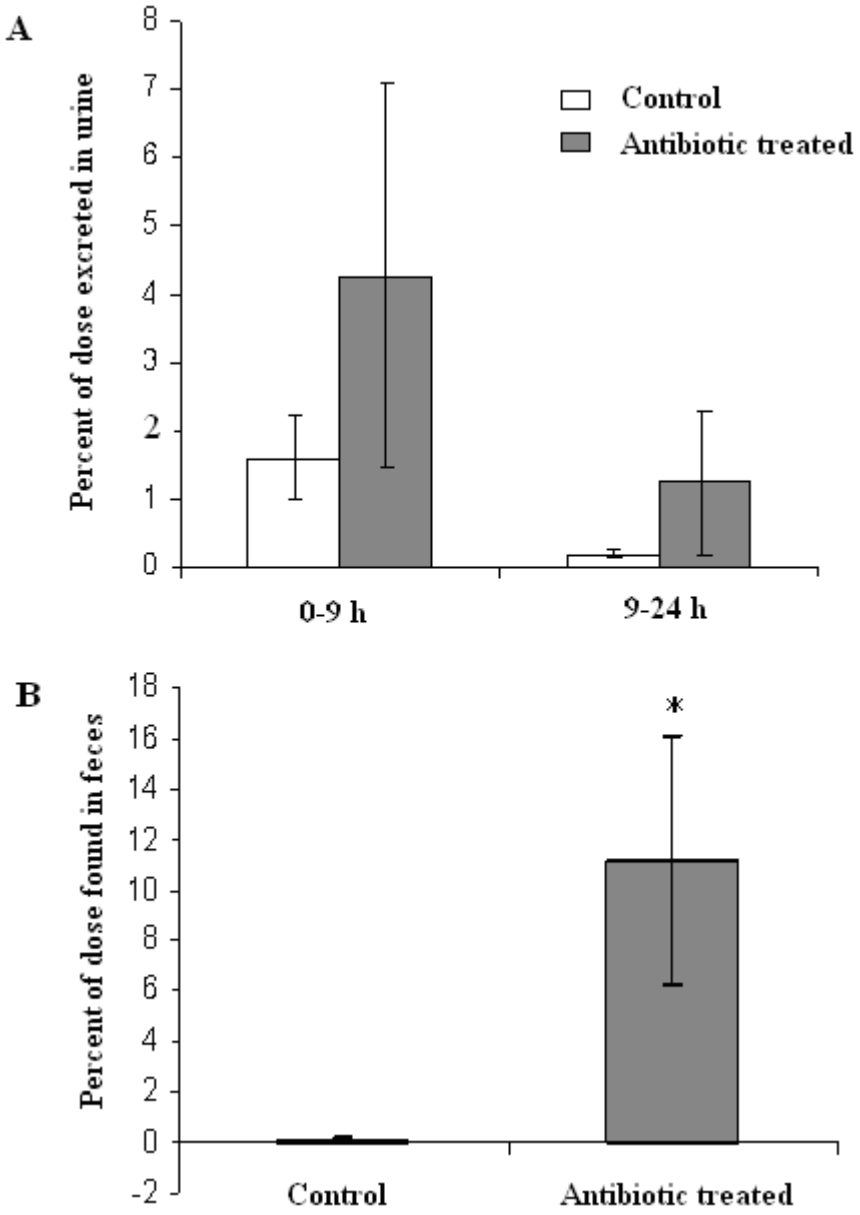

Figure 3.16. The percentage of GlcN oral dose excreted in urine (A) and found in the feces (B) of control and antibiotic-treated rats (n = 9/group). The antibiotic treated group was orally administered a combination of 100 mg/kg neomycin trisulphate, 50 mg/kg tetracycline HCl, and 50 mg/kg bacitracin twice daily for two days. The control group received saline using the same regimen. In the third day both groups were given 200 mg/kg GlcN orally. *Significant difference from control ($p < 0.05$). The data represent the means ± SD.

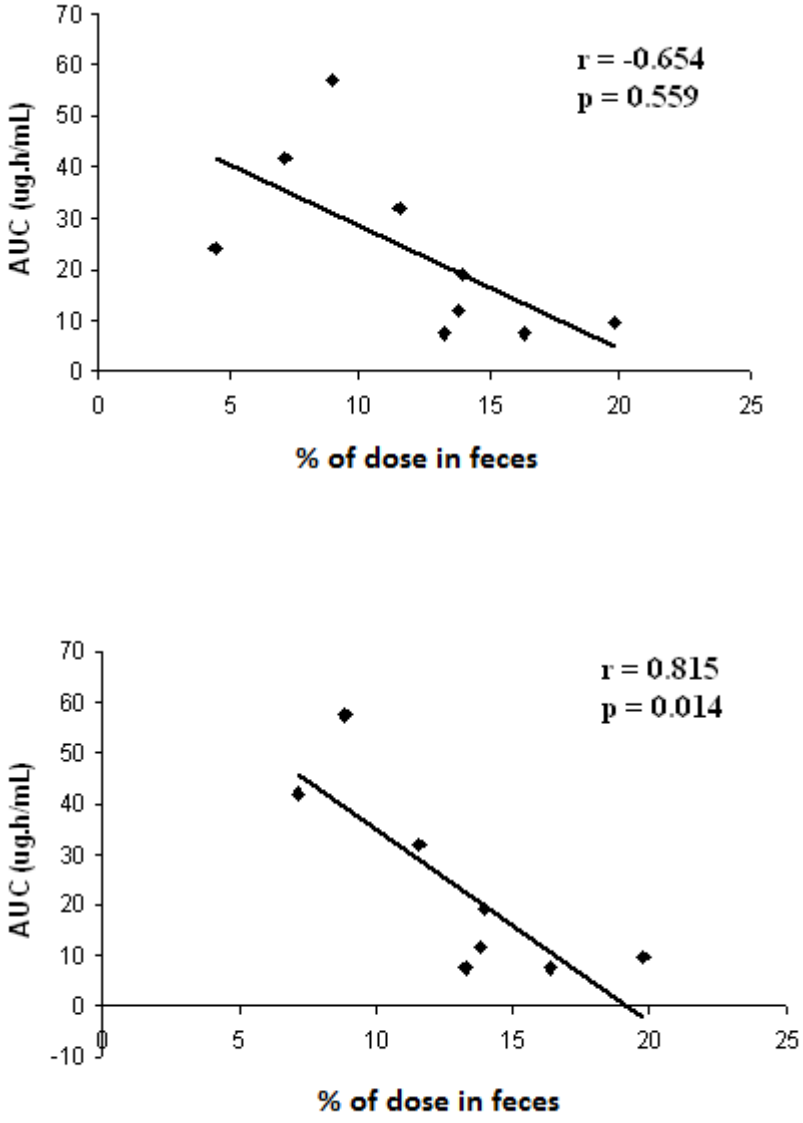

Figure 3.17. A negative correlation between GlcN $AUC_{0\text{-}9}$ after the oral administration of 200 mg/kg GlcN to antibiotic-treated rats, and the percent of the administered dose eliminated in feces 24 h post-dose. The relationship was only significant ($p = 0.014$) when an outlier identified by the Cook's distance approach was excluded.

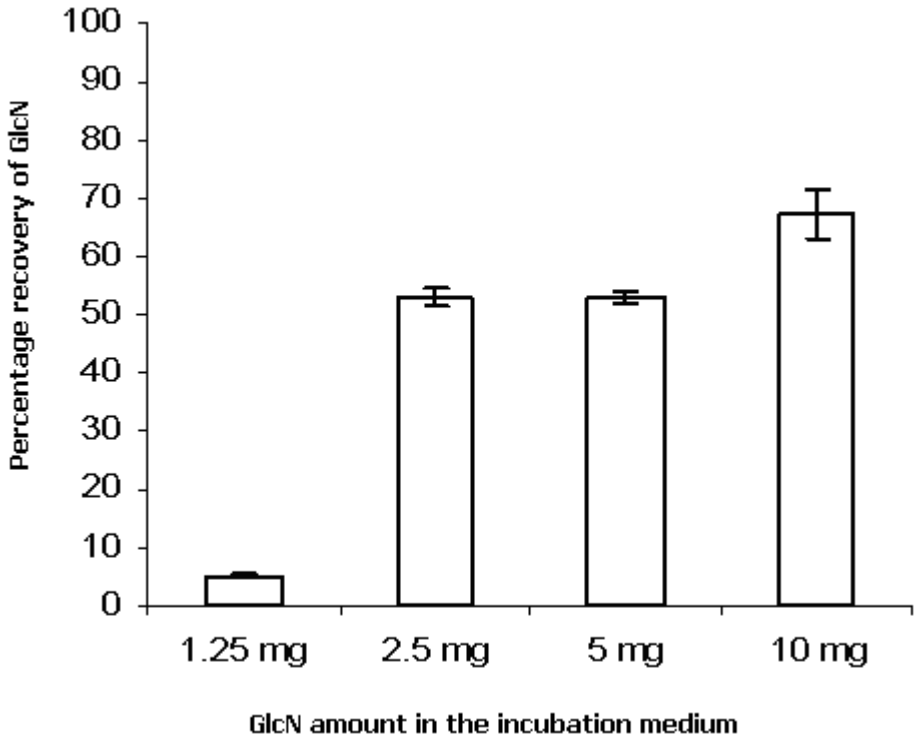

Figure 3.18. Percentage recovery of GlcN from incubation with the rat feces. GlcN aqueous solutions were incubated with 1 g rat feces for 24 h at room temperature. The experiment was done in triplicate. The data represent the means ± SD.

Table 3.4. Pharmacokinetic parameters (means ± SD) following oral administration of GlcN in control and antibiotic-treated rats.

Parameter	Control	Antibiotic treated
n	9	9
C_o, μg/mL	0.21 ± 0.29	0.34 ± 0.39
C_{max}, μg/mL	6.61 ± 2.70	9.41 ± 6.46
T_{max}, h	0.81 ± 0.21	1.03 ± 0.49
AUC_{0-9}, μg.h/mL	12.03 ± 5.96	23.44 ± 18.79
t½, h	2.21± 2.00	2.09 ± 0.62
% of dose in urine (0-9 h)	1.60 ± 0.61	4.30 ± 2.81
% of dose in urine (9-24 h)	0.20 ± 0.06	1.20 ± 1.04
% of dose in 24 h feces	0.11 ± 0.15	11.18 ± 4.90*

n, number of rats; T_{max}, time to reach maximum plasma concentration; C_o, plasma concentration at time zero; C_{max}, maximum plasma concentration; AUC_{0-9}; area under plasma concentration-time curve; t½, elimination half life; *, significant difference from control ($p < 0.05$).

3.3.6. The effect of food on the oral bioavailability of GlcN

The peak plasma concentration was attained faster and with a higher value for Cmax in the fasted group compared to the fed group (average Tmax and Cmax was 0.8 ± 0.2 h and 6.92 ± 4.95 μg/ml for the fasted group vs. 1.25 ± 0.5 h and 2.76 ± 1.41 μg/ml for the fed group). None of the above values show a statistically significant difference. Similarly, the AUC_{0-6} values were not significantly different between the fasted (8.14 ± 6.56 μg.h/ml) and fed group (6.47 ± 2.88 μg.h/ml). Nevertheless, the variability within the fasted group was much higher than that observed when the compound was administered with food (Figure 3.19).

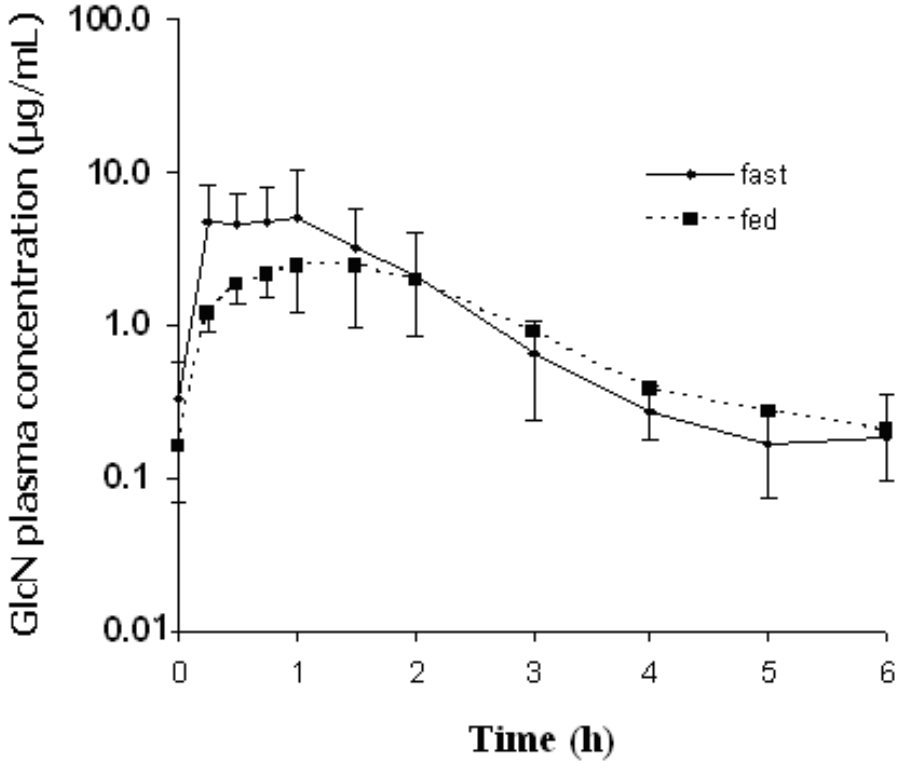

Figure 3.19. The effect of food on the plasma concentration-time curve of the orally administered GlcN. 200 mg/kg GlcN were given orally to fasted and fed rat group (n = 5) in a randomized cross-over fashion. The data represent the means ± SD.

3.3.7. The effect of verapamil on the oral bioavailability of GlcN

Treating the rat with verapamil (calcium channel blocker and P-glycoprotein inhibitor) 2 h prior to GlcN administration significantly delays the time to reach the peak plasma concentration (Figure 3.20A). The Cmax and the AUC_{0-6} were highly increased in the pretreated groups compared to the control, but the difference was statistically non-significant (Table 3.5).

On the other hand, administering cyclosporine A (P-glycoprotein inhibitor) results in multiple peaking in the plasma concentration-time curve, which make it impossible to observe the terminal phase during the time range of the experiment (6h) (Figure 3.20B). Calculating the pharmacokinetic parameters reveal that the difference is not significant compared to those obtained from the control rat group (Table 3.5). No significant difference in the amount excreted in rat urine in 9 h post dose was also observed with both rat groups.

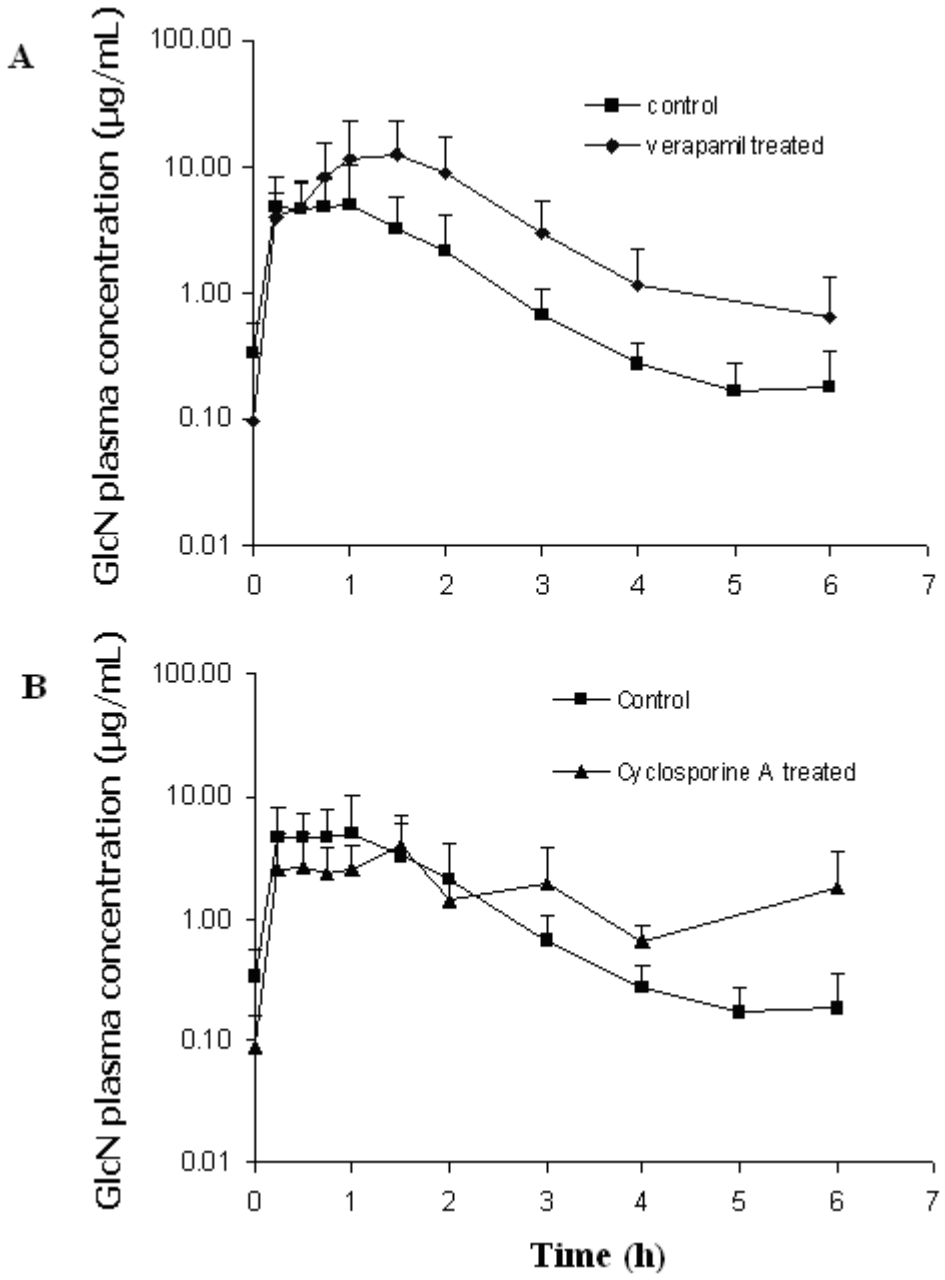

Figure 3.20. The effect of co-administration of verapamil (A) or cyclosporine A (Sandimmune®) (B), on the plasma concentration-time curve of the orally administered GlcN. The rat groups were given 25 mg/kg verapamil (n = 5) or 30 mg/kg cyclosporine A (n = 4), 2 h before the oral administration of 200 mg/kg GlcN. The data represent the means ± SD.

