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An in *vitro* Method to Determine Intestinal Bioavailability of Glucosamine Salt Mixture

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Abstract

Glucosamine is an amino sugar commonly used to improve joint health. It is often available for consumers as specialized supplements, the matrixes of which are formulated with components that facilitate enhancing functionality of the bioactive glucosamine. The primary objective of this study was to determine the in *vitro* bioaccessibility and bioavailability of a commercial glucosamine sulphate supplement, formulated with a mineral clay mixture. We used a modified a 3-step *in vitro* digestion procedure that included oral, gastric, and gastrointestinal digestions to assess bioaccessibility. Bioavailability followed using a $Caco_2$ cell permeability test. Glucosamine bioaccessibility was not affected by gastric digestion and only marginally affected by gastrointestinal digestion (e.g., > 90% recovery). Bioavailability was dramatically lower, averaging approximately 15%, but similar for both the glucosamine reference standard and clay mineral mix glucosamine formulated product. Our in *vitro* bioavailability measurement of glucosamine, corrected for bioaccessibility, agree with values from *in vivo* rodent models. We conclude that the *in vitro* 3-step digestion of glucosamine, used to mimic gastrointestinal digestion, followed by the $Caco_2$ permeability assay represents an alternative method to assess digestibility and bioavailability of formulated glucosamine products.

Keywords: Glucosamine; Clay Mineral Mix; Bioaccessibility; Bioavailability

Introduction

Amino sugars, such as glucosamine (2-amino-2-deoxy-β - D - glucopyranose), are essential for glycoprotein and glycolipid biosynthesis in mucosal membranes [1]. Glucosamine salts are valued commodities for treatment of osteoarthritis (OA), a common form of arthritis in seniors and the leading cause for disability in this demographic population [2]. In adults aged 60 or older, 10% male and 13% female suffer from osteoarthritis, and this is increasing as the obesity rate in North America worsens [3]. Once consumed, glucosamine salts are hydrolysed in the gastrointestinal trace to produce free glucosamine [4]. Glucosamine is used for glucosamine-6-phosphate and N-acetylglucosamine synthesis, with glucosamine-6-phosphate being derived from glutamine and fructose-6-phosphate by glutamine-fructose-6-phosphate transaminase. Glutamine-fructose-6-phosphate is ultimately converted to uridine diphosphate N-acetylglucosamine, a component of glycoproteins, peptidoglycans, and glycosaminoglycans which have reported anti-inflammatory and chondroprotective properties in humans [5]. Glucosamine is quickly absorbed, extensively distributed, rapidly metabolized and excreted after oral administration; however, previous animal studies indicate that it has low bioavailability. Some commercial glucosamine products are formulated with cation-based mineral clays that are designed to further enhance anti-inflammatory properties and improve relief from arthritic pain. There is no information if the presence of cation based mineral clays contribute to reduced bioaccessibility/ bioavailability of glucosamine [6].

The human intestinal $Caco_2$ cell, derived from human colon carcinoma, when differentiated, resembles a functional enterocyte with effective tight junctions, a number of transporters and microvilli for absorption process [7]. Only recently has *in vitro* $Caco_2$ permeability testing been done to include a pre-bioaccessibility evaluation to accurately estimate net bioavailability of bioactives and toxins [8-9]. The purpose of this study was to develop an *in vitro* assay that would accurately determine glucosamine bioavailability, thus representing a viable alternative to *in vivo* animal testing.

