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Determination of glucosamine and chondroitin in supplement by UPLC-TUV

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Abstract. An ultra-performance liquid chromatography (UPLC) method for determination of glucosamine and chondroitin in pharmaceuticals was validated as column Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 µm) and column BEH HILIC (2.1 × 100 mm, 1.7 µm); detector TUV at 255 nm; mobile phase was methanol-water for glucosamine and methanol–5 mM ammonium acetate (pH 8) for chondroitin. The study was carried out after derivatization with 9-fluorenylmethyloxycarbonyl chloride for glucosamine determination. According to the International Conference on Harmonization (ICH) guidelines, the developed method was validated. The proposed method was proved simple, rapid, precise, and accurate to analyze glucosamine and chondroitin in functional foods on practical application.

Keywords*: glucosamine, chondroitin, derivatization with FMOC- Cl, UPLC-TUV.*

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1. Introduction

Osteoarthritis is a chronic disease considered a global organ failure and characterized by irremediable damage to significant tissues of the joint, namely cartilage, synovial membrane, and subchondral bone. The disease pathogenesis was observed, including articular cartilage loss, synovial membrane inflammation, and subchondral bone alteration (Martel-Pelletier, 2005).

Glucosamine, 2-amino-2-deoxy-D-glucose, is an essential part of chitin and mucopolysaccharides. Large complexes of negatively charged carbohydrate chains are incorporated into mucous secretions, connective tissue, ligaments, and cartilage. Glucosamine is also a widely used compound for reducing symptoms of osteoarthritis. According to U.S. National Institutes of Health (NIH) and other scientists' research, side effects of glucosamine were significantly less common than non-steroidal anti-inflammatory drugs (NSAIDs), significantly less than one-third compared to ibuprofen (a commonly used of this group of drugs). Its safety was proved to be acceptable under using conditions and does not affect glucose metabolism (Anderson, 2005; Stanley, 2004). Chondroitin sulfate is a sulfated glycosaminoglycan which is a long, unbranched chain made from a repeating disaccharide structure of glucuronic acid and N-acetyl galactosamine residues. It is also one of the most widely used compounds in managing osteoarthritis patients. Chondroitin sulfate has been shown to change the death process of a chondrocyte, improving the anabolic/catabolic balance of the extracellular cartilage matrix, reducing some proinflammatory and catabolic factors and, in subchondral bone osteoblasts, increasing the ratio of OPG/ RANKL (Martel-Pelletier, 2005).

Moreover, the long-term administration of oral CS is proved to be safe, well-tolerated, and fully indicated to control the pain symptoms and increase the mobility of knee osteoarthritis patients (Uebelhart, 2008). Ultra-performance liquid chromatography (UPLC) has become one of the most frequently applied approaches in fast chromatographic separations (Liang, 2010).

Hydrophilic interaction liquid chromatography (HILIC) uses traditional polar stationary phases, commonly applied to determinate polar compounds, such as pharmaceuticals, biopharmaceuticals, organic contaminants in environmental samples, and food components in foods (Redón, 2020). Pre-column derivatization is an efficient method that produces stable and highly fluorescent derivatives for analysis, and the most commonly used derivatization reagent for this purpose is 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) (Jámbor, 2009; Molnár-Perl, 2011). However, these methods all take a long time and have complex processing.

There have been many methods to quantify glucosamine and chondroitin on different subjects by many techniques such as electrophoresis, high-performance liquid chromatography combined with different detectors (HPLC-RID, UV, PDA, FD), MS/MS) (Harmita, 2017; Huang, 2011; Kosman, 2017). In the United States Pharmacopoeia (USP, 40), glucosamine and chondroitin are quantified separately with two methods, PDA probe high-performance liquid chromatography, and potentiometric titration, respectively. While in the British Pharmacopoeia (BP, 2016), quantification of glucosamine and chondroitin by potentiometric titration (USP, 40). According to Vietnam Pharmacopoeia V (VMPH, 2018), glucosamine is determined by liquid chromatography with UV probe, aminopropylsilyl silica gel column, but chondroitin still does not have a process for quantification. However, these methods all take a long time and have complex processing. Therefore, this study aims to establish a quantitative and straightforward glucosamine chondroitin process, less time analysis, by UPLC-TUV and pre-column derivatization with FMOC-Cl.