Table 3.5. Pharmacokinetic parameters (means ± SD) following the oral administration of 200 mg/kg of GlcN in control rat group and in rats pretreated with verapamil (25 mg/kg) or cyclosporine A (Sandimmune®) (30 mg/kg)

Parameter	control	GlcN + verapamil	GlcN + cyclosporine A
n	5	5	4
C_o, μg/mL	0.33 ± 0.24	0.13 ± 0.20	0.09 ± 0.08
C_{max}, μg/mL	6.89 ± 4.98	13.58 ± 11.91	4.47 ± 2.00
T_{max}, h	0.7 ± 0.33	1.3 ± 0.27*	2.75 ± 2.40
AUC_{0-6}, μg.h/mL	8.14 ± 6.56	25.83 ± 19.44	9.19 ± 4.29
t½, h	1.01 ± 0.27	3.02 ± 4.47	na
% of dose in urine (0-9 h)	1.60 ± 0.61	3.12 ± 1.79	1.19 ± 0.45

n, number of rats; T_{max}, time to reach maximum plasma concentration; C_{max}, maximum plasma concentration; AUC_{0-9}; area under plasma concentration-time curve; t½, elimination half life; na, not applicable**;** *, significant difference from control ($p < 0.05$).

3.4. Discussion

The first attempt to study GlcN oral pharmacokinetics in humans was made by Setnikar *et al.*, using the radiolabeled compound (Setnikar & Rovati, 2001). The study found that 11.3% of the orally administered radioactivity is eliminated in the feces over 120 h post dose; hence, assumed 88.7% oral absorption of the compound. However, the plasma data reveal oral bioavailability of only 44%; hence, suggested significant hepatic first-pass metabolism behind the pre-systemic loss of the compound (Setnikar & Rovati, 2001).

Setnikar *et al.* also assumed that GlcN is completely absorbed from the gut based on the physical properties of the compound, as GlcN is a water soluble compound with a low molecular weight (MW = 179). Its pka value is 6.91, which indicates that around 54% of the compound would be un-ionized in the small intestine (pH = 6.8) and 46% would be ionized. This degree of ionization should favours its absorption from the small intestine (Setnikar *et al.*, 1986).

Previous studies on the rat showed that the radiolabeled compound is extensively excreted in the expired air as $^{14}CO_2$, only when the compound is administered orally, but not intravenously (Robinson, 1968; Setnikar *et al.*, 1984), Little information is available about the liver's ability to metabolize GLcN to CO_2. Plagemann and Erbe studied the conversion of GlcN to CO_2 and lactate on cultured Novikoff rat hepatoma cells using the radiolabeled compound and reported only 2-3% conversion to CO_2. This percentage increased slightly with increasing GlcN concentration in the incubation media. However, most of the

radioactivity is incorporated into glycoproteins and glycolipids (70-80% as glycoprotein) (Plagemann & Erbe, 1973).

The liver's limited ability to metabolize GlcN to CO_2 necessitated re-investigating the liver as the site of the first-pass metabolism of GlcN. Using rats as a suitable animal model (Chiou *et al.*, 2000), our lab has reported equal exposure following i.v. and i.p. doses of the compound, but only 19% bioavailability after oral administration. That ruled out the involvement of the liver and pointed to the gut as the site of the pre-systemic loss of GlcN (Aghazadeh-Habashi *et al.*, 2002b).

Our previous observation, however, had been made using a large dose (i.e., 350 mg/kg), hence raising the possibility of saturation of the hepatic enzymes following the i.p. administration as proposed for other drugs (Nickerson & Toler, 1997). Such a situation may mask the liver's role in the process and underestimate its contribution in metabolizing GlcN. Our present data demonstrate complete bioavailability following small i.p. doses of 10 and 50 mg/kg (Table 3.1, Fig 3.1), hence unequivocally confirming that, indeed, the gut rather than the liver is responsible for the pre-systemic loss of GlcN. Several factors may be involved in the loss of drugs in the gut, including chemical degradation, poor absorption, metabolism, and/or uptake by the gut microflora.

It is reported that the intestinal absorption of GlcN is mediated by facilitative transporters (Tesoriere *et al.*, 1972). The transporter-mediated uptake of GlcN was also observed in the brain and hepatic tissues (Plagemann & Erbe, 1973; Tan *et al.*, 1977). Recent studies suggested that glucose facilitative

transporters GLUTs are involved in GlcN uptake by the cells (Uldry *et al.*, 2002). The involvement of a transporter in absorption raises the possibility of non-linear pharmacokinetics. Rat studies on the radiolabeled GlcN suggested linear absorption kinetics, as a linear relationship was observed between the oral doses (100, 1000, and 2700 mg) and the corresponding $AUC_{0\text{-}144}$, the urinary excretion, and the respiratory elimination (Setnikar & Rovati, 2001). Nevertheless; the study did not differentiate between the parent compound and its metabolic products. Based on the limited human data, GlcN appears to have non-linear pharmacokinetics. Persiani *et al.* suggested linear pharmacokinetics for the 750-1500 mg dosage range followed by a reduced relative absorption for 3000 mg doses. A close examination of their data, however, suggests a linear relationship between the dose and the AUC for 750, 1500, and 3000 mg doses that is preceded by a saturated phase (Persiani *et al.*, 2005). The line passing through the data crosses the AUC axis at a point substantially higher than the origin (Figure 1.6). This seems to suggest the involvement of two parallel processes, a process that is capacity-limited (i.e., is saturated with low doses) and a linear process that is operative following higher doses. Further human data are needed to clarify this point.

The present *in vivo* results generated using rat, demonstrates a linear relationship between the orally administered dose and the average AUC (Figure 3.6). The linearity was also confirmed by a linear relationship that we found between the dose and the GlcN excreted in urine (Table 3.3). Our *in vitro* studies on the everted rat segments also demonstrated a linear relationship between GlcN

concentration in the incubation media and the accumulation rate from the mucosal to the serosal fluid. Both experiments indicated that GlcN intestinal absorption is linear and not capacity-limited. However, our observation does not rule out the possibility of efficient transporters involvement that is only saturated at extremely high doses. At the same time we found that GlcN is permeable though the different parts of the rat gut, but the highest permeability was observed in the proximal intestine, mainly the duodenum (Figure 3.7). This may suggest a facilitated absorption, as the possibility of region-selective passive diffusion is unlikely. The contribution of passive diffusion together with facilitative transporters in the absorption of GlcN may also exist, as has been suggested previously in GlcN uptake by rat hepatoma cells (Plagemann & Erbe, 1973).

The observation that in transporting GlcN, the duodenum is more efficient than other gut segments does not necessarily suggest the former to be the major site of absorption for the compound. This is likely because of the short residency of the unabsorbed fraction of the dose therein. As depicted in Figure 3.4, for a typical individual rat, following oral administration, the plasma GlcN concentration remains during the length of the experiment indicative of a gradual and continual absorption along the GIT. In addition to the continual absorption of the orally administered doses, the plasma GlcN concentration demonstrates a substantial fluctuation that is masked when the mean data are presented (Figure 3.1). The observation suggests that the true elimination t½ of GlcN can only be determined following i.v. or i.p. doses, since identifying the log-linear terminal

phase of the concentration-time curve is not feasible because the compound continues absorption following oral doses.

Following i.v. administration the plasma GlcN concentration declines rapidly in the first 1.5 h (Figure 3.4). Although the plasma levels are still measurable at 1.5 h (0.27 ± 0.34 μg/mL) to 6 h (0.33 ± 0.31 μg/mL) post i.v. dosing, the levels are in the range of the basal concentration, which make it impossible to calculate the terminal t½ using the plasma data. On the other hand, the urinary data following 10 mg/kg of i.v. doses appears to be more reliable in this context, as we noticed that GlcN is excreted in the urine with large amounts even after it disappears from the plasma. Using the urinary excretion curve plot, GlcN t½ was found to be 4.96 ± 3.45 h, which is substantially longer than the values estimated from the plasma data (Tables 3.1).

It is reported that GlcN is a substrate for glucose facilitative transporters (GLUT1, 2 & 4), and its affinity to GLUT2 is much higher than glucose. Furthermore, GlcN uptake by hepatocytes was found to be entirely mediated by GLUT2 (Uldry *et al.*, 2002). In the intestinal mucosa, GLUT2 is abundantly present at the basolateral membrane and considered to be the main transporter that mediates the transport of glucose, galactose, and fructose from enterocytes to the bloodstream. At a high luminal glucose concentration, GLUT2 rapidly increases on the apical membrane (t½ less than 5 min) and became the major route for glucose intestinal absorption (Drozdowski & Thomson, 2006; Kellett *et al.*, 2008). We examined whether this transporter also mediates GlcN intestinal absorption. Using the everted rat segments, we inhibited the GLUT2 transporter

with 0.1 mM cytochalasin B, and measured the rate and extent of the absorption of GlcN with and without an inhibitor. We did not observe any significant difference in the GlcN transport. While when we inhibited GLUT2 with quercetin, we observed a significant ($p < 0.05$) decrease in the rate and extent of the GlcN mucosal to serosal absorption. However, GlcN absorption continued with a constant rate over the time period of the experiment.

The discrepancy in the effect of the two examined inhibitors could be attributed to the competitive inhibition nature of cytochalasin B, which is highly affected by its concentration in the incubation medium (Ebstensen & Plagemann, 1972; Lachaal *et al.*, 2000). In our experiment, we tested the effect of 0.1 mM, a concentration that has been used in cell cultures (Guillam *et al.*, 1998; Hosokawa & Thorens, 2002). This concentration may be insufficient to cause inhibition under our experimental conditions. In fact, most of the studies that investigated the inhibitory effect of cytochalasin B on animal tissues used a higher concentration (0.2-20 mM) (Helliwell & Kellett, 2002; Nakamura *et al.*, 2003). Increasing the concentration was not feasible for us due to the large amounts of the inhibitor required.

Alternatively, we tested the effect of quercetin dehydrate, reported to be a potent, specific, non-competitive inhibitor of GLUT2, and able to inhibit the transporter activity at lower concentrations (Kwon *et al.*, 2007). Our data suggest that GLUT2 is involved in the passage of GlcN through the gut wall, as quercetin dihydrate significantly inhibits the process. The continuous accumulation of GlcN in the serosal fluid in the presence of a GLUT2 inhibitor may point to the

contribution of passive diffusion or other transporters in the absorption process. As GlcN has an amino group in its structure we suspected that it could be a substrate to amino acid transporters. The transport of amino acids across the intestinal brush border membrane is mediated by sodium-dependent transporters (Broer, 2008). To examine of this kind of transporters participate in GlcN intestinal absorption, we measured GlcN transport across the everted rat gut segments in sodium free Krebs-Henseleit buffer and in normal buffer (Ganapathy & Radhakrishnan, 1980; Russell *et al.*, 1988). Our results showed no significant difference, indicating that sodium-dependent transporters are not involved in the intestinal absorption of GlcN, which matches a previous findings (Tesoriere *et al.*, 1972).

We also examined the possibility of presence of interaction between glucose and GlcN on the intestinal transporters. Biggee *et al.* followed the increase of plasma GlcN in 16 OA patients for 3 h after the oral administration of 1500 mg of GlcN sulphate with and without 75 g of glucose (during a glucose tolerance test) and noticed a delay in the plasma appearance of GlcN accompanied with non significant increase in its plasma level (Biggee *et al.*, 2007a), The authors attributed the delayed absorption to the competition of glucose and GlcN on the intestinal transporters. They suggested that glucose also competed with GlcN on hepatic glucose transporters and decreased its hepatic uptake, resulting in the observed increase in the plasma level (Biggee *et al.*, 2007a). Actually, the competitive inhibition of GlcN uptake by glucose and cytochalasin B (GLUT2 inhibitor) was reported earlier in studies conducted on hepatoma cells and brain

synaptosomes (Ebstensen & Plagemann, 1972; Tan *et al.*, 1977). We expected to see a similar effect for glucose in the intestinal tissues. However, incubating the everted segments with different concentrations of glucose (zero, 10 mM, and 100 mM) did not show any significant alteration in the accumulation rate or the cumulative amount of GlcN in the serosal fluid (Figure 3.12).

Glucose uptake by mammalian hepatocytes is known to be entirely mediated by GLUT2, which is located at the cell membrane and responsible for glucose's uptake and release to and from the blood (Uldry & Thorens, 2004). In the mammalian brain synaptosomes, GLUT3 is considered the main transporter of glucose. Both transporters (GLUT2 & 3) are facilitative and insulin-independent; they transport glucose within the concentration gradient. The driving force for glucose uptake in both cases is its rapid phosphorylation inside the cells (Radziuk & Pye, 2001). GlcN is also rapidly phosphorylated inside the cells before its incorporation into macromolecules (Plagemann & Erbe, 1973); hence glucose competition with GlcN may occur either at the transporters level or at the phosphorylation level.

Glucose intestinal absorption is more complicated. Glucose is actively transported into the enterocytes by sodium/glucose co-transporters (SGLT1), which is located at the brush border membrane. Glucose is then delivered to the bloodstream by the glucose facilitative transporter GLUT2, which is located mainly at the basolateral membrane and transports glucose, fructose, and galactose. However, GLUT2 is also present in the apical membrane with low levels (Kellett & Helliwell, 2000). At high luminal glucose levels (over 30-50

mM), SGLT1 is saturated and glucose uptake from the intestinal lumen is continued by GLUT2, which is rapidly inserted at the brush border membrane (Drozdowski & Thomson, 2006). If GLUT2 is participating in absorbing GlcN, it would be hard to detect the competition, since at low glucose levels GLUT2 is not the main transporter for glucose and its affinity to GlcN is 20 times higher than its affinity for glucose (Uldry *et al.*, 2002). At higher glucose levels, however, GLUT2 is over-expressed at the absorption site, which could mask the concentration effect. In fact, it was reported that increasing glucose luminal concentration can enhance the absorption of fructose, which shares the same transporter with glucose. GLUT5 absorbs fructose from the intestinal lumen and GLUT2 delivers it to the blood stream. In fructose malabsorption, GLUT5 is down-regulated. Co-administering glucose, however, enhances the intestinal absorption of fructose when GLUT2 is inserted at the apical membrane (Jones *et al.*, 2011), which supports our finding that there is a lack of competition between glucose and GlcN on the intestinal absorption.

On the other hand, we observed that incubation with the everted rat gut segment for 60 min leads to loss of around 14% of the GlcN initially added to the incubation medium. It is believed that exogenous GlcN is subjected to rapid phosphorylation inside the cells to form GlcN-6-P, which subsequently enters into the HBP to form proteoglycans, glycolipids, and glycoproteins (Anderson *et al.*, 2005). In addition, GlcN-6-P can provide energy to the tissues through its deamination and conversion to fructose-6-P by GNPDA. The deamination process is frequently observed and well studied in bacteria (Oliva *et al.*, 1995; Tanaka *et*

al., 2005). In mammals, however, this pathway's contribution to the GlcN metabolism is not clear. Kohn *et al.* determined the production of $^{14}CO_2$ in the expired air of food-deprived rats after they were administered i.p. radiolabeled GlcN with and without oral glucose. They noticed that most of the radioactivity was either incorporated into glycoprotein and sialic acid or excreted in the urine, while not more than 6% was excreted in the expired air (Kohn *et al.*, 1962). The preferential incorporation of GlcN in macromolecules over its deamination and oxidation was also reported in the rat hepatoma cell line (Plagemann & Erbe, 1973).

Mammalian GNPDA has found to exist with high density in the kidney and small intestine of the rat (Wolosker *et al.*, 1998) mainly at the apical portion of the small intestine epithelial cells and the epithelium of the proximal convoluted tubules. The restricted distribution of GNPDA in the cells with high metabolic rates reflects its rule in providing them with enough energy (Wolosker *et al.*, 1998). There is a possibility that orally administered GlcN is utilized by the intestinal tissue for energy production, especially at low luminal glucose levels leading to the observed low oral bioavailability. In the present study, however, we did not detect any significant difference in the extent of accumulation of GlcN in the serosal fluid of the everted rat segments in presence of 10 mM or 100 mM glucose and in the complete absence of glucose (Figure 3.12).