Materials and Methods

Materials

D-(+)-glucosamine sulfate and N-9-fluorenyl-methoxycarbonyloxyl succinimide (FMOC-Su) were purchased from Sigma Aldrich (St. Louis, MO, USA). Mineral clay samples were collected from six different locations in the Sierra Mountains (USA) and supplied by SierraSil Health Inc. Canada. Pancreatin and pepsin enzymes (pig), bovine serum albumin (BSA), NaH_2PO_4, mucin (pig), D-(+)-glucosamine, lipase (pig), α-amylase (*Bacillus* species), urea and bile salts (bovine), modified eagle medium (DMEM) containing 4.5 g/L glucose, penicillin-streptomycin solution (10,000 U penicillin and 10 mg streptomycin per mL), Hank's Balanced Salt Solution (HBSS) and glycine were also obtained from Sigma-Aldrich (St. Louis, MO, USA), KH_2PO_4, $MgCl_2 \bullet 6H_2O$, KCl and uric acid were obtained from VWR. $Caco_2$ cells (HTB-37TM) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum and trypsin-EDTA were purchased from Invitrogen (Burlington, ON. Canada). The mineral clay samples used for the matrix were air-dried, disaggregated sieved (2 mm) to remove

large particles, and lastly, small particles greater than 250 μm in diameter were excluded by screening. Glucosamine sulphate powder was premixed with the clay sample and the mixture kept in cold room until the simulated bioavailability experiments were conducted.

HPLC Analysis of Glucosamine

Glucosamine was analyzed from a glucosamine sulfate standard and glucosamine sulfate formulated in clay mineral mix using the AOAC official method 2005.01 [10]. Briefly, samples were derivatized using N-(9-fluorenylmethoxy-carbonyloxy)-succinimide (FMOC-Su), 97% pure, in a heated water bath (60 °C) for 30 minutes. HPLC analysis was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Wilmington, DE) equipped with a Phenomenex Prodigy (MidBore™ ODS-3 Column (100 Å, 5 μm, 150 × 3.2 mm) and Diode Array Detector (detection wavelength, 265 nm). The column temperature was 30 °C. The mobile phase A was water containing 0.05% trifluoroacetic acid, pH of 2.4, while mobile phase B was 100% acetonitrile. Samples were eluted with a gradient fluid of 30% B from 0-6 minutes; 30-100% B from 6-11 minutes; 100-30% B from 11-13 minutes; 30% B from 13-15 minutes at a flow rate of 0.8 mL/min. The injection volume was 10 μL. Calibration curves of known standard D-(+)-glucosamine hydrochloride used to calculate the glucosamine sulfate in both pure and digested samples.

In Vitro Simulated Bioaccessibility

Different simulation digestive fluids were prepared as described previously for gastric and gastrointestinal digestion using a 3-step Unified Bioaccessibility method (UBM) [9, 11]. The simulated saliva fluid contained amylase, mucin and uric acid. The simulated gastric fluid (bovine serum albumin, mucin and pepsin) was made in HDPE and the pH adjusted to 1.0 using HCl. The simulated duodenal fluid contained $CaCl_2$, bovine serum albumin pancreatin and lipase were made in HDPE, and adjusted to pH 8.0 using NaOH. Samples were prewarmed for 1 hour prior to exposure to the simulated oral digestion, where simulated salivary fluid (pH 6.5) was added to the samples and manually shaken for 30 seconds. This was immediately followed by transferring an aliquot of the saliva digestion to the simulated gastric solution (pH 1.0) which was placed in a shaking incubator set at 37 °C and 150 RPM for 1 hour. A set volume of simulated duodenal fluid (pH 8.0 ± 0.2), and bile fluid (pH 7.4 ± 0.2) of bile fluid were then added to the samples removed from the digestion phase to simulate gastrointestinal digestion. These samples were also incubated in the shaking incubator set at 37 °C at 150 RPM for another 4 hours. When the experiments were completed, sample tubes were removed and placed in a boiling water bath for 5 minutes to inactivate the enzymes. Digested sample pH was readjusted to pH 7, derivatized as outline above to determine total glucosamine content.