2. Materials and Methods

2.1. Chemicals and materials

The reference samples of glucosamine hydrochloride (≥ 99%) and chondroitin sulfate (≥ 99%) were purchased from Sigma (St. Louis, MO, USA). Fluorenylmethyloxycarbonyl chloride derivatization reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, and HPLC-grade water were acquired from Honeywell (North Carolina, USA). Ammonium acetate, acid formic, sodium hydroxide, sodium borate was purchased from Merck (Darmstadt, Germany). The tablets contain two active ingredients: glucosamine and chondroitin.

2.2. Instrumentation

A UPLC Acquity H-Class system (Waters Corporation, Massachusetts, USA) equipped with a TUV detector.

2.3. Methods

According to Qian et al. (2013) and the structures and polarities of analytes, chondroitin was quantified by HILIC column, and glucosamine was determined by UPLC-TUV with a C_{18} column after derivatization with 9-fluorenylmethyloxycarbonyl chloride (Michel, 2004; Qian, 2013). The chromatographic parameters, including elution solvents, mobile phase ratios, detective wavelengths, flow rates, and column temperatures, were investigated. Meanwhile, the FMOC-Cl derivatization conditions have optimized reaction temperatures (30 °C, 50 °C, and 70 °C).

Preparation of stock solutions. Primary stock solutions of glucosamine and chondroitin are prepared with methanol at 1000 µg/mL. Working solutions for analysis and validation are suitably diluted from stock solutions.

Preparation of reagent solutions. FMOC-Cl is dissolved in acetonitrile at 1500 µg/mL.

Methods validation. According to ICH guidelines, the proposed method was validated for selectivity, linearity, the limit of detection (LOD), quantification (LOQ), precision, and accuracy.

3. Results and Discussion

3.1. Methods development

The high polarity of the determined compounds made it challenging to optimize chromatographic separation (Huang, 2006; Václavíková, 2015). On the other hand, the molecular weight of glucosamine and chondroitin is also a problem for HPLC quantification. Establishing and optimizing the analysis process, therefore, becomes much more necessary. According to Government Chemist (2012), chondroitin determination has several typical approaches, such as high-performance liquid (HPLC) methods based on size exclusion and using amine column or ion exchange HPLC. Meanwhile, glucosamine also has been

researched by an extensive range of methods like gas chromatography, liquid chromatography-tandem mass spectral, and one of the most effective methods that have been reported is high- performance chromatography method involving derivatization with FMOC-Cl (Huang, 2006).

3.1.1. Chondroitin quantification

The survey results showed that aliquots of the process samples (10 µL) were injected into column-a BEH HILIC column (2.1 × 100 mm, 1.7 µm) with mobile phase, a 50:50 mixture of acetonitrile and 5 mM ammonium acetate (pH 8), was delivered at 0.25 mL/min and samples were detected at a wavelength of 255 nm. This mobile phase mixture brings back an exemplary chromatogram of chondroitin, which is an excellent and symmetrical peak.

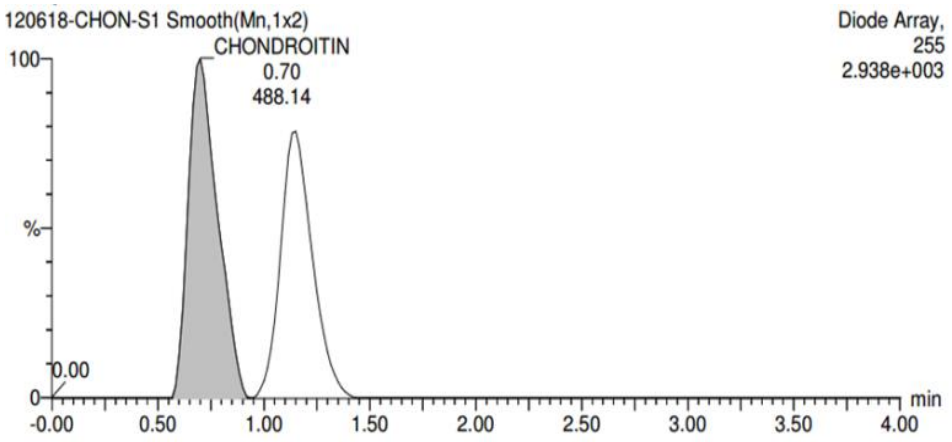

Figure 1. Chondroitin chromatogram (Mobile phase: acetonitrile: 5 mM ammonium acetate (pH 8) (50:50))