On the other hand, mucin, the main intestinal secretion, is a very high molecular-weight glycoprotein secreted mainly by intestinal goblet cells and to a lesser extent by enterocytes. It is an O-linked glycan, which means that it has

UDP-GalNAc attached to its protein backbone (Robbe *et al.*, 2003). GlcN is essential for mucin formation as UDP-GalNAc is only formed endogenously from the epimerization of UDP-GlcNAc (Thoden *et al.*, 2001). The incorporation of exogenous GlcN into the intestinal mucous secretion was reported previously after the radiolabeled compound was administered by i.p. and i.v. (Forstner, 1970). In that study, which was performed on rats, the radioactivity was detectable in the intestinal glycoprotein of the brush border membrane 30 min after injection, and reached its peak at 90 min. The radioactivity started to appear in the intestinal lumen 150 min post administration. The contribution of exogenous GlcN in the formation of the intestinal glycoprotein (Forstner, 1970), and our observation that 14% of GlcN is lost during our 60 min incubation, may suggest that part of the oral dose is used by the enterocytes, most probably to form mucin. More detailed study in GlcN biotransformation by the intestinal tissues is required.

Moreover, it is well known that some bacterial species (e.g., E. coli and lactic acid bacteria) are able to deaminate GlcN-6-P and convert it to fructose-6-P by GNPDA and subsequently using it as an energy and carbon source (Koser *et al.*, 1961; Oliva *et al.*, 1995). In the intestine, anaerobic and facultative aerobic bacteria are abundantly present and increase along the intestine towards the distal lumen (particularly the ileum and colon). Intestinal flora plays an important role in fermenting and degrading the indigestible polysaccharides (cellulose, pectin, and xylan) and glycoconjugates (mucin, heparin, chondroitin sulphate, and hyaluronic acid) (Musso *et al.*, 2011). We suspected that intestinal microflora

contribute in decreasing the oral bioavailability of GlcN. We treated our rats with antibiotics combination of 100 mg/kg neomycin sulphate, 50 mg/kg tetracycline HCl and 50 mg/kg bacitracin twice daily for two days before the administration of GlcN. This antibiotic combination and regimen has been widely used in rat model to eradicate intestinal flora in order to examine the role of microflora in drug metabolism (Gingell *et al.*, 1971; Zachariah & Juchau, 1974; Remmel *et al.*, 1981; Sasaki *et al.*, 1997). The data generated from the comparison between GlcN pharmacokinetics in antibiotic treated rats and control rats, suggest that the intestinal flora is significantly involved in efficiently clearing GlcN. We found 11.18 ± 4.9 % (range 3.89%-19.78 %) of the administered dose in the feces of the antibiotic-treated rats compared to 0.11 ± 0.15 % (range 0-0.4 %) in controls. This was accompanied by a doubling, albeit an insignificant one, of the oral bioavailability and the percentage of the dose excreted in the urine. We attribute this lack of statistical significance to the observed variability. Nevertheless, there appears to be a negative relationship between the bioavailability and the amount found in the feces in the antibiotic-treated rat (Figure 3.17). In other words, a higher extent of absorption reduces the amount found in the feces. Treating the rat with antibiotics can facilitate elimination from the gut (antibiotic-associated diarrhoea) (Loeschke *et al.*, 1980)) which decreases the contact time of the compound with the absorptive mucosa and facilitates its elimination from the GIT. In our experiment, antibiotic-treated rats did not experience diarrhoea; however, both groups passed soft stool, which may partially explain the lack of a significant increase in the oral bioavailability. The dependency of the

bioavailability on the presence of microflora is further confirmed by the observation that the *in vitro* incubation of GlcN with rat feces resulted in substantial loss of the compound (Figure 3.18).

Early works on the effect of intestinal flora on GlcN oral bioavailability were inconclusive (Capps *et al.*, 1966; Robinson, 1968). In one study, the authors used radiolabeled GlcN and measured the exhaled radioactivity by control and antibiotic-treated rats. They found that the pulmonary excretion of radioactivity in the latter group suggested a greater exposure in the antibiotic-treated rats (Capps *et al.*, 1966) . In contrast, the other study reported no significant difference in the excreted radioactivity between the antibiotic-treated and control groups (Robinson, 1968). In the current study, both the *in vitro* and *in vivo* results indicated that GlcN is, indeed, taken up by the gut flora, a process that contributes to the low oral bioavailability of the compound.

We went further to investigate other factors that may add in lowering the oral bioavailability of GlcN and, hence, affect its therapeutic benefits. GlcN is well tolerated by patients; however, it is preferred to be taken with food to avoid potential gastric upset (mainly bloating, diarrhea, and flatulence) (Tapadinhas *et al.*, 1982). In our study we examined the influence of food intake on the oral bioavailability of GlcN and found no potential interaction (Figure 3.19).

GlcN does not bind to plasma or synovial proteins (Setnikar *et al.*, 1986; Persiani *et al.*, 2009b) and does not inhibit or activate liver microsomal enzymes (Persiani *et al.*, 2009a); hence it is assumed that it has no potential for drug interaction. Except with warfarin, where GlcN co-administration led to a

significant increase in INR and increased the risk of bleeding (Knudsen & Sokol, 2008), no serious drug-drug interaction was reported with GlcN. Nevertheless, other medications may influence GlcN pharmacokinetics. Recently, Jackson *et al.* reported a significant decrease in the oral bioavailability of GlcN HCl when co-administered with CS (Jackson *et al.*, 2010), which was an interesting observation as the combination is believed to have superior clinical activity (Clegg *et al.*, 2006). In the current study we tested the effect of verapamil on GlcN HCl oral pharmacokinetics. Verapamil is a calcium channel blocker used mainly to treat cardiovascular diseases including hypertension, angina, and arrhythmia. As cardiovascular diseases are common complications among elderly (Mittelmark *et al.*, 1993), the main users of GlcN, we chose to investigate the influence of verapamil concurrent administration on our compound. Moreover, verapamil may have a beneficial effect on OA. Some reported data suggested that intracellular Ca^{2+} levels play an important role in regulating GAG production (Lee & Ping, 1990; Eifler *et al.*, 2006), and that verapamil can improve OA by reducing the HA export and prevent aggrecan loss from the articular cartilage by inhibiting p-glycoprotein (Prehm, 2005).

In our study, we gave the rat single oral dose of verapamil HCl, equivalent to 25 mg/kg verapamil. This dose has been used in our lab to investigate verapamil pharmacological effect on the electrocardiogram (ECG) of the heart in pre-adjuvant arthritis rat model (Hanafy *et al.*, 2008; Hanafy *et al.*, 2010). Our results showed that the AUC_{0-6} increased in three out of five rats pretreated with verapamil (a range of 5.76 - 52.54 µg.h/mL compared to a range of 3.13-15.40

µg.h/mL in the control rats). The same observation was seen in the amount excreted in the urine over a period of 9 h., see Table 3.5. Although the differences did not reach statistical significance, they showed a trend toward higher bioavailability. Increasing the number of rats could confirm our observations; however, with this level of individual variability we calculated that the minimum number of rats required for statistically significant results is 19 in each group.

Several studies reported a pronounced increase in the oral bioavailability of some medications (mainly anticancer drugs) when co-administered with verapamil, due to its inhibitory effect on P-glycoprotein efflux transporters (Tannergren *et al.*, 2003; Choi & Li, 2005; Bansal *et al.*, 2009). GlcN is a hydrophilic compound with a relatively low molecular weight, those properties, do not support being a substrate for P-glycoprotein transporters (Raub, 2006); however, the substantial increase in the AUC of GlcN in some rats pretreated with verapamil led us to investigate the possibility that efflux transporters are involved in limiting the oral bioavailability of GlcN. We gave another group of rats, an oral dose of cyclosporine A, a drug which is widely used in animal models to inhibit P-glycoprotein (Britten *et al.*, 2000; Qadir *et al.*, 2005; Liow *et al.*, 2007) before the oral administration of GlcN. We gave a dose of 30 mg/kg, which is a medium dose as there was no reported specific dose for studying the P-glycoprotein inhibitory effect of cyclosporine A in the rat (the reported dose range from 5-60 mg/kg (Britten *et al.*, 2000; Yan *et al.*, 2010)). The results showed no increase in the AUC or urinary excretion of the compound compared to the control, which

indicates that p-glycoprotein inhibition is not behind the observable increase in the oral bioavailability of GlcN when co-administered with verapamil and another mechanism may involved.

By reviewing the literature we found some evidence that calcium channels are involved in regulating colonic mucin production, and that administering verapamil and other calcium channel blockers can significantly inhibit mucin secretion (Barcelo *et al.*, 2001). There was also evidence about inhibiting GlcN incorporation into proteoglycans (GAG) in immature rat sertoli cells, and that calcium channel blockers can decrease the production of the extracellular matrix of connective tissues (Lee & Ping, 1990; Fagnen *et al.*, 1999). This means that verapamil may elevate GlcN plasma concentration by inhibiting the compound incorporation into glycoprotein and proteoglycans. The previous information questioned the use the plasma concentration of GlcN to reflect its biological activity. The exact mechanism of GlcN in improving OA is still not well understood, if we consider it as a precursor of GAG in the connective tissue, therefore inhibiting its incorporation into macromolecules is expected to decrease its benefits for treating OA, even if its plasma concentration remains high. However, if the anti-inflammatory properties of GlcN are mediating its activity in OA, then, co-administration with verapamil may have duel beneficial effect by increasing the level of GlcN at site of inflammation and inhibiting HA export (Prehm, 2005). Our study of GlcN-verapamil interaction is considered a preliminary study and further investigations are recommended in the future work.

We noticed a severe fluctuation (multiple peaking) in the GlcN plasma concentration after cyclosporine A was administered. The elimination phase did not seem to be reached within the experiment's time period (Figure 3.20B). In the formulation of cyclosporine A (Sandimmune®), the drug is dissolved in Cremophor EL (polyoxyethylated castor oil) and 32.9% alcohol, which is a lipid-based formulation that may affect gastric emptying and GlcN delivery to the absorption site (Davies *et al.*, 2010). Actually, the Tmax was severely delayed up to six hours in one rat, but the average Tmax was not significantly different from that obtained in the control rats. Further investigation of the effect of a high-fat meal on GlcN pharmacokinetics is required.

CHAPTER 4

The study limitation

There are some limitations of our study.

1. GlcN is endogenously present in the rat plasma. The basal level is typically around 0.2 µg/mL (in all rats tested; mean, 0.29 ± 0.52 µg/mL; range, 0 to 1.1 µg/mL, median, 0.12 µg/mL). Due to the observed variability, in our study we did not account for the basal levels of GlcN in calculating the AUC and the total amount excreted in the urine over the experiment time period which could led to overestimation of their values. However, in our comparative experiments the observed differences should be reliable as the potential error associated with the basal levels are expected to be applied to all arms of the study.
2. In the study of the involvement of glucose transporters in GlcN transport across the everted rat gut, we investigated the possibility of GLUT2 involvement. This transporter is mainly located at the basolateral membrane and with low levels on the apical membrane of the enterocytes (Drozdowski & Thomson, 2006; Kellett *et al.*, 2008). The low expression of GLUT2 on the apical membrane may hinder the ability of the tested inhibitors to act on the transporter. Nevertheless, Quercetin dihydrate is absorbed by the enterocytes and can exert its effect on the basolateral membrane (Kwon *et al.*, 2007). We did not investigate the possibility of the involvement of GLUT5, which is mainly located at the apical

membrane due to the lack of specific inhibitor for this transporter. More studies on GLUT5 ability to transport GlcN are recommended.

3. During studying the contribution of intestinal microflora on GlcN oral bioavailability, we treated one group of rats with an antibiotic combination of neomycin trisulphate, tetracycline HCl and bacitracin twice daily for two days before the administration of GlcN dose. Although this combination has been reported previously (Gingell *et al.*, 1971; Zachariah & Juchau, 1974; Remmel *et al.*, 1981; Sasaki *et al.*, 1997), we did not investigate if this combination efficiently eliminates the entire intestinal microflora under our experimental conditions. We gave the antibiotic treated rats GlcN oral dose 12 h after the last antibiotic dose, we did not give the rat antibiotics during the experiment to avoid any possible interaction. However, the intestinal microflora may partially recover during this time period and contribute in the observed variability in our results.

CHAPTER 5

Conclusion

In the current thesis we were able to improve a simple sensitive HPLC assay of GlcN in human and rat plasma with a LLOQ of 50 ng/mL. The method was further applied in our study of GlcN absorption kinetics.

Our results confirmed that the liver has no role in decreasing the oral bioavailability of GlcN, and that the gut is the site responsible for the compound's first-pass metabolism. Capacity-limited transporters are not behind the low oral bioavailability of GlcN, as both *in vivo* and *in vitro* studies showed clearly that GlcN intestinal absorption is linear; nevertheless, we found evidence on the involvement of glucose facilitative transporter GLUT2 in GlcN intestinal absorption. Passive diffusion and/or high capacity facilitative transporters may contribute to GlcN intestinal transport and led to the observed linear absorption kinetics. Our *in vitro* results showed that the highest permeability of GlcN is likely to be from the duodenum, yet most of the orally administered compound is absorbed from the jejunum, due to its large surface area. Moreover, the intestinal absorption is independent on sodium and glucose luminal levels.

A considerable amount of the compound is lost during the incubation with the intestinal tissue. This indicates that one mechanism by which the gut decreases the oral bioavailability of GlcN is the degradation or biotransformation of the compound by the gut tissues. At the same time, intestinal flora has the ability to fully ferment any unabsorbed GlcN that reaches the lower gut, which adds to its limited oral bioavailability.

No significant difference was found in the bioavailability of GlcN when co-administered with food or verapamil; however, the pronounced increase in the AUC_{0-6} observed in some rats pretreated with verapamil should be considered. Further investigation is required.

It is important to mention that the biotransformation or degradation of GlcN by the intestinal tissue or the gut flora can decrease its oral bioavailability, but it does not necessitate decrease in the therapeutic activity. To date, the mechanism by which GlcN improves OA and RA is not well understood (Biggee *et al.*, 2006). It is not clear if GlcN exerts its effect directly in the articular cartilage and synovial fluid, or whether its effect is a result of some metabolic cascades occurring in other parts of the body. Several studies have reported a strong correlation between RA pathogenesis and the intestinal tissue integrity, particularly the mucosal defence layer integrity (Hvatum *et al.*, 2006; Scheinecker & Smolen, 2011). Although a similar relationship has not been observed with OA, the disease mostly affects the elderly population and changes in intestinal tissue proliferation rate and cell maturation are common with aging, lead to pronounced changes in the way nutrients are digested and absorbed (Woudstra & Thomson, 2002). Intestinal bacterial composition also changes with ageing, and some strains are increased at the expense of others (Hopkins *et al.*, 2001). These alterations highly affect patient immunity and disease susceptibility. Recent studies revealed that disturbances in the intestinal microflora balance can predispose obesity and type II diabetes (Musso *et al.*, 2011). Re-establishing this balance and improving intestinal tissue integrity may protect the body from aging diseases (Bengmark,

2006; Vranesic-Bender, 2010; Kaushal & Kansal, 2011). Our results do not provide information about the kinds of bacteria that uptake GlcN, or if the compound can re-establish an intestinal bacterial balance; however these points required further investigation.

Future directions and studies

In order to better understand the gut's role in GlcN oral bioavailability and therapeutic activity, several studies can be done;

1. A study of the kinetics of GlcN degradation or biotransformation by the intestinal tissues.
2. A more detailed investigation about the effect of verapamil and other commonly co-administered drugs or nutrient supplements on GlcN pharmacokinetics and pharmacodynamics.
3. A study of the effect of repeated oral doses of GlcN on the integrity and permeability of the gut in the elderly and OA patients.
4. Screening of the intestinal flora in healthy, OA and RA patients and the influence of repeated oral administration of GlcN on the intestinal flora.

CHAPTER 6

References

Adebowale A, Du J, Liang Z, Leslie JL & Eddington ND (2002) The bioavailability and pharmacokinetics of glucosamine hydrochloride and low molecular weight chondroitin sulfate after single and multiple doses to beagle dogs. *Biopharm Drug Dispos* **23**, 217-225.

Adebowale AO CD, Liang Z, Eddington ND. (2000) Analysis of glucosamine and chondroitin sulfate content in marketed products and the caco-2 permeability of chondroitin sulfate raw materials. *J Am Nutraceutical Assoc* **3**, 37-44.

Aghazadeh-Habashi A & Jamali F (2011) The glucosamine controversy; a pharmacokinetic issue. *J Pharm Pharm Sci* **14**, 264-273.

Aghazadeh-Habashi A, Sattari S, Pasutto F & Jamali F (2002a) High performance liquid chromatographic determination of glucosamine in rat plasma. *J Pharm Pharm Sci* **5**, 176-180.

Aghazadeh-Habashi A, Sattari S, Pasutto F & Jamali F (2002b) Single dose pharmacokinetics and bioavailability of glucosamine in the rat. *J Pharm Pharm Sci* **5**, 181-184.

Akamatsu N, Nakajima H & Miyata S (1976) Glucosamine metabolism in regenerating rat liver. *Biochem J* **158**, 589-592.

Albert SG, Oiknine RF, Parseghian S, Mooradian AD, Haas MJ & McPherson T (2007) The effect of glucosamine on Serum HDL cholesterol and apolipoprotein AI levels in people with diabetes. *Diabetes Care* **30**, 2800-2803.

Anderson JW, Nicolosi RJ & Borzelleca JF (2005) Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. *Food Chem Toxicol* **43**, 187-201.

Anello M, Spampinato D, Piro S, Purrello F & Rabuazzo AM (2004) Glucosamine-induced alterations of mitochondrial function in pancreatic beta-cells: possible role of protein glycosylation. *Am J Physiol Endocrinol Metab* **287**, E602-608.

Aronson NN, Jr. (1982) Use of radioactive glucosamine in the perfused rat liver to prepare alpha 1-acid glycoprotein (orosomucoid) with 3H- or 14C-labelled sialic acid and N-acetylglucosamine residues. *Biochem J* **203**, 141-148.