Bioaccessibility was assessed from the percentage of glucosamine that remained performed as follows:

$$Bioaccessibility(\%) = \frac{glucosamine\ in\ digested\ fluid}{total\ glucosamine\ added} \times 100\% \quad (1)$$

In vitro Bioavailability

We used 21-day differentiated $Caco_2$ cells (22 to 29 passages) according to the methods described by Chen et al.to determine the in vitro bioavailability of glucosamine following prior digestion[9]. Briefly, Caco-2 cells were seeded onto 6-well translucent Transwell inserts (24 mm diameter, 0.4 μm pore size, high-density polyethylene terephthalate membrane, BD Biosciences, San Jose, CA, USA) at a density of 2.5 × 105 cells/cm². and allowed to grow for 3 weeks in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS) 100 μg/ml of penicillin and 100 μg/ml of streptomycin at 37 °C under a 5% CO_2 atmosphere. Media was changed every 2–3 days, and cells were sub-cultured weekly by trypsin-EDTA treatment.

For all experiments, the culture medium used to hold the Caco-2 cells was removed on both sides of the Transwell® insert, and 1.5 mL of digested fluid, or reference sample was added back to the apical side. HBSS (2.6 mL) was added to the basolateral side of the membrane chamber. The cell cultures were incubated at 37 °C and 5% CO_2 for 2 hr to allow glucosamine transport. Media collected from the basolateral solution was used to determine glucosamine enterocyte permeability, while samples collected from the apical side were used to determine cellular uptake of glucosamine.All experiments were conducted using in triplicate. The viability of Caco-2 cells at time of experiment was measured using MTT redox assay [9].

In this study, permeability is defined as the percent of glucosamine recovered from the basolateral side of the membrane, compared to the total amount present in the digested fluid. Bioavailability was defined as the product present available at oral digestion. Uptake of glucosamine by cells was calculated by total glucosamine present in the digested fluid, subtracted by glucosamine found in apical fluid and basolateral fluid. Total absorption of glucosamine was calculated as the total of permeable glucosamine and glucosamine taken up by the cell.

Calculations for $Caco_2$ uptake were as follows:

$$\text{Permeability } (\%) = \frac{\text{amount of glucosamine in the basolateral side}}{\text{amount of glucosamine present in digested fluid}} \times 100\% \qquad (2)$$

Cellular uptake (%)
$$= (\text{total glucosamine in digested fluid} - \text{glucosamine in apical side} - \text{glucosamine in basolateral side})$$
$$\times 100\% \qquad (3)$$

Absorption (%) = Permeability + Cellular uptakeBioavailability (%)
$$= \text{absorption} \times \text{bioaccessibility} \qquad (4)$$
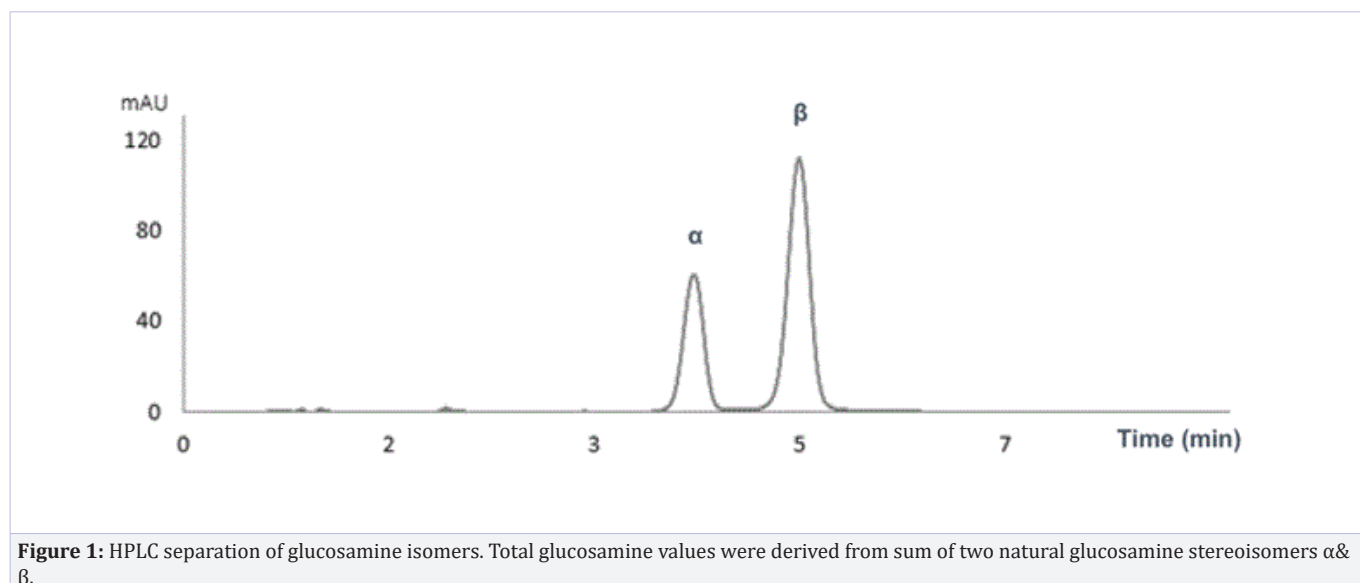
Statistical Analysis