Asanbaeva A, Masuda K, Thonar EJ, Klisch SM & Sah RL (2007) Mechanisms of cartilage growth: modulation of balance between proteoglycan and collagen in vitro using chondroitinase ABC. *Arthritis Rheum* **56**, 188-198.

Bansal T, Mishra G, Jaggi M, Khar RK & Talegaonkar S (2009) Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats. *European Journal of Pharmaceutical Sciences* **36**, 580-590.

Barcelo A, Claustre J, Abello J, Chayvialle JA & Plaisancie P (2001) Selective involvement of calcium and calcium channels in stimulated mucin secretion from rat colon. *Scand J Gastroenterol* **36**, 1339-1343.

Baron AD, Zhu JS, Zhu JH, Weldon H, Maianu L & Garvey WT (1995) Glucosamine induces insulin resistance in vivo by affecting GLUT 4 translocation in skeletal muscle. Implications for glucose toxicity. *J Clin Invest* **96**, 2792-2801.

Bassleer C, Rovati L & Franchimont P (1998) Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. *Osteoarthritis Cartilage* **6**, 427-434.

Beaudry F & Vachon P (2008) Determination of glucosamine in horse plasma by liquid chromatography tandem mass spectrometry. *Biomed Chromatogr* **22**, 1-4.

Bekesi JG & Winzler RJ (1969) The effect of D-glucosamine on the adenine and uridine nucleotides of sarcoma 180 ascites tumor cells. *J Biol Chem* **244**, 5663-5668.

Bekesi JG & Winzler RJ (1970) Inhibitory effects of D-glucosamine on the growth of Walker 256 carcinosarcoma and on protein, RNA, and DNA synthesis. *Cancer Res* **30**, 2905-2912.

Bengmark S (2006) Impact of nutrition on ageing and disease. *Curr Opin Clin Nutr Metab Care* **9**, 2-7.

Berninsone PM (2006) Carbohydrates and glycosylation. *WormBook*, 1-22.

Biggee BA, Blinn CM, McAlindon TE, Nuite M & Silbert JE (2006) Low levels of human serum glucosamine after ingestion of glucosamine sulphate relative to capability for peripheral effectiveness. *Ann Rheum Dis* **65**, 222-226.

Biggee BA, Blinn CM, Nuite M, McAlindon TE & Silbert JE (2007a) Changes in serum levels of glucosamine and sulphate after ingestion of glucosamine sulphate with and without simultaneous ingestion of glucose. *Ann Rheum Dis* **66**, 1403-1404.

Biggee BA, Blinn CM, Nuite M, Silbert JE & McAlindon TE (2007b) Effects of oral glucosamine sulphate on serum glucose and insulin during an oral glucose tolerance test of subjects with osteoarthritis. *Ann Rheum Dis* **66**, 260-262.

Black C, Clar C, Henderson R, MacEachern C, McNamee P, Quayyum Z, Royle P & Thomas S (2009) The clinical effectiveness of glucosamine and chondroitin supplements in slowing or arresting progression of osteoarthritis of the knee: a systematic review and economic evaluation. *Health Technol Assess* **13**, 1-148.

Block JA, Oegema TR, Sandy JD & Plaas A (2010) The effects of oral glucosamine on joint health: is a change in research approach needed? *Osteoarthritis Cartilage* **18**, 5-11.

Brasky TM, Lampe JW, Slatore CG & White E (2011) Use of glucosamine and chondroitin and lung cancer risk in the VITamins And Lifestyle (VITAL) cohort. *Cancer Causes Control* **22**, 1333-1342.

Britten CD, Baker SD, Denis LJ, Johnson T, Drengler R, Siu LL, Duchin K, Kuhn J & Rowinsky EK (2000) Oral paclitaxel and concurrent cyclosporin A: targeting clinically relevant systemic exposure to paclitaxel. *Clin Cancer Res* **6**, 3459-3468.

Broer S (2008) Amino acid transport across mammalian intestinal and renal epithelia. *Physiol Rev* **88**, 249-286.

Buse MG (2006) Hexosamines, insulin resistance, and the complications of diabetes: current status. *Am J Physiol Endocrinol Metab* **290**, E1-E8.

Capps JC, Shetlar MR & Bradford RH (1966) Hexosamine metabolism. I. The absorption and metabolism, in vivo, of orally administered D-glucosamine and N-acetyl-D-glucosamine in the rat. *Biochim Biophys Acta* **127**, 194-204.

Chan PS, Caron JP & Orth MW (2005a) Effect of glucosamine and chondroitin sulfate on regulation of gene expression of proteolytic enzymes and their inhibitors in interleukin-1-challenged bovine articular cartilage explants. *Am J Vet Res* **66**, 1870-1876.

Chan PS, Caron JP, Rosa GJ & Orth MW (2005b) Glucosamine and chondroitin sulfate regulate gene expression and synthesis of nitric oxide and prostaglandin E(2) in articular cartilage explants. *Osteoarthritis Cartilage* **13**, 387-394.

Chen D, Zhang Z, Cao J, Zhang D & Jia B (2010) [Effect of glucosamine hydrochloride capsules on articular cartilage of rabbit knee joint in osteoarthritis]. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* **24**, 287-291.

Chesnokov V, Sun C & Itakura K (2009) Glucosamine suppresses proliferation of human prostate carcinoma DU145 cells through inhibition of STAT3 signaling. *Cancer Cell Int* **9**, 25.

Chiou WL, Ma C, Chung SM, Wu TC & Jeong HY (2000) Similarity in the linear and non-linear oral absorption of drugs between human and rat. *Int J Clin Pharmacol Ther* **38**, 532-539.

Choi CS, Lee FN & Youn JH (2001) Free fatty acids induce peripheral insulin resistance without increasing muscle hexosamine pathway product levels in rats. *Diabetes* **50**, 418-424.

Choi J-S & Li X (2005) The effect of verapamil on the pharmacokinetics of paclitaxel in rats. *European Journal of Pharmaceutical Sciences* **24**, 95-100.

Chu SC, Yang SF, Lue KH, Hsieh YS, Lee CY, Chou MC & Lu KH (2006) Glucosamine sulfate suppresses the expressions of urokinase plasminogen activator and inhibitor and gelatinases during the early stage of osteoarthritis. *Clin Chim Acta* **372**, 167-172.

Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, Bradley JD, Bingham CO, 3rd, Weisman MH, Jackson CG, Lane NE, Cush JJ, Moreland LW, Schumacher HR, Jr., Oddis CV, Wolfe F, Molitor JA, Yocum DE, Schnitzer TJ, Furst DE, Sawitzke AD, Shi H, Brandt KD, Moskowitz RW & Williams HJ (2006) Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. *N Engl J Med* **354**, 795-808.

Coggon D, Reading I, Croft P, McLaren M, Barrett D & Cooper C (2001) Knee osteoarthritis and obesity. *Int J Obes Relat Metab Disord* **25**, 622-627.

Comb DG & Roseman S (1958) Glucosamine metabolism. IV. Glucosamine-6-phosphate deaminase. *J Biol Chem* **232**, 807-827.

Cooksey RC & McClain DA (2002) Transgenic mice overexpressing the rate-limiting enzyme for hexosamine synthesis in skeletal muscle or adipose tissue exhibit total body insulin resistance. *Ann N Y Acad Sci* **967**, 102-111.

d'Abusco AS, Calamia V, Cicione C, Grigolo B, Politi L & Scandurra R (2007) Glucosamine affects intracellular signalling through inhibition of mitogen-activated protein kinase phosphorylation in human chondrocytes. *Arthritis Res Ther* **9**, R104.

D'Alessandris C, Andreozzi F, Federici M, Cardellini M, Brunetti A, Ranalli M, Del Guerra S, Lauro D, Del Prato S, Marchetti P, Lauro R & Sesti G (2004) Increased O-glycosylation of insulin signaling proteins results in their impaired activation and enhanced susceptibility to apoptosis in pancreatic beta-cells. *FASEB J* **18**, 959-961.

Dahl IM & Husby G (1985) Hyaluronic acid production in vitro by synovial lining cells from normal and rheumatoid joints. *Ann Rheum Dis* **44**, 647-657.

Davies NM, Takemoto JK, Brocks DR & Yáñez JA (2010) Multiple Peaking Phenomena in Pharmacokinetic Disposition. *Clinical Pharmacokinetics* **49**, 351-377 310.2165/11319320-000000000-000000000.

Descombes A & Haerdi W (1992) HPLC separation of catecholamines after derivatization with 9-fluorenylmethyl chloroformate. *Chromatographia* **33**, 83-86.

Dieppe P, Chard J, Lohmander S & Smith C (2002) Osteoarthritis. *Clin Evid*, 1071-1090.

Dieppe PA, Cushnaghan J & Shepstone L (1997) The Bristol 'OA500' study: progression of osteoarthritis (OA) over 3 years and the relationship between clinical and radiographic changes at the knee joint. *Osteoarthritis Cartilage* **5**, 87-97.

Dodge GR & Jimenez SA (2003) Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. *Osteoarthritis Cartilage* **11**, 424-432.

Dostrovsky NR, Towheed TE, Hudson RW & Anastassiades TP (2011) The effect of glucosamine on glucose metabolism in humans: a systematic review of the literature. *Osteoarthritis Cartilage* **19**, 375-380.

Drozdowski LA & Thomson AB (2006) Intestinal sugar transport. *World J Gastroenterol* **12**, 1657-1670.

Du J, White N & Eddington ND (2004) The bioavailability and pharmacokinetics of glucosamine hydrochloride and chondroitin sulfate after oral and intravenous single dose administration in the horse. *Biopharm Drug Dispos* **25**, 109-116.

Duan W, Paka L & Pillarisetti S (2005) Distinct effects of glucose and glucosamine on vascular endothelial and smooth muscle cells: evidence

for a protective role for glucosamine in atherosclerosis. *Cardiovasc Diabetol* **4**, 16.

Ebstensen RD & Plagemann PG (1972) Cytochalasin B: inhibition of glucose and glucosamine transport. *Proc Natl Acad Sci U S A* **69**, 1430-1434.

Eifler RL, Blough ER, Dehlin JM & Haut Donahue TL (2006) Oscillatory fluid flow regulates glycosaminoglycan production via an intracellular calcium pathway in meniscal cells. *J Orthop Res* **24**, 375-384.

Einarsson S, Josefsson B & Lagerkvist S (1983) Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* **282**, 609-618.

Esko JD, Kimata K & Lindahl U (2009) Proteoglycans and Sulfated Glycosaminoglycans.

Fagnen G, Phamantu NT, Bocquet J & Bonnamy PJ (1999) Inhibition of transmembrane calcium influx induces decrease in proteoglycan synthesis in immature rat Sertoli cells. *J Cell Biochem* **76**, 322-331.

Feldmann M & Maini RN (1999) The role of cytokines in the pathogenesis of rheumatoid arthritis. *Rheumatology (Oxford)* **38 Suppl 2**, 3-7.

Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, Kington RS, Lane NE, Nevitt MC, Zhang Y, Sowers M, McAlindon T, Spector TD, Poole AR, Yanovski SZ, Ateshian G, Sharma L, Buckwalter JA, Brandt KD & Fries JF (2000) Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Ann Intern Med* **133**, 635-646.

Felson DT, Naimark A, Anderson J, Kazis L, Castelli W & Meenan RF (1987) The prevalence of knee osteoarthritis in the elderly. The Framingham Osteoarthritis Study. *Arthritis Rheum* **30**, 914-918.

Firestein GS (2005) Pathogenesis of rheumatoid arthritis: how early is early? *Arthritis Res Ther* **7**, 157-159.

Forstner GG (1970) (1-14C)glucosamine incorporation by subcellular fractions of small intestinal mucosa. Identification by precursor labeling of three functionally distinct glycoprotein classes. *J Biol Chem* **245**, 3584-3592.

Friedman SJ & Skehan P (1980) Membrane-active drugs potentiate the killing of tumor cells by D-glucosamine. *Proc Natl Acad Sci U S A* **77**, 1172-1176.

Gabriel SE (2001) The epidemiology of rheumatoid arthritis. *Rheum Dis Clin North Am* **27**, 269-281.

Ganapathy V & Radhakrishnan AN (1980) Sodium-dependent inhibition of amino acid and dipeptide transport by harmaline in monkey small intestine. *Biochem Pharmacol* **29**, 713-716.

Gingell R, Bridges JW & Williams RT (1971) The role of the gut flora in the metabolism of prontosil and neoprontosil in the rat. *Xenobiotica* **1**, 143-156.

Giraud I, Rapp M, Maurizis JC & Madelmont JC (2000) Application to a cartilage targeting strategy: synthesis and in vivo biodistribution of (14)C-labeled quaternary ammonium-glucosamine conjugates. *Bioconjug Chem* **11**, 212-218.

Golaz O, Wilkins MR, Sanchez JC, Appel RD, Hochstrasser DF & Williams KL (1996) Identification of proteins by their amino acid composition: an evaluation of the method. *Electrophoresis* **17**, 573-579.

Gouze JN, Gouze E, Popp MP, Bush ML, Dacanay EA, Kay JD, Levings PP, Patel KR, Saran JP, Watson RS & Ghivizzani SC (2006) Exogenous glucosamine globally protects chondrocytes from the arthritogenic effects of IL-1beta. *Arthritis Res Ther* **8**, R173.

Grassi W, De Angelis R, Lamanna G & Cervini C (1998) The clinical features of rheumatoid arthritis. *Eur J Radiol* **27 Suppl 1**, S18-24.

Guan Y, Tian Y, Li Y, Yang Z, Jia Y, Hang T & Wen A (2011) Application of a liquid chromatographic/tandem mass spectrometric method to a kinetic study of derivative glucosamine in healthy human urine. *J Pharm Biomed Anal* **55**, 181-186.

Guillam MT, Burcelin R & Thorens B (1998) Normal hepatic glucose production in the absence of GLUT2 reveals an alternative pathway for glucose release from hepatocytes. *Proc Natl Acad Sci U S A* **95**, 12317-12321.

Gustavsson B & Betnér I (1990) Fully automated amino acid analysis for protein and peptide hydrolysates by precolumn derivatization with 9-fluorenyl methylchloroformate and 1-aminoadamantane. *Journal of Chromatography A* **507**, 67-77.

Han IH, Choi SU, Nam DY, Park YM, Kang MJ, Kang KH, Kim YM, Bae G, Oh IY, Park JH, Ye JS, Choi YB, Kim DK, Lee J & Choi YW (2010) Identification and assessment of permeability enhancing vehicles for transdermal delivery of glucosamine hydrochloride. *Arch Pharm Res* **33**, 293-299.

Hanafy S, Dagenais NJ, Dryden WF & Jamali F (2008) Effects of angiotensin II blockade on inflammation-induced alterations of pharmacokinetics and pharmacodynamics of calcium channel blockers. *Br J Pharmacol* **153**, 90-99.

Hanafy S, El-Kadi AO & Jamali F (2010) Drug-disease interaction: reduced verapamil response in isoproterenol-induced myocardial injury in rats. *Pharmacology* **86**, 196-202.

Hebert LF, Jr., Daniels MC, Zhou J, Crook ED, Turner RL, Simmons ST, Neidigh JL, Zhu JS, Baron AD & McClain DA (1996) Overexpression of glutamine:fructose-6-phosphate amidotransferase in transgenic mice leads to insulin resistance. *J Clin Invest* **98**, 930-936.

Helliwell PA & Kellett GL (2002) The active and passive components of glucose absorption in rat jejunum under low and high perfusion stress. *J Physiol* **544**, 579-589.

Herrero-Beaumont G, Ivorra JA, Del Carmen Trabado M, Blanco FJ, Benito P, Martin-Mola E, Paulino J, Marenco JL, Porto A, Laffon A, Araujo D, Figueroa M & Branco J (2007) Glucosamine sulfate in the treatment of knee osteoarthritis symptoms: a randomized, double-blind, placebo-controlled study using acetaminophen as a side comparator. *Arthritis Rheum* **56**, 555-567.

Hochberg MC, Lethbridge-Cejku M, Scott WW, Jr., Reichle R, Plato CC & Tobin JD (1995) The association of body weight, body fatness and body fat distribution with osteoarthritis of the knee: data from the Baltimore Longitudinal Study of Aging. *J Rheumatol* **22**, 488-493.

Hopkins MJ, Sharp R & Macfarlane GT (2001) Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. *Gut* **48**, 198-205.

Hosokawa M & Thorens B (2002) Glucose release from GLUT2-null hepatocytes: characterization of a major and a minor pathway. *Am J Physiol Endocrinol Metab* **282**, E794-801.

Hua J, Suguro S, Hirano S, Sakamoto K & Nagaoka I (2005) Preventive actions of a high dose of glucosamine on adjuvant arthritis in rats. *Inflamm Res* **54**, 127-132.

Huang TM, Cai L, Yang B, Zhou MX, Shen YF & Duan GL (2006a) Liquid chromatography with electrospray ionization mass spectrometry method for the assay of glucosamine sulfate in human plasma: validation and application to a pharmacokinetic study. *Biomed Chromatogr* **20**, 251-256.

Huang TM, Deng CH, Chen NZ, Liu Z & Duan GL (2006b) High performance liquid chromatography for the determination of glucosamine sulfate in human plasma after derivatization with 9-fluorenylmethyl chloroformate. *J Sep Sci* **29**, 2296-2302.

Husby G (1985) Amyloidosis and rheumatoid arthritis. *Clin Exp Rheumatol* **3**, 173-180.

Hvatum M, Kanerud L, Hallgren R & Brandtzaeg P (2006) The gut-joint axis: cross reactive food antibodies in rheumatoid arthritis. *Gut* **55**, 1240-1247.

Hwang SY, Shin JH, Hwang JS, Kim SY, Shin JA, Oh ES, Oh S, Kim JB, Lee JK & Han IO (2010) Glucosamine exerts a neuroprotective effect via suppression of inflammation in rat brain ischemia/reperfusion injury. *Glia* **58**, 1881-1892.

Igarashi M, Kaga I, Takamori Y, Sakamoto K, Miyazawa K & Nagaoka I (2011) Effects of glucosamine derivatives and uronic acids on the production of glycosaminoglycans by human synovial cells and chondrocytes. *Int J Mol Med* **27**, 821-827.

Jackson CG, Plaas AH, Sandy JD, Hua C, Kim-Rolands S, Barnhill JG, Harris CL & Clegg DO (2010) The human pharmacokinetics of oral ingestion of glucosamine and chondroitin sulfate taken separately or in combination. *Osteoarthritis Cartilage* **18**, 297-302.

Jiang L, Tian W, Wang Y, Rong J, Bao C, Liu Y, Zhao Y & Wang C (2011) Body mass index and susceptibility to knee osteoarthritis: A systematic review and meta-analysis. *Joint Bone Spine*.

Johnson SR, Archibald A, Davis AM, Badley E, Wright JG & Hawker GA (2007) Is self-reported improvement in osteoarthritis pain and disability reflected in objective measures? *J Rheumatol* **34**, 159-164.

Jones HF, Butler RN & Brooks DA (2011) Intestinal fructose transport and malabsorption in humans. *Am J Physiol Gastrointest Liver Physiol* **300**, G202-206.

Kanwischer M, Kim SY, Kim JS, Bian S, Kwon KA & Kim DD (2005) Evaluation of the physicochemical stability and skin permeation of glucosamine sulfate. *Drug Dev Ind Pharm* **31**, 91-97.

Kaplan MJ (2006) Cardiovascular disease in rheumatoid arthritis. *Curr Opin Rheumatol* **18**, 289-297.

Kaushal D & Kansal VK (2011) Age-related decline in macrophage and lymphocyte functions in mice and its alleviation by treatment with probiotic Dahi containing Lactobacillus acidophilus and Bifidobacterium bifidum. *J Dairy Res* **78**, 404-411.

Kellett GL, Brot-Laroche E, Mace OJ & Leturque A (2008) Sugar absorption in the intestine: the role of GLUT2. *Annu Rev Nutr* **28**, 35-54.

Kellett GL & Helliwell PA (2000) The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane. *Biochem J* **350 Pt 1**, 155-162.

Kelly GS (1998) The role of glucosamine sulfate and chondroitin sulfates in the treatment of degenerative joint disease. *Altern Med Rev* **3**, 27-39.

Kim JH, Huh JE, Baek YH, Lee JD, Choi DY & Park DS (2011) Effect of Phellodendron amurense in protecting human osteoarthritic cartilage and chondrocytes. *J Ethnopharmacol* **134**, 234-242.

Kim MM, Mendis E, Rajapakse N & Kim SK (2007) Glucosamine sulfate promotes osteoblastic differentiation of MG-63 cells via anti-inflammatory effect. *Bioorg Med Chem Lett* **17**, 1938-1942.

Knox P, Levick JR & McDonald JN (1988) Synovial fluid--its mass, macromolecular content and pressure in major limb joints of the rabbit. *Q J Exp Physiol* **73**, 33-45.

Knudsen JF & Sokol GH (2008) Potential glucosamine-warfarin interaction resulting in increased international normalized ratio: case report and review of the literature and MedWatch database. *Pharmacotherapy* **28**, 540-548.

Kohn P, Winzler J & Hoffman RC (1962) Metabolism of D-glucosamine and N-acetyl-D-glucosamine in the intact rat. *J Biol Chem* **237**, 304-308.

Kopec JA, Rahman MM, Berthelot JM, Le Petit C, Aghajanian J, Sayre EC, Cibere J, Anis AH & Badley EM (2007) Descriptive epidemiology of osteoarthritis in British Columbia, Canada. *J Rheumatol* **34**, 386-393.

Koser SA, Tribby I & Stuedell JT (1961) Glucosamine utilization by some lactic acid bacteria. *J Infect Dis* **108**, 324-332.

Kuettner KE, Pauli BU, Gall G, Memoli VA & Schenk RK (1982) Synthesis of cartilage matrix by mammalian chondrocytes in vitro. I. Isolation, culture characteristics, and morphology. *J Cell Biol* **93**, 743-750.

Kulmatycki KM & Jamali F (2005) Drug disease interactions: role of inflammatory mediators in disease and variability in drug response. *J Pharm Pharm Sci* **8**, 602-625.

Kwon O, Eck P, Chen S, Corpe CP, Lee JH, Kruhlak M & Levine M (2007) Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *FASEB J* **21**, 366-377.

Lachaal M, Rampal AL, Ryu J, Lee W, Hah J & Jung CY (2000) Characterization and partial purification of liver glucose transporter GLUT2. *Biochim Biophys Acta* **1466**, 379-389.

Lafontaine-Lacasse M, Dore G & Picard F (2011) Hexosamines stimulate apoptosis by altering SIRT1 action and levels in rodent pancreatic beta-cells. *J Endocrinol* **208**, 41-49.

Largo R, Martinez-Calatrava MJ, Sanchez-Pernaute O, Marcos ME, Moreno-Rubio J, Aparicio C, Egido J & Herrero-Beaumont G (2009) Effect of a high dose of glucosamine on systemic and tissue inflammation in an experimental model of atherosclerosis aggravated by chronic arthritis. *Am J Physiol Heart Circ Physiol* **297**, H268-276.

Laverty S, Sandy JD, Celeste C, Vachon P, Marier JF & Plaas AH (2005) Synovial fluid levels and serum pharmacokinetics in a large animal model following treatment with oral glucosamine at clinically relevant doses. *Arthritis Rheum* **52**, 181-191.

Lee RC & Ping JA (1990) Calcium antagonists retard extracellular matrix production in connective tissue equivalent. *J Surg Res* **49**, 463-466.

Liang Z, Leslie J, Adebowale A, Ashraf M & Eddington ND (1999) Determination of the nutraceutical, glucosamine hydrochloride, in raw materials, dosage forms and plasma using pre-column derivatization with ultraviolet HPLC. *J Pharm Biomed Anal* **20**, 807-814.

Liow JS, Lu S, McCarron JA, Hong J, Musachio JL, Pike VW, Innis RB & Zoghbi SS (2007) Effect of a P-glycoprotein inhibitor, Cyclosporin A, on the disposition in rodent brain and blood of the 5-HT1A receptor radioligand, [11C](R)-(-)-RWAY. *Synapse* **61**, 96-105.

Lippiello L, Woodward J, Karpman R & Hammad TA (2000) In vivo chondroprotection and metabolic synergy of glucosamine and chondroitin sulfate. *Clin Orthop Relat Res*, 229-240.

Loeschke K, Kautz U & Lohrs U (1980) Effects of antibiotics on caecal electrolyte transport and morphology in rats. Contribution to the pathogenesis of antibiotic-associated diarrhoea. *Klin Wochenschr* **58**, 383-385.

Love DC & Hanover JA (2005) The hexosamine signaling pathway: deciphering the "O-GlcNAc code". *Sci STKE* **2005**, re13.

Maccioni HJ, Giraudo CG & Daniotti JL (2002) Understanding the stepwise synthesis of glycolipids. *Neurochem Res* **27**, 629-636.

Markenson JA (2011) Review: glucosamine and chondroitin, alone or in combination, do not clinically improve knee or hip pain in osteoarthritis. *Ann Intern Med* **154**, JC3-4.

Marshall S, Bacote V & Traxinger RR (1991) Complete inhibition of glucose-induced desensitization of the glucose transport system by inhibitors of mRNA synthesis. Evidence for rapid turnover of glutamine:fructose-6-phosphate amidotransferase. *J Biol Chem* **266**, 10155-10161.

Masson E, Lagarde M, Wiernsperger N & El Bawab S (2006) Hyperglycemia and glucosamine-induced mesangial cell cycle arrest and hypertrophy: Common or independent mechanisms? *IUBMB Life* **58**, 381-388.

McCarthy G, O'Donovan J, Jones B, McAllister H, Seed M & Mooney C (2007) Randomised double-blind, positive-controlled trial to assess the efficacy of glucosamine/chondroitin sulfate for the treatment of dogs with osteoarthritis. *Vet J* **174**, 54-61.

McClain DA, Lubas WA, Cooksey RC, Hazel M, Parker GJ, Love DC & Hanover JA (2002) Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. *Proc Natl Acad Sci U S A* **99**, 10695-10699.

Mehta NG (1980) ABO(H) blood group antigens of the human erythrocyte membrane: Contribution of glycoprotein and glycolipid. *Journal of Membrane Biology* **52**, 17-24.

Meulyzer M, Vachon P, Beaudry F, Vinardell T, Richard H, Beauchamp G & Laverty S (2008) Comparison of pharmacokinetics of glucosamine and synovial fluid levels following administration of glucosamine sulphate or glucosamine hydrochloride. *Osteoarthritis Cartilage* **16**, 973-979.

Meulyzer M, Vachon P, Beaudry F, Vinardell T, Richard H, Beauchamp G & Laverty S (2009) Joint inflammation increases glucosamine levels attained in synovial fluid following oral administration of glucosamine hydrochloride. *Osteoarthritis Cartilage* **17**, 228-234.

Miller KL & Clegg DO (2011) Glucosamine and chondroitin sulfate. *Rheum Dis Clin North Am* **37**, 103-118.

Mittelmark MB, Psaty BM, Rautaharju PM, Fried LP, Borhani NO, Tracy RP, Gardin JM & O'Leary DH (1993) Prevalence of cardiovascular diseases among older adults. The Cardiovascular Health Study. *Am J Epidemiol* **137**, 311-317.

Molnar Z & Bekesi JG (1972) Cytotoxic effects of D-glucosamine on the ultrastructures of normal and neoplastic tissues in vivo. *Cancer Res* **32**, 756-765.

Mroz PJ & Silbert JE (2004) Use of 3H-glucosamine and 35S-sulfate with cultured human chondrocytes to determine the effect of glucosamine concentration on formation of chondroitin sulfate. *Arthritis Rheum* **50**, 3574-3579.

Muniyappa R (2011) Glucosamine and osteoarthritis: time to quit? *Diabetes Metab Res Rev* **27**, 233-234.

Muniyappa R, Karne RJ, Hall G, Crandon SK, Bronstein JA, Ver MR, Hortin GL & Quon MJ (2006) Oral glucosamine for 6 weeks at standard doses does not cause or worsen insulin resistance or endothelial dysfunction in lean or obese subjects. *Diabetes* **55**, 3142-3150.

Musso G, Gambino R & Cassader M (2011) Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. *Annu Rev Med* **62**, 361-380.

Naito K, Watari T, Furuhata A, Yomogida S, Sakamoto K, Kurosawa H, Kaneko K & Nagaoka I (2010) Evaluation of the effect of glucosamine on an experimental rat osteoarthritis model. *Life Sci* **86**, 538-543.

Nakamura H, Masuko K, Yudoh K, Kato T, Kamada T & Kawahara T (2007) Effects of glucosamine administration on patients with rheumatoid arthritis. *Rheumatol Int* **27**, 213-218.

Nakamura K, Takayama K, Nagai T & Maitani Y (2003) Regional intestinal absorption of FITC-dextran 4,400 with nanoparticles based on beta-sitosterol beta-D-glucoside in rats. *J Pharm Sci* **92**, 311-318.

Newsome G (2002) Guidelines for the management of rheumatoid arthritis: 2002 update. *J Am Acad Nurse Pract* **14**, 432-437.

Nickerson DF & Toler SM (1997) Intraperitoneal and intraportal administration of droloxifene to the Sprague-Dawley rat: assessing the first-pass effect. *Xenobiotica* **27**, 627-632.

Noack W, Fischer M, Forster KK, Rovati LC & Setnikar I (1994) Glucosamine sulfate in osteoarthritis of the knee. *Osteoarthritis Cartilage* **2**, 51-59.

Oegema TR, Jr., Deloria LB, Sandy JD & Hart DA (2002) Effect of oral glucosamine on cartilage and meniscus in normal and chymopapain-injected knees of young rabbits. *Arthritis Rheum* **46**, 2495-2503.

Oliva G, Fontes MR, Garratt RC, Altamirano MM, Calcagno ML & Horjales E (1995) Structure and catalytic mechanism of glucosamine 6-phosphate deaminase from Escherichia coli at 2.1 A resolution. *Structure* **3**, 1323-1332.

Pashkova E, Pirogov A, Bendryshev A, Ivanaynen E & Shpigun O (2009) Determination of underivatized glucosamine in human plasma by high-performance liquid chromatography with electrochemical detection: application to pharmacokinetic study. *J Pharm Biomed Anal* **50**, 671-674.

Pastorini E, Vecchiotti S, Colliva C, Persiani S, Rotini R, Roatti G, Zaccarelli L, Rovati LC & Roda A (2011) Identification and quantification of glucosamine in rabbit cartilage and correlation with plasma levels by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry. *Anal Chim Acta* **695**, 77-83.

Patti ME, Virkamaki A, Landaker EJ, Kahn CR & Yki-Jarvinen H (1999) Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance of early postreceptor insulin signaling events in skeletal muscle. *Diabetes* **48**, 1562-1571.

Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G & Rovati LC (2002) Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. *Arch Intern Med* **162**, 2113-2123.

Persiani S, Canciani L, Larger P, Rotini R, Trisolino G, Antonioli D & Rovati LC (2009a) In vitro study of the inhibition and induction of human cytochromes P450 by crystalline glucosamine sulfate. *Drug Metabol Drug Interact* **24**, 195-209.

Persiani S, Matthews A, Larger P, Hall M, Rotini R, Trisolino G, Antonioli D, Zaccarelli L & Rovati LC (2009b) Glucosamine binding to proteins in plasma and synovial fluid and blood cell/plasma partitioning in mouse and man in vitro. *Drug Metabol Drug Interact* **24**, 211-227.

Persiani S, Roda E, Rovati LC, Locatelli M, Giacovelli G & Roda A (2005) Glucosamine oral bioavailability and plasma pharmacokinetics after increasing doses of crystalline glucosamine sulfate in man. *Osteoarthritis Cartilage* **13**, 1041-1049.

Persiani S, Rotini R, Trisolino G, Rovati LC, Locatelli M, Paganini D, Antonioli D & Roda A (2007) Synovial and plasma glucosamine concentrations in osteoarthritic patients following oral crystalline glucosamine sulphate at therapeutic dose. *Osteoarthritis Cartilage* **15**, 764-772.

Peuhkuri K, Vapaatalo H & Korpela R (2010) Even low-grade inflammation impacts on small intestinal function. *World J Gastroenterol* **16**, 1057-1062.

Plagemann PG & Erbe J (1973) Transport and metabolism of glucosamine by cultured Novikoff rat hepatoma cells and effects on nucleotide pools. *Cancer Res* **33**, 482-492.

Popov N (1985) Effects of D-galactosamine and D-glucosamine on retention performance of a brightness discrimination task in rats. *Biomed Biochim Acta* **44**, 611-622.

Pouwels MJ, Jacobs JR, Span PN, Lutterman JA, Smits P & Tack CJ (2001) Short-term glucosamine infusion does not affect insulin sensitivity in humans. *J Clin Endocrinol Metab* **86**, 2099-2103.

Pouwels MJ, Span PN, Tack CJ, Olthaar AJ, Sweep CG, van Engelen BG, de Jong JG, Lutterman JA & Hermus AR (2002) Muscle uridine diphosphate-hexosamines do not decrease despite correction of hyperglycemia-induced insulin resistance in type 2 diabetes. *J Clin Endocrinol Metab* **87**, 5179-5184.

Prehm P (2005) Inhibitors of hyaluronan export prevent proteoglycan loss from osteoarthritic cartilage. *J Rheumatol* **32**, 690-696.

Qadir M, O'Loughlin KL, Fricke SM, Williamson NA, Greco WR, Minderman H & Baer MR (2005) Cyclosporin A is a broad-spectrum multidrug resistance modulator. *Clin Cancer Res* **11**, 2320-2326.

Qu CJ, Karjalainen HM, Helminen HJ & Lammi MJ (2006) The lack of effect of glucosamine sulphate on aggrecan mRNA expression and (35)S-sulphate incorporation in bovine primary chondrocytes. *Biochim Biophys Acta* **1762**, 453-459.

Quastel JH & Cantero A (1953) Inhibition of tumour growth by D-glucosamine. *Nature* **171**, 252-254.

Radziuk J & Pye S (2001) Hepatic glucose uptake, gluconeogenesis and the regulation of glycogen synthesis. *Diabetes Metab Res Rev* **17**, 250-272.

Raub TJ (2006) P-glycoprotein recognition of substrates and circumvention through rational drug design. *Mol Pharm* **3**, 3-25.

Remmel RP, Pohl LR & Elmer GW (1981) Influence of the intestinal microflora on the elimination of warfarin in the rat. *Drug Metab Dispos* **9**, 410-414.

Robbe C, Capon C, Maes E, Rousset M, Zweibaum A, Zanetta JP & Michalski JC (2003) Evidence of regio-specific glycosylation in human intestinal mucins: presence of an acidic gradient along the intestinal tract. *J Biol Chem* **278**, 46337-46348.

Robinson GB (1968) Distribution of isotopic label after the oral administration of free and bound 14C-labelled glucosamine in rats. *Biochem J* **108**, 275-280.