All assays were conducted in duplicate or triplicate and means were calculated with a standard deviation (Minitab for Windows, version 13.32; State College, PA, USA). Differences between glucosamine reference sample and the clay mineral mix product were assessed using student's t-test.

Results

Figure 1 shows the HPLC chromatogram for quantitative glucosamine determination. The eluted glucosamine typically showed two peaks on the chromatogram at the 4th and 5th minute, respectively. The interconversion of 2 natural glucosamine stereoisomers (α and β), in aqueous solution is not preventable, therefore the sum of the two areas under both curves was done to calculate the glucosamine content.

The bioaccessibility of glucosamine reference standard alone neared 100%, for gastric and gastrointestinal digestion, respectively (Figure 2). The presence of the different clay minerals that represented the matrix of the formulated glucosamine reduced bioaccessibility only marginally (e.g. 1-3%), at both gastric and the gastrointestinal digestion. The mineral clay composition known to contain a complex mixture of divalent cations had no apparent effect on the extent on the recovery of glucosamine following simulated digestion[6].

The percent permeability of the glucosamine-mineral mix using the Caco$_2$ monolayer, corrected for gastrointestinal digestion averaged 9.40±1.16%, which was not different to the glucosamine reference standard. Similarly, the glucosamine-mineral mix had an average Caco$_2$ cellular uptake of 6.11±3.51%, which again resembled the glucosamine sulfate standard (5.4%) (Figure 3). The source of the clay material was a notable factor for the wide range in glucosamine bioavailability observed in vitro (e.g., range = 10.3–21.5%).
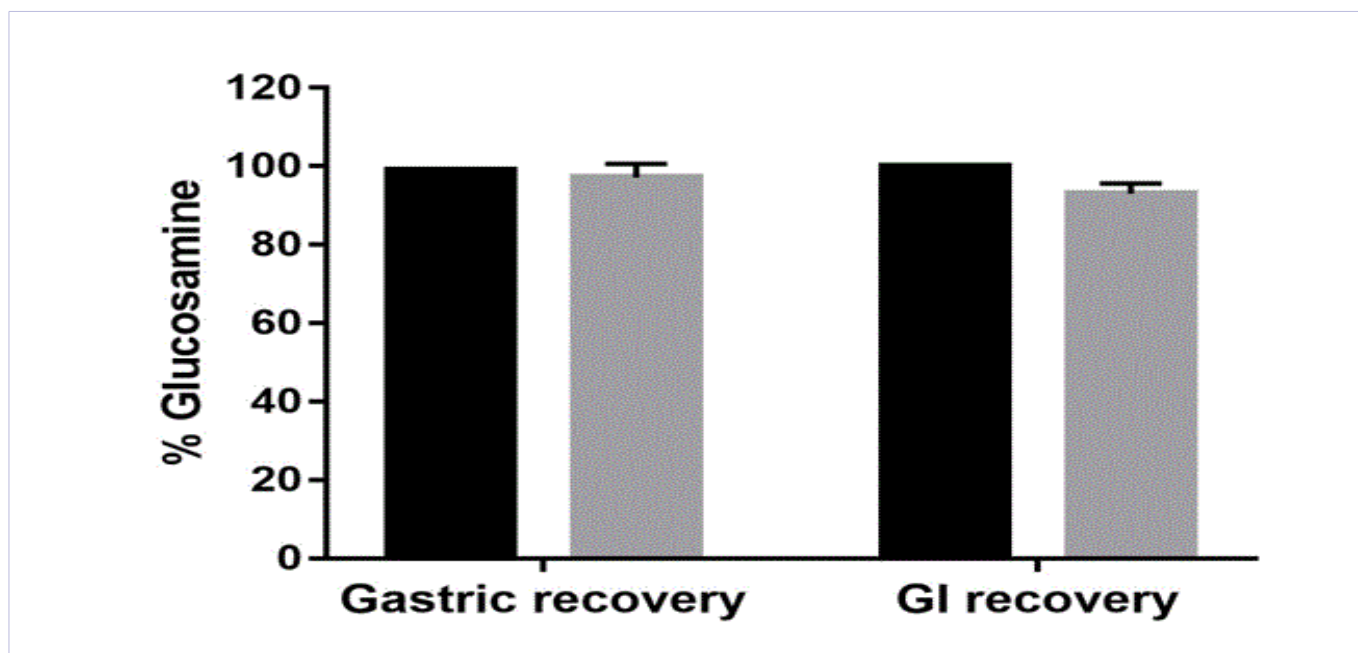