Roda A, Sabatini L, Barbieri A, Guardigli M, Locatelli M, Violante FS, Rovati LC & Persiani S (2006) Development and validation of a sensitive HPLC-

ESI-MS/MS method for the direct determination of glucosamine in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **844**, 119-126.

Roschmann RA & Rothenberg RJ (1987) Pulmonary fibrosis in rheumatoid arthritis: a review of clinical features and therapy. *Semin Arthritis Rheum* **16**, 174-185.

Rossetti L, Hawkins M, Chen W, Gindi J & Barzilai N (1995) In vivo glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats. *J Clin Invest* **96**, 132-140.

Russell AL (1999) Glycoaminoglycan (GAG) deficiency in protective barrier as an underlying, primary cause of ulcerative colitis, Crohn's disease interstitial cystitis and possibly Reiter's syndrome. *Med Hypotheses* **52**, 297-301.

Russell AS, Aghazadeh-Habashi A & Jamali F (2002) Active ingredient consistency of commercially available glucosamine sulfate products. *J Rheumatol* **29**, 2407-2409.

Russell JB, Strobel HJ, Driessen AJ & Konings WN (1988) Sodium-dependent transport of neutral amino acids by whole cells and membrane vesicles of Streptococcus bovis, a ruminal bacterium. *J Bacteriol* **170**, 3531-3536.

Saag KG, Teng GG, Patkar NM, Anuntiyo J, Finney C, Curtis JR, Paulus HE, Mudano A, Pisu M, Elkins-Melton M, Outman R, Allison JJ, Suarez Almazor M, Bridges SL, Jr., Chatham WW, Hochberg M, MacLean C, Mikuls T, Moreland LW, O'Dell J, Turkiewicz AM & Furst DE (2008) American College of Rheumatology 2008 recommendations for the use of nonbiologic and biologic disease-modifying antirheumatic drugs in rheumatoid arthritis. *Arthritis Rheum* **59**, 762-784.

Salvatore S, Heuschkel R, Tomlin S, Davies SE, Edwards S, Walker-Smith JA, French I & Murch SH (2000) A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Aliment Pharmacol Ther* **14**, 1567-1579.

Sasaki I, Tamura T, Shibakawa T, Fujita T, Murakami M, Yamamoto A & Muranishi S (1997) Metabolism of azetirelin, a new thyrotropin-releasing hormone (TRH) analogue, by intestinal microorganisms. *Pharm Res* **14**, 1004-1007.

Sawitzke AD, Shi H, Finco MF, Dunlop DD, Harris CL, Singer NG, Bradley JD, Silver D, Jackson CG, Lane NE, Oddis CV, Wolfe F, Lisse J, Furst DE, Bingham CO, Reda DJ, Moskowitz RW, Williams HJ & Clegg DO (2010) Clinical efficacy and safety of glucosamine, chondroitin sulphate, their combination, celecoxib or placebo taken to treat osteoarthritis of the knee: 2-year results from GAIT. *Ann Rheum Dis* **69**, 1459-1464.

Schachter H (1986) Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. *Biochem Cell Biol* **64**, 163-181.

Scheinecker C & Smolen JS (2011) Rheumatoid arthritis in 2010: from the gut to the joint. *Nat Rev Rheumatol* **7**, 73-75.

Scroggie DA, Albright A & Harris MD (2003) The effect of glucosamine-chondroitin supplementation on glycosylated hemoglobin levels in patients with type 2 diabetes mellitus: a placebo-controlled, double-blinded, randomized clinical trial. *Arch Intern Med* **163**, 1587-1590.

Setnikar I, Cereda R, Pacini MA & Revel L (1991) Antireactive properties of glucosamine sulfate. *Arzneimittelforschung* **41**, 157-161.

Setnikar I, Giacchetti C & Zanolo G (1986) Pharmacokinetics of glucosamine in the dog and in man. *Arzneimittelforschung* **36**, 729-735.

Setnikar I, Giachetti C & Zanolo G (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, Palumbo R, Canali S & Zanolo G (1993) Pharmacokinetics of glucosamine in man. *Arzneimittelforschung* **43**, 1109-1113.

Setnikar I & Rovati LC (2001) Absorption, distribution, metabolism and excretion of glucosamine sulfate. A review. *Arzneimittelforschung* **51**, 699-725.

Shankar RR, Zhu JS & Baron AD (1998) Glucosamine infusion in rats mimics the beta-cell dysfunction of non-insulin-dependent diabetes mellitus. *Metabolism* **47**, 573-577.

Shikhman AR, Brinson DC, Valbracht J & Lotz MK (2009) Differential metabolic effects of glucosamine and N-acetylglucosamine in human articular chondrocytes. *Osteoarthritis Cartilage* **17**, 1022-1028.

Simon RR, Marks V, Leeds AR & Anderson JW (2011) A comprehensive review of oral glucosamine use and effects on glucose metabolism in normal and diabetic individuals. *Diabetes Metab Res Rev* **27**, 14-27.

Sivojelezova A, Koren G & Einarson A (2007) Glucosamine use in pregnancy: an evaluation of pregnancy outcome. *J Womens Health (Larchmt)* **16**, 345-348.

Smith JB & Haynes MK (2002) Rheumatoid arthritis--a molecular understanding. *Ann Intern Med* **136**, 908-922.

Sowers MR & Karvonen-Gutierrez CA (2010) The evolving role of obesity in knee osteoarthritis. *Curr Opin Rheumatol* **22**, 533-537.

Spector TD, Dacre JE, Harris PA & Huskisson EC (1992) Radiological progression of osteoarthritis: an 11 year follow up study of the knee. *Ann Rheum Dis* **51**, 1107-1110.

Stuber K, Sajko S & Kristmanson K (2011) Efficacy of glucosamine, chondroitin, and methylsulfonylmethane for spinal degenerative joint disease and degenerative disc disease: a systematic review. *J Can Chiropr Assoc* **55**, 47-55.

Stumpf JL & Lin SW (2006) Effect of glucosamine on glucose control. *Ann Pharmacother* **40**, 694-698.

Sweeney C, Mackintosh D & Mason RM (1993) UDP-sugar metabolism in Swarm rat chondrosarcoma chondrocytes. *Biochem J* **290 (Pt 2)**, 563-570.

Tallia AF & Cardone DA (2002) Asthma exacerbation associated with glucosamine-chondroitin supplement. *J Am Board Fam Pract* **15**, 481-484.

Tan CH, Peterson NA & Raghupathy E (1977) D-Glucosamine uptake by rat brain synaptosomes. *Biochim Biophys Acta* **464**, 459-462.

Tanaka T, Takahashi F, Fukui T, Fujiwara S, Atomi H & Imanaka T (2005) Characterization of a novel glucosamine-6-phosphate deaminase from a hyperthermophilic archaeon. *J Bacteriol* **187**, 7038-7044.

Tannergren C, Petri N, Knutson L, Hedeland M, Bondesson U & Lennernas H (2003) Multiple transport mechanisms involved in the intestinal absorption and first-pass extraction of fexofenadine[ast]. *Clin Pharmacol Ther* **74**, 423-436.

Tapadinhas MJ, Rivera IC & Bignamini AA (1982) Oral glucosamine sulphate in the management of arthrosis: report on a multi-centre open investigation in Portugal. *Pharmatherapeutica* **3**, 157-168.

Tesoriere G, Dones F, Magistro D & Castagnetta L (1972) Intestinal absorption of glucosamine and N-acetylglucosamine. *Experientia* **28**, 770-771.

Theodosakis J (1997) *The arthritis cure : the medical miracle that can halt, reverse, and may even cure osteoarthritis / Jason Theodosakis, Brenda Adderly and Barry Fox*. Sydney :: Macmillan.

Thoden JB, Wohlers TM, Fridovich-Keil JL & Holden HM (2001) Human UDP-galactose 4-epimerase. Accommodation of UDP-N-acetylglucosamine within the active site. *J Biol Chem* **276**, 15131-15136.

Tiraloche G, Girard C, Chouinard L, Sampalis J, Moquin L, Ionescu M, Reiner A, Poole AR & Laverty S (2005) Effect of oral glucosamine on cartilage degradation in a rabbit model of osteoarthritis. *Arthritis Rheum* **52**, 1118-1128.

Tobon GJ, Youinou P & Saraux A (2010) The environment, geo-epidemiology, and autoimmune disease: Rheumatoid arthritis. *J Autoimmun* **35**, 10-14.

Traxinger RR & Marshall S (1989) Role of amino acids in modulating glucose-induced desensitization of the glucose transport system. *J Biol Chem* **264**, 20910-20916.

Tsai CY, Lee TS, Kou YR & Wu YL (2009) Glucosamine inhibits IL-1beta-mediated IL-8 production in prostate cancer cells by MAPK attenuation. *J Cell Biochem* **108**, 489-498.

Uitterlinden EJ, Jahr H, Koevoet JL, Jenniskens YM, Bierma-Zeinstra SM, Degroot J, Verhaar JA, Weinans H & van Osch GJ (2006) Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. *Osteoarthritis Cartilage* **14**, 250-257.

Uitterlinden EJ, Koevoet JL, Verkoelen CF, Bierma-Zeinstra SM, Jahr H, Weinans H, Verhaar JA & van Osch GJ (2008) Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants. *BMC Musculoskelet Disord* **9**, 120.

Uldry M, Ibberson M, Hosokawa M & Thorens B (2002) GLUT2 is a high affinity glucosamine transporter. *FEBS Lett* **524**, 199-203.

Uldry M & Thorens B (2004) The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Arch* **447**, 480-489.

van der Kraan PM, Vitters EL, de Vries BJ & van den Berg WB (1990) High susceptibility of human articular cartilage glycosaminoglycan synthesis to changes in inorganic sulfate availability. *J Orthop Res* **8**, 565-571.

Vogt W, Egeler E, Sommer W, Eisenbeiss F & Meyer HD (1987) High-performance liquid chromatographic determination of hormonal peptides and their fluorenylmethoxycarbonyl derivatives. *J Chromatogr* **400**, 83-89.

Vranesic-Bender D (2010) The role of nutraceuticals in anti-aging medicine. *Acta Clin Croat* **49**, 537-544.

Wandel S, Juni P, Tendal B, Nuesch E, Villiger PM, Welton NJ, Reichenbach S & Trelle S (2010) Effects of glucosamine, chondroitin, or placebo in patients with osteoarthritis of hip or knee: network meta-analysis. *BMJ* **341**, c4675.

Wang SX, Laverty S, Dumitriu M, Plaas A & Grynpas MD (2007) The effects of glucosamine hydrochloride on subchondral bone changes in an animal model of osteoarthritis. *Arthritis Rheum* **56**, 1537-1548.

Wang X, Chen X, Chen L, Wang B, Peng C, He C, Tang M, Zhang F, Hu J, Li R, Zhao X & Wei Y (2008) Optimizing high-performance liquid chromatography method for quantification of glucosamine using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization in rat plasma: application to a pharmacokinetic study. *Biomed Chromatogr* **22**, 1265-1271.

Weiden S & Wood IJ (1958) The fate of glucosamine hydrochloride injected intravenously in man. *J Clin Pathol* **11**, 343-349.

Wilson TH & Wiseman G (1954) The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. *J Physiol* **123**, 116-125.

Wolosker H, Kline D, Bian Y, Blackshaw S, Cameron AM, Fralich TJ, Schnaar RL & Snyder SH (1998) Molecularly cloned mammalian glucosamine-6-phosphate deaminase localizes to transporting epithelium and lacks oscillin activity. *FASEB J* **12**, 91-99.

Woudstra T & Thomson AB (2002) Nutrient absorption and intestinal adaptation with ageing. *Best Pract Res Clin Gastroenterol* **16**, 1-15.

Wu YL, Kou YR, Ou HL, Chien HY, Chuang KH, Liu HH, Lee TS, Tsai CY & Lu ML (2010) Glucosamine regulation of LPS-mediated inflammation in human bronchial epithelial cells. *Eur J Pharmacol* **635**, 219-226.

Yan YD, Kim DH, Sung JH, Yong CS & Choi HG (2010) Enhanced oral bioavailability of docetaxel in rats by four consecutive days of pre-treatment with curcumin. *Int J Pharm* **399**, 116-120.

Yang S, Zou LY, Bounelis P, Chaudry I, Chatham JC & Marchase RB (2006) Glucosamine administration during resuscitation improves organ function after trauma hemorrhage. *Shock* **25**, 600-607.

Yki-Jarvinen H, Daniels MC, Virkamaki A, Makimattila S, DeFronzo RA & McClain D (1996) Increased glutamine:fructose-6-phosphate amidotransferase activity in skeletal muscle of patients with NIDDM. *Diabetes* **45**, 302-307.

Yu JG, Boies SM & Olefsky JM (2003) The effect of oral glucosamine sulfate on insulin sensitivity in human subjects. *Diabetes Care* **26**, 1941-1942.

Yun J, Tomida A, Nagata K & Tsuruo T (1995) Glucose-regulated stresses confer resistance to VP-16 in human cancer cells through a decreased expression of DNA topoisomerase II. *Oncol Res* **7**, 583-590.

Zachariah PK & Juchau MR (1974) The role of gut flora in the reduction of aromatic nitro-groups. *Drug Metab Dispos* **2**, 74-78.

Zhang L, Liu WS, Han BQ, Peng YF & Wang DF (2006a) Antitumor activities of D-glucosamine and its derivatives. *J Zhejiang Univ Sci B* **7**, 608-614.

Zhang LJ, Huang TM, Fang XL, Li XN, Wang QS, Zhang ZW & Sha XY (2006b) Determination of glucosamine sulfate in human plasma by precolumn derivatization using high performance liquid chromatography with fluorescence detection: its application to a bioequivalence study. *J Chromatogr B Analyt Technol Biomed Life Sci* **842**, 8-12.

Zhang W, Nuki G, Moskowitz RW, Abramson S, Altman RD, Arden NK, Bierma-Zeinstra S, Brandt KD, Croft P, Doherty M, Dougados M, Hochberg M, Hunter DJ, Kwoh K, Lohmander LS & Tugwell P (2010) OARSI recommendations for the management of hip and knee osteoarthritis: part III: Changes in evidence following systematic cumulative update of research published through January 2009. *Osteoarthritis Cartilage* **18**, 476-499.

Zhong S, Zhong D & Chen X (2007) Improved and simplified liquid chromatography/electrospray ionization mass spectrometry method for the analysis of underivatized glucosamine in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **854**, 291-298.

Zhu Y, Zou J, Xiao D, Fan H, Yu C, Zhang J, Yang J & Guo D (2009) Bioequivalence of two formulations of glucosamine sulfate 500-mg capsules in healthy male Chinese volunteers: an open-label, randomized-sequence, single-dose, fasting, two-way crossover study. *Clin Ther* **31**, 1551-1558.

APPENDIX

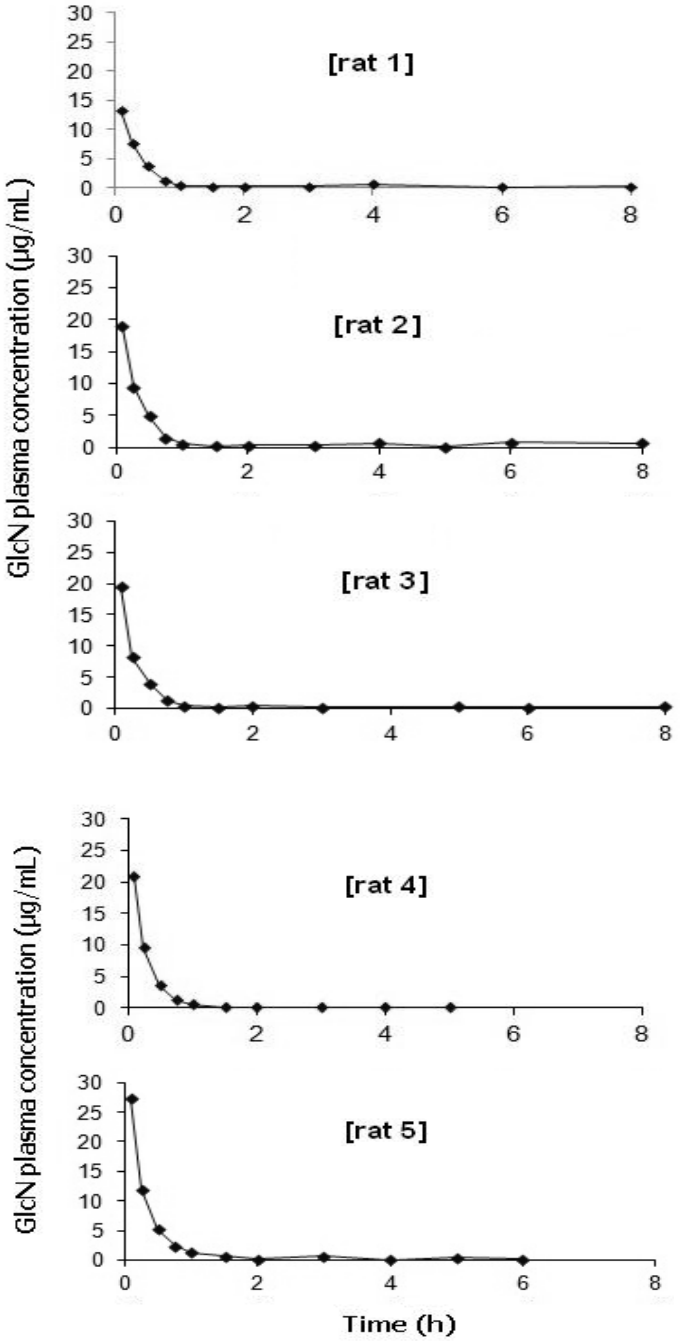

Figure i. The plasma concentration-time curve of GlcN in individual Sprague Dawley rats after i.v. administration of 10 mg/kg dose.

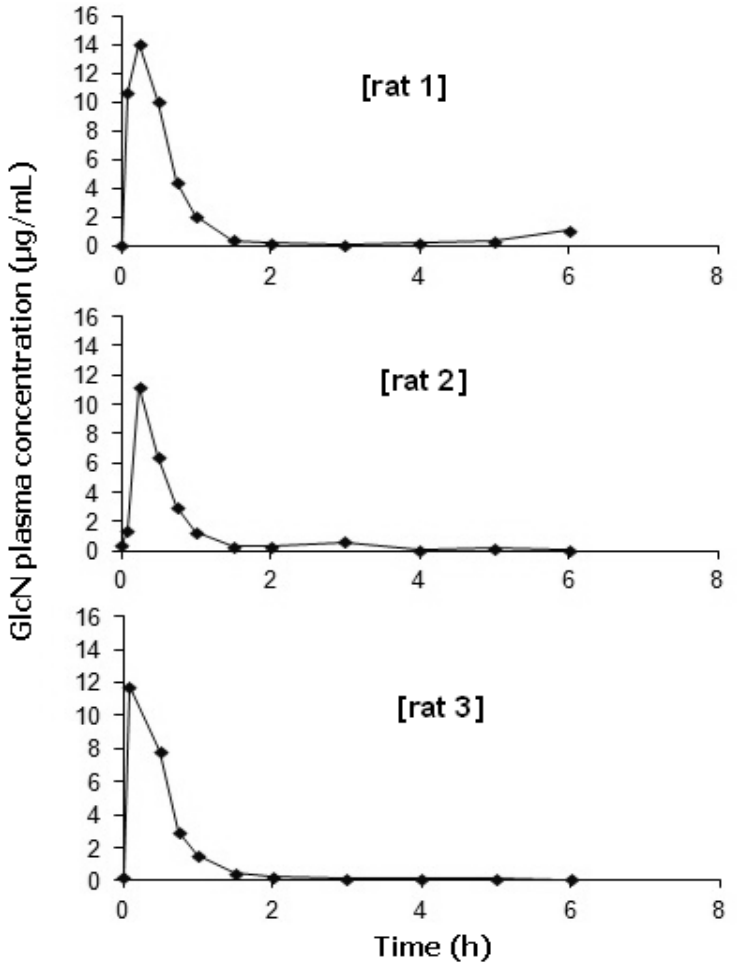

Figure ii. The plasma concentration-time curve of GlcN in individual Sprague Dawley rats after i.p. administration of 10 mg/kg dose.

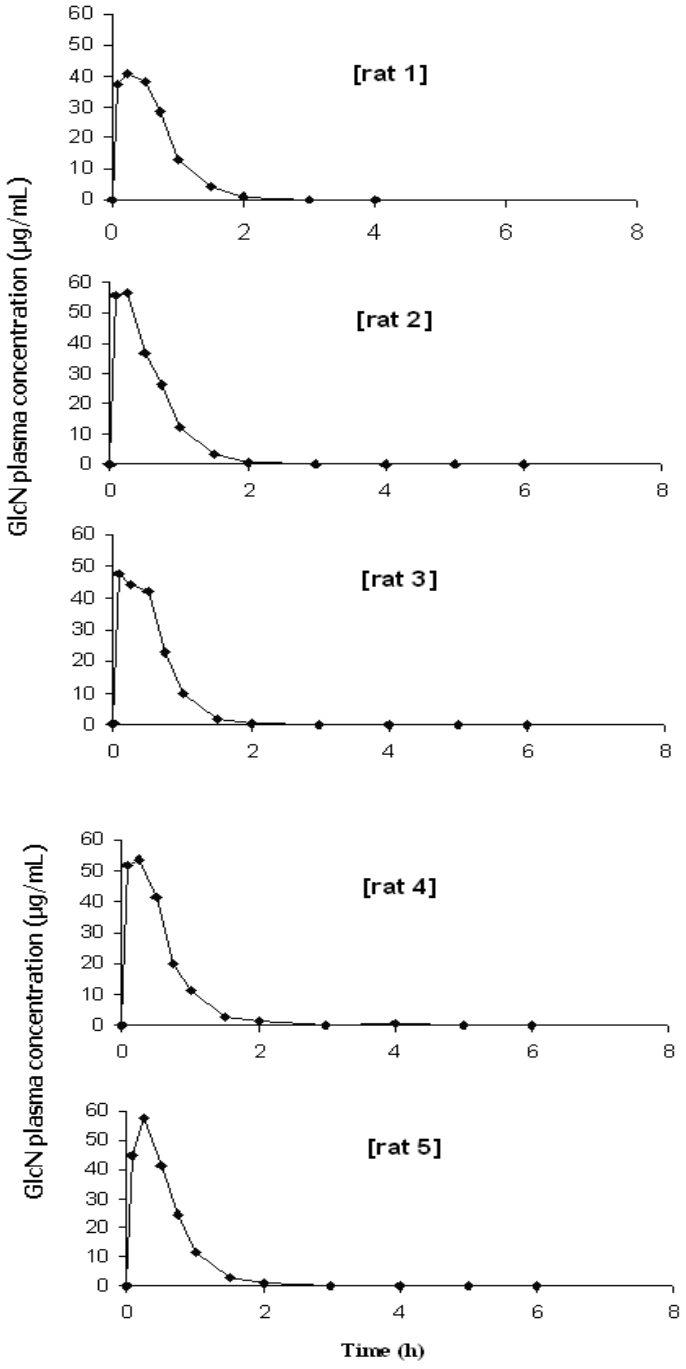

Figure iii. The plasma concentration-time curve of GlcN in individual Sprague Dawley rats after i.p. administration of 50 mg/kg dose.

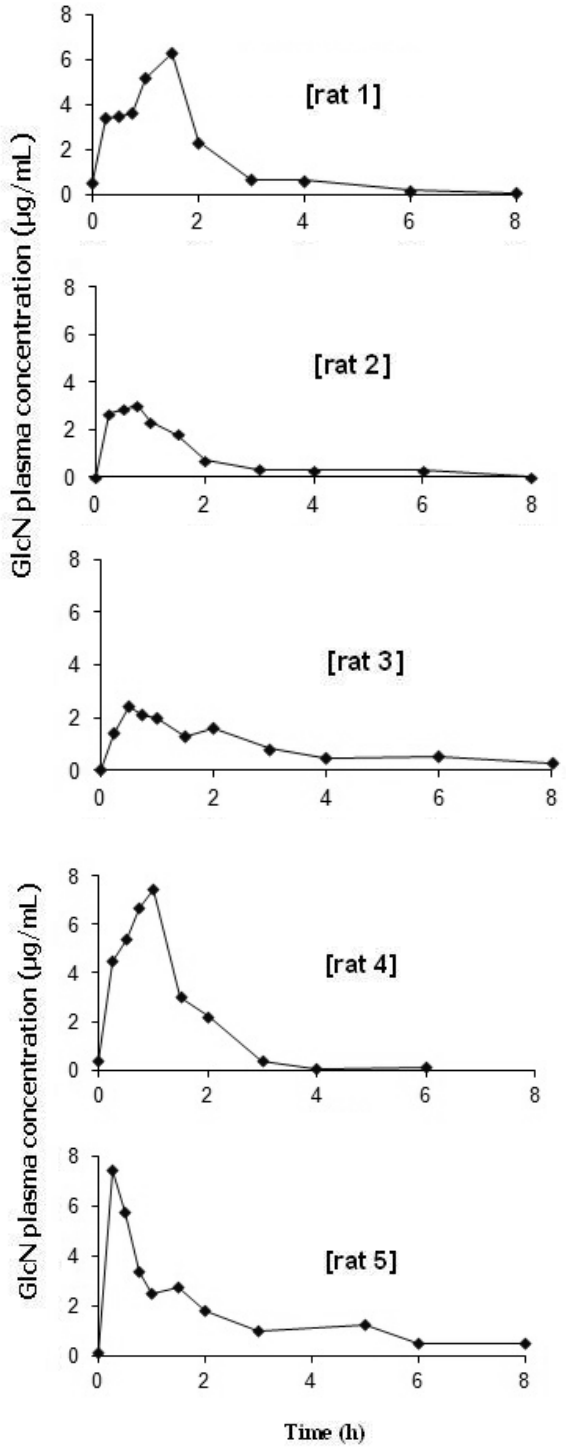

Figure iv. The plasma concentration-time curve of GlcN in individual Sprague Dawley rats after oral administration of 200 mg/kg dose.

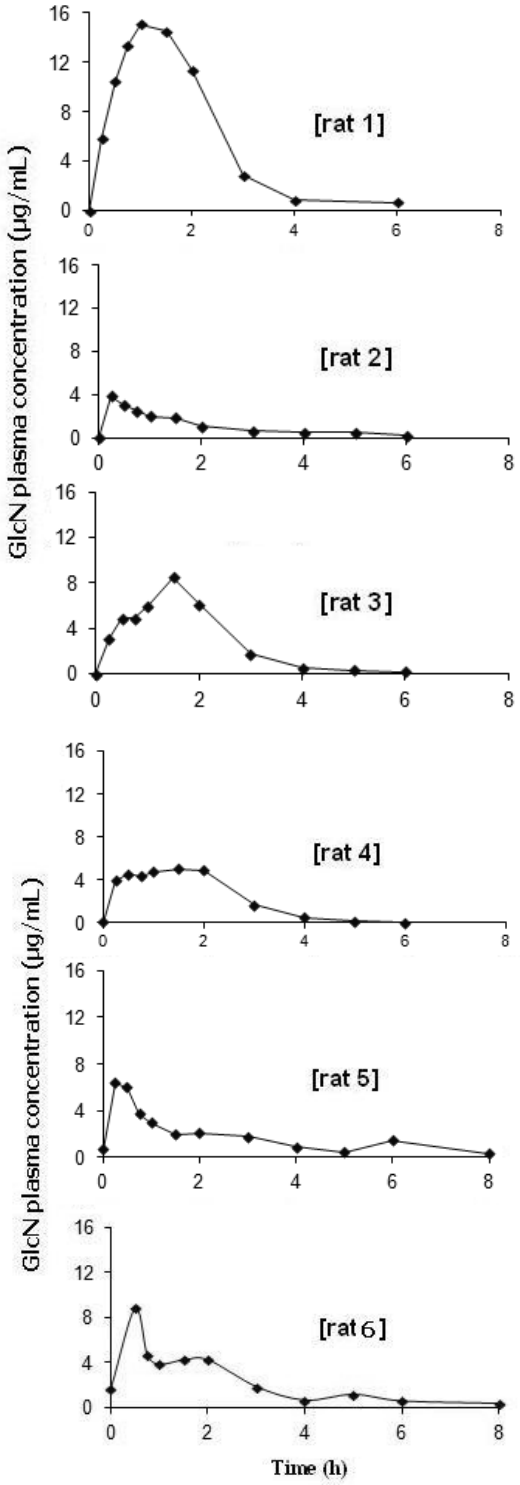

Figure v. The plasma concentration-time curve of GlcN in individual Sprague Dawley rats after oral adminisrtation of 400 mg/kg dose.

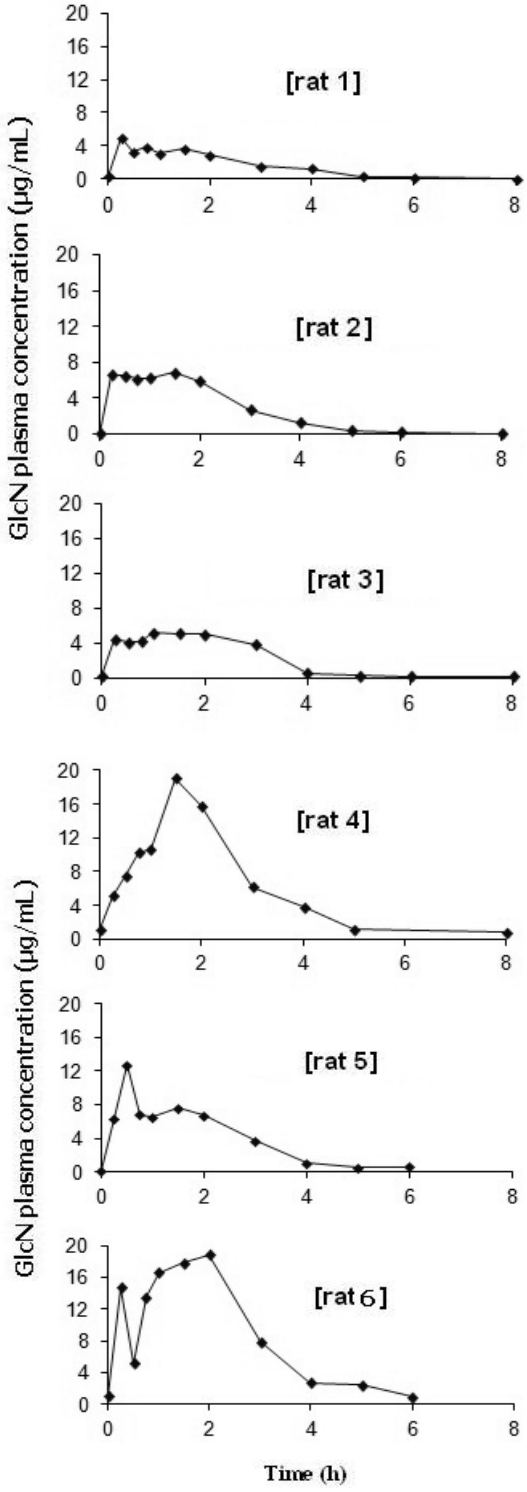

Figure vi. The plasma concentration-time curve of GlcN in individual Sprague Dawley rats after oral adminisrtation of 600 mg/kg dose.

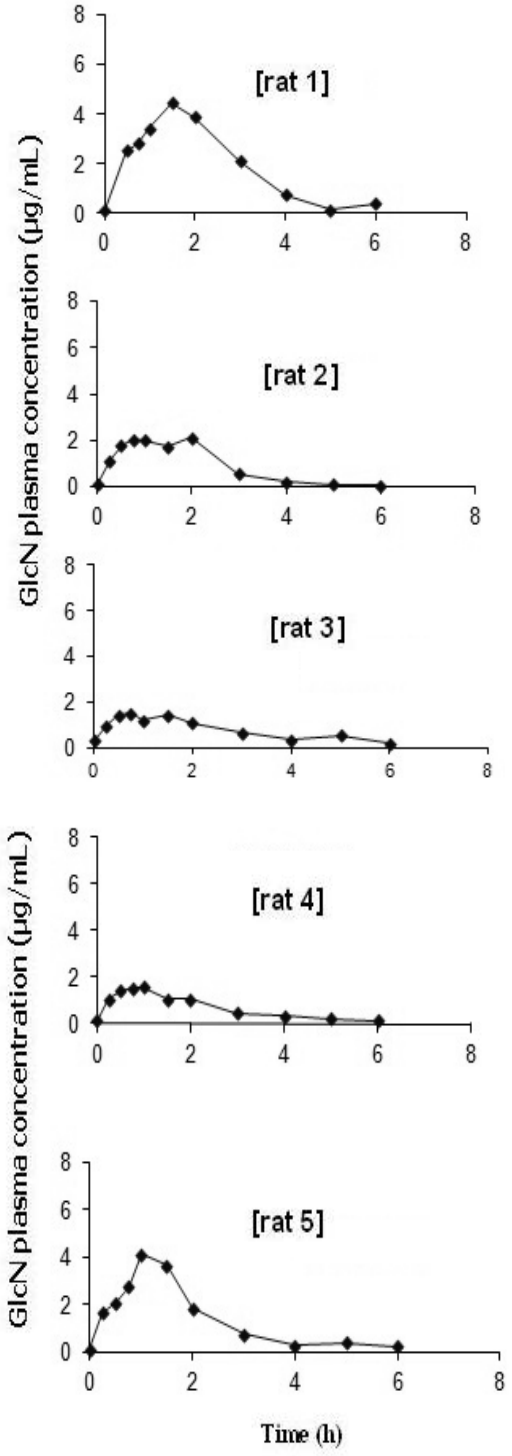

Figure vii. The effect of food on the oral bioavailability of GlcN**.** The plasma concentration-time curve of GlcN in individual Sprague Dawley rats after oral adminisrtation of 200 mg/kg dose to fed rats as a part of the cross-over study. Rats were having a free acess to food before and during the experiment.