Figure 1: HPLC separation of glucosamine isomers. Total glucosamine values were derived from sum of two natural glucosamine stereoisomers α& β.

Figure 2: *In vitro* bioaccessibility of glucosamine from gastric and gastrointestinal digestion of control (black) and mineral clay matrix (grey). Values represent mean ± SD (n=6). No statistical difference between glucosamine standard and mineral mix samples were found. The control refers to the glucosamine sulfate standard, while the matrix refers to the glucosamine sulfate mineral clay formulation.

Figure 3: Caco$_2$ cell permeability, cellular uptake and absorption of glucosamine post gastrointestinal digestion, from control (black) and mineral clay matrix (grey). Values represent mean ± SD (n=6).No statistical difference between glucosamine standard and mineral mix samples were found. The control refers to the glucosamine sulfate standard, while the matrix refers to the glucosamine sulfate mineral clay formulation.
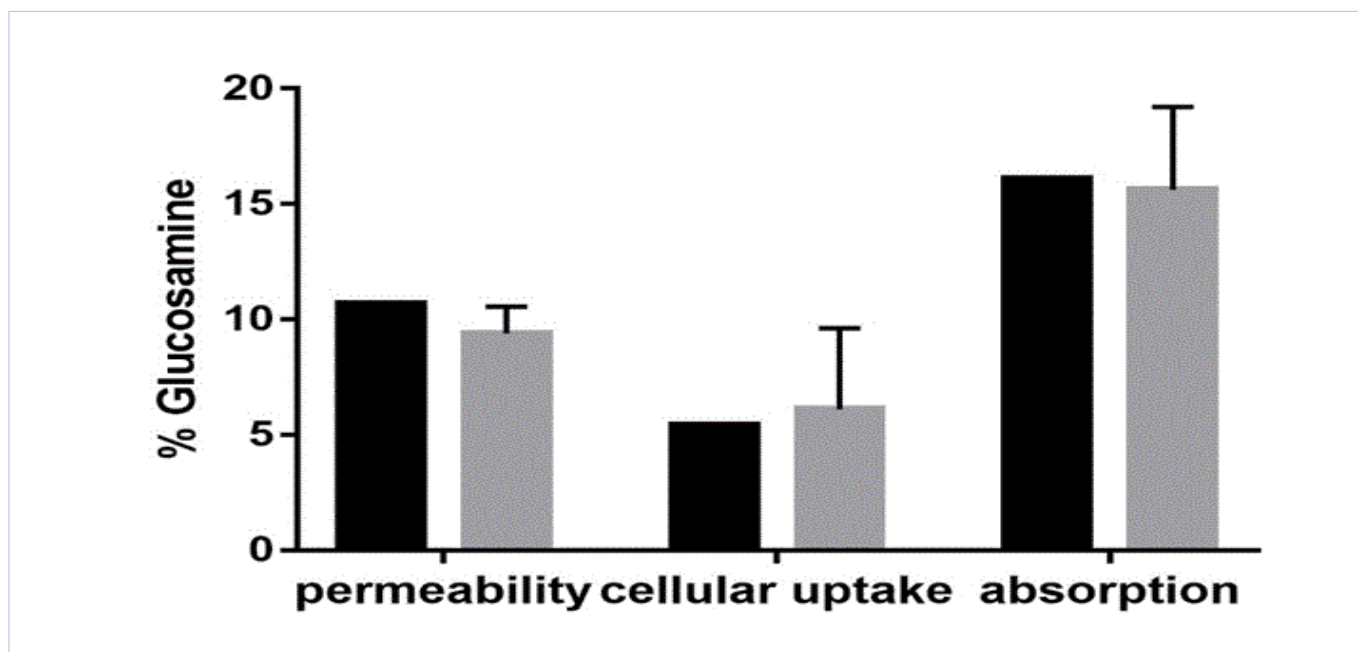
Discussion