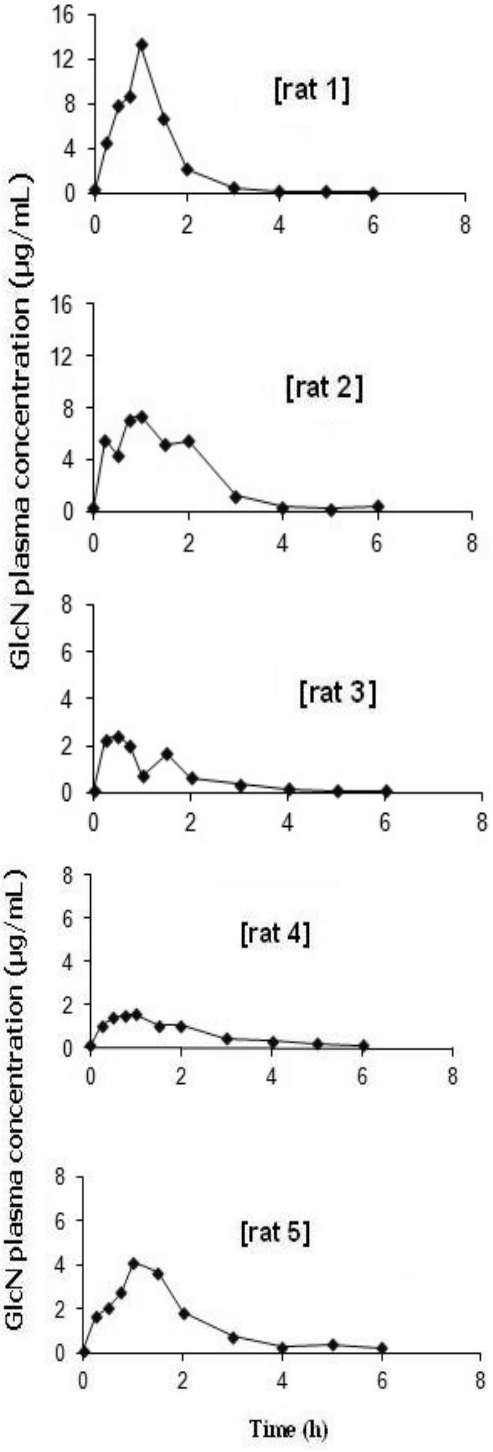

Figure viii. The effect of food on the oral bioavailability of GlcN. The plasma concentration-time curve of GlcN in individual Sprague Dawley rats after oral adminisrtation of 200 mg/kg dose to fasted rats as a part of the cross-over study. Rats were deprived of food 12 h before GlcN administration.

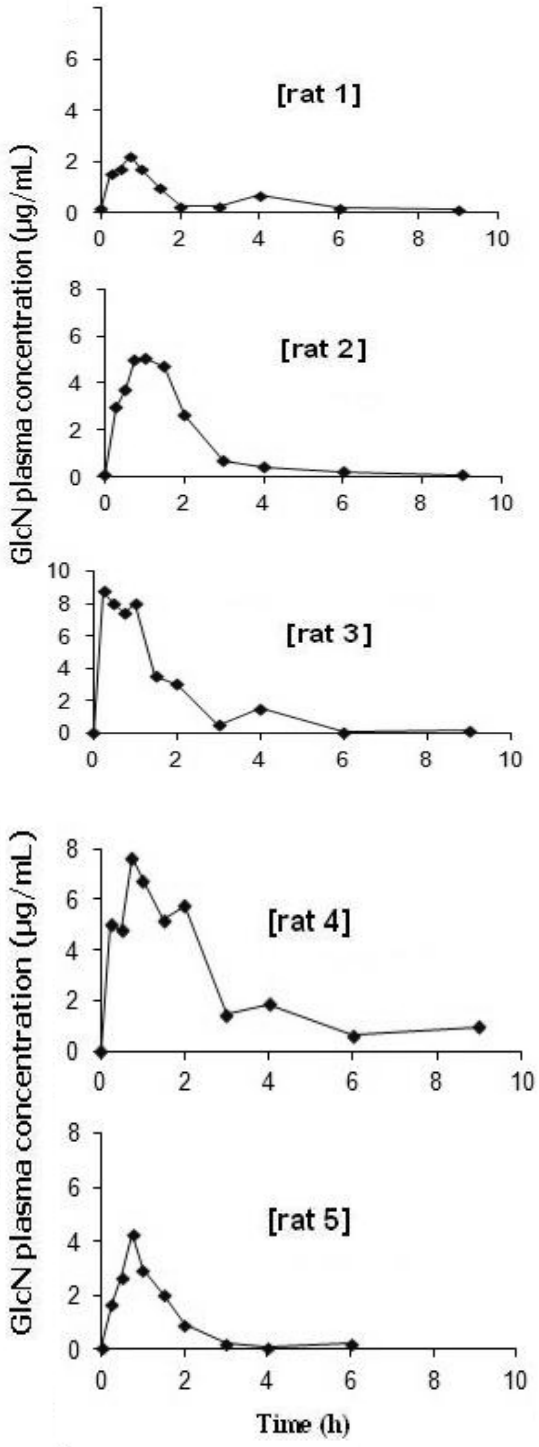

Figure ix. The plasma concentration-time curve of GlcN in the individual control Sprague Dawley rats (rat 1- rat 5). The rats were given 0.5 mL saline orally twice daily for two days before the oral administration of 200 mg/kg GlcN.

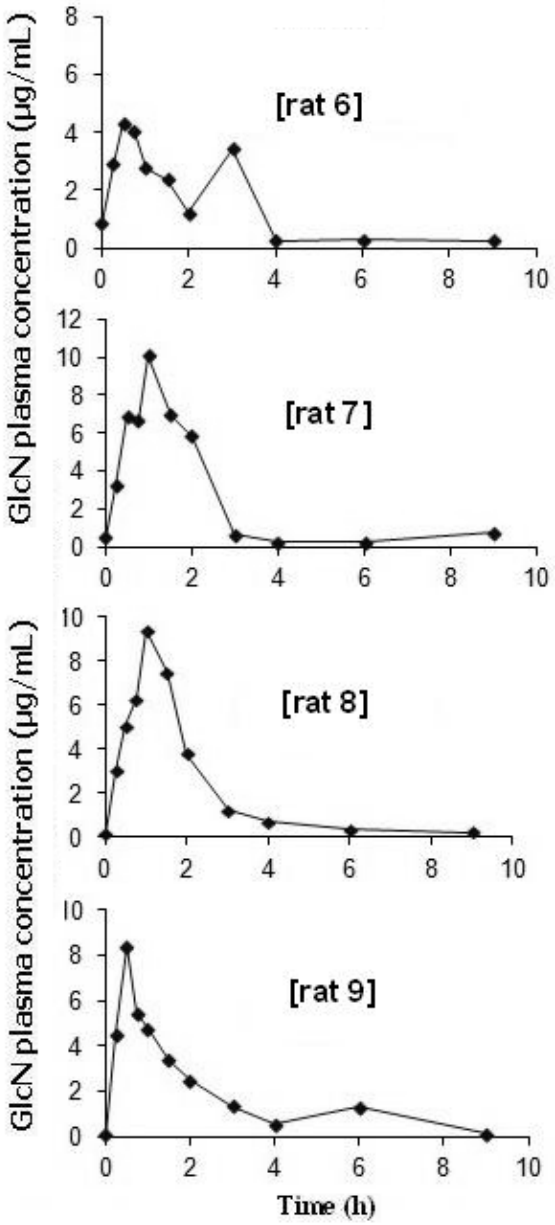

Figure x. The plasma concentration-time curve of GlcN in the control Sprague Dawley rats (rat 6 - rat 9). The rats were given 0.5 mL saline orally twice daily for two days before the oral administration of 200 mg/kg GlcN.

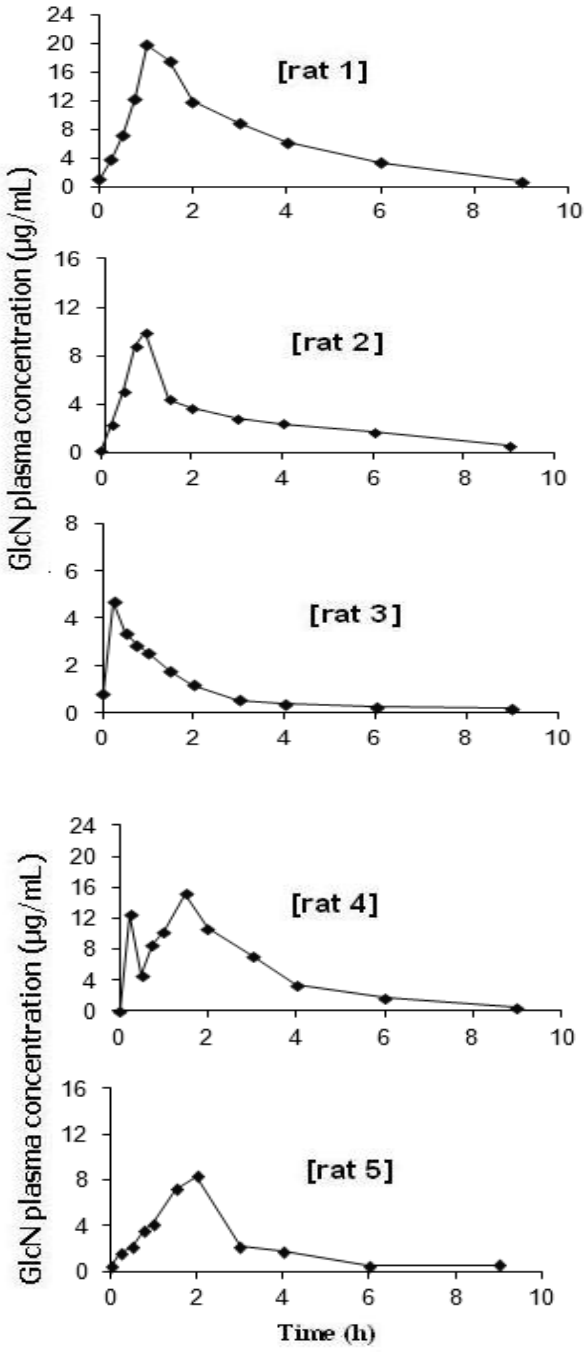

Figure xi. The plasma concentration-time curve of GlcN in the individual antibiotic treated Sprague Dawley rats (rat 1-rat 5). The rats were orally administered a combination of 100 mg/kg neomycin trisulphate, 50 mg/kg tetracycline HCl, and 50 mg/kg bacitracin twice daily for two days before the oral administration of 200 mg/kg GlcN.

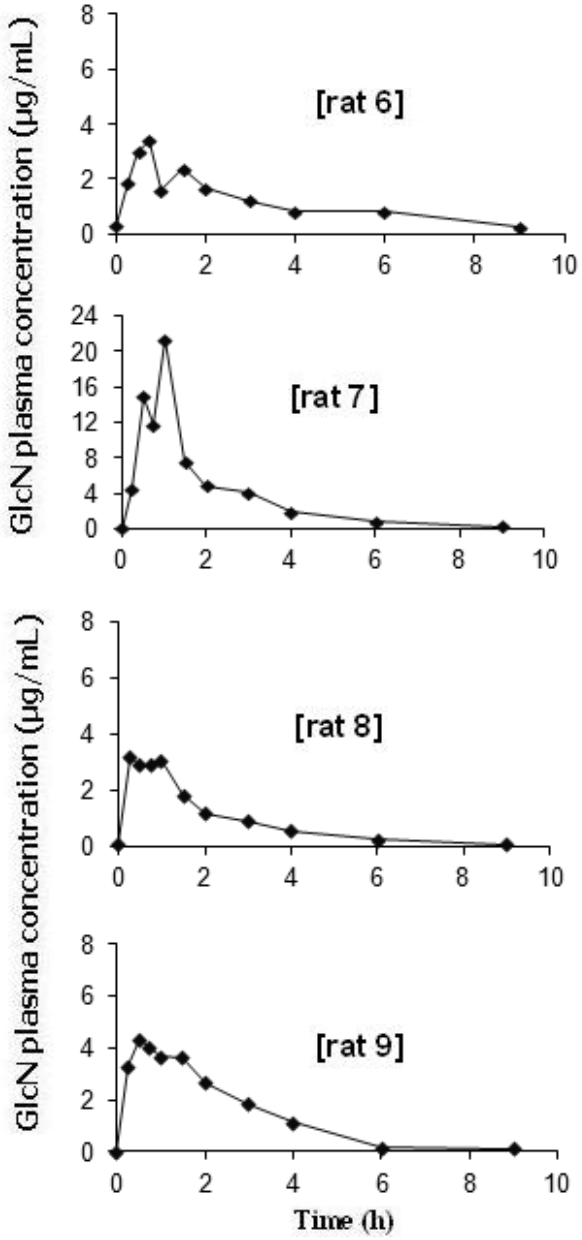

Figure xii. The plasma concentration-time curve of GlcN in the individual antibiotic treated Sprague Dawley rats (rat 6-rat 9). The rats were orally administered a combination of 100 mg/kg neomycin trisulphate, 50 mg/kg tetracycline HCl, and 50 mg/kg bacitracin twice daily for two days before the oral administration of 200 mg/kg GlcN.

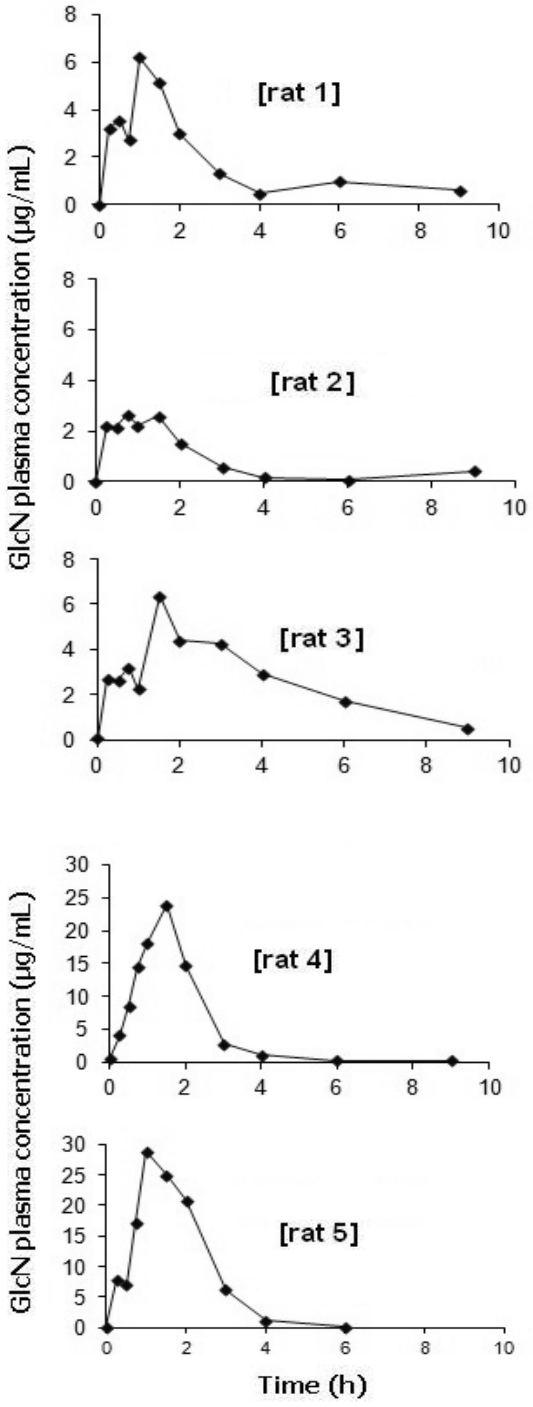

Figure xiii. The effect of co-administration of verapamil on the plasma concentration-time curve of the orally administered GlcN. The rats were given 25 mg/kg verapamil 2 h before the oral administration of 200 mg/kg GlcN.

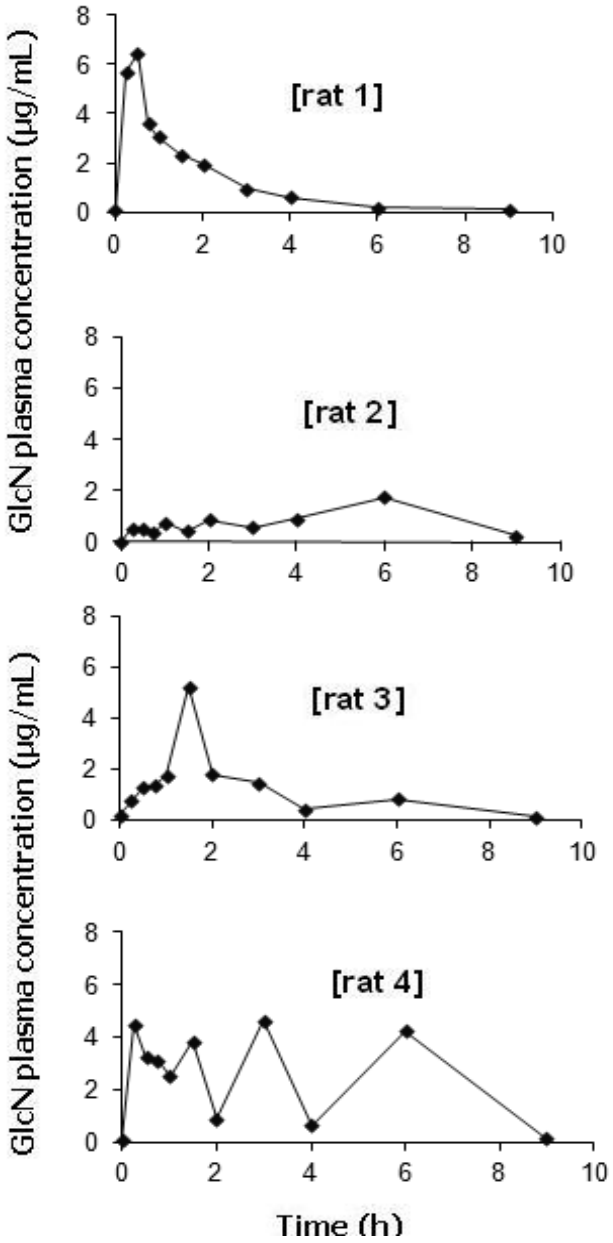

Figure xiv. The effect of co-administration of cyclosporine A (Sandimmune®) on the plasma concentration-time curve of the orally administered GlcN. The rats were given 30 mg/kg cyclosporine A, 2 h before the oral administration of 200 mg/kg GlcN.