The *in vitro* bioaccessibility assay of the glucosamine reference standard indicated no appreciable loss during neither gastric nor intestinal digestion.Glucosamine samples prepared with the clay mineral mix to formulate the commercial supplement had comparatively marginally lower bioaccessibility following gastrointestinal digestion. The fact that the coefficient of variation for both digestion phases was less than 10%, indicated that different locations from which the mineral clay sampled to formulate the glucosamine supplement, was not a factor to non-uniformity observed in bioaccessibility between supplement batches.

Bioavailability determined using differentiated $Caco_2$ monolayers prepared on Transwell® inserts produced results that showed limited glucosamine permeability, low cellular uptake and thus a low percent bioavailability for both the pure glucosamine reference standard and the commercial glucosamine product formulated with a clay mineral mix. Thus, the minor bioaccessibility loss of glucosamine in both cases could not be attributed to the low bioavailability that was measured subsequently. Our results agree with reports that there is limited uptake of glucosamine by intestinal cells [12-13]. Once corrected for bioaccessibility, our estimate of glucosamine bioavailability using the $Caco_2$ cell monolayer permeability assay was within a range of values reported from *in vivo* experiments (e.g., 5–26%), using different animals including dogs, horses, rats [14-18]. Formerly, low bioavailability of glucosamine was attributed to low solubility of glucosamine in aqueous solutions, using different salt forms, such as hydrochloride or sulfate[19]. This characteristic would explain to some extent our results using the $Caco_2$ permeability method, where solubility of the analyte is an essential component of paracellular absorption. Others have reported glucosamine to be a poor substrate for glucose transports required to ensure transcellular intestinal uptake [19]. Finally, former in vivo experiments that designed experiments to enable apparent absorption of glucosamine that involved accounting for fecal metabolite content have reported glucosamine utilization to approach 100% [16]. This interesting finding reflects the influence of the gut microbiome and thus capacity to metabolize glucosamine in the large intestine; however,no information is available of the bioactivity of gut metabolites[20-21].

In conclusion, our findings demonstrate a useful alternative to *in vivo* methods to measure glucosamine bioavailability. The fact that the differentiated $Caco_2$ cell produced results that were in generally agreement with *in vivo* literature values indicates that the *in vitro* cell-based assay can be used as an alternative to animal studies for determine glucosamine bioavailability, thus eliminating animal costs and ethical animal welfare issues.

Acknowledgements

Declarations

The authors have no declarations to report. The cell culture work was approved by UBC biosafety (UBC 42:2019).

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