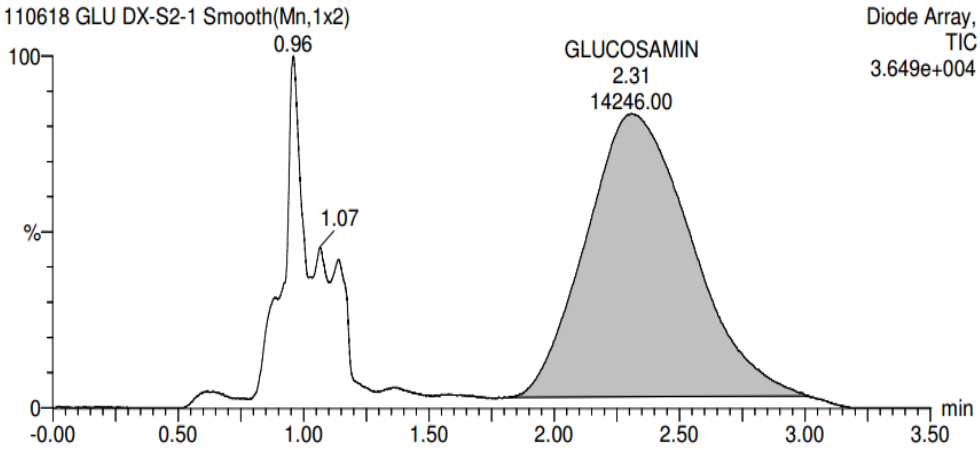

Figure 2. Glucosamine chromatogram under optimal conditions

3.1.2. Glucosamine determination

The following factors were investigated: reaction temperature of derivatization, quantitative wavelength, mobile phase ratio, respectively. Depending on the stability of glucosamine-FMOC-Cl over time and a result shown on the chromatogram, reaction temperature has been chosen. The maximum absorption peak of the derived glucosamine is 259 nm; conducting a survey, at 255

nm, the derived peak signal is good, with little background noise, so the quantitation wavelength is 255 nm. The chosen mobile phase was also accepted as a reasonable and symmetrical peak requirement.

The optimal chromatographic conditions for glucosamine were selected based on the research of Huang (2006), the investigation factor of derivatization and quantification has been chosen. The results showed the most potential condition is using the indirect method of pre-column derivatization by hydrolysis with alkaline agents and FMOC-Cl reagent at the reaction temperature of 50 °C, quantified on the TUV probe UPLC system with the Acquity UPLC BEH C18 column (2.1 × 100mm, 1.7 µm); detection wavelength 255 nm; flow rate 0.25 mL/min; sample injection volume 10 µL. Mobile phase A is acetonitrile; mobile phase B is water. The mobile phase ratio was adjusted according to the gradient elution program [time (minutes)/%A] as follows 0-2.5/35, 2.5-4/60.

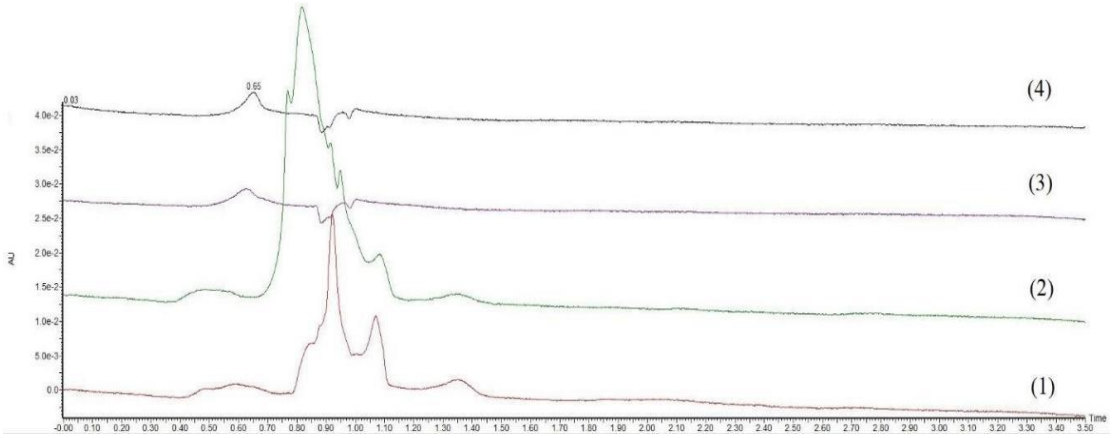

Figure 3. Chromatogram (1) reactive solvent, (2) unreacted glucosamine sample (3) sample solvent, (4) mobile phase sample

3.2. System suitability testing

System suitability testing was assessed by analyzing six replicates of glucosamine - FMOC-Cl standard at 25 g/mL and chondroitin standard at 300 µg/mL. The results in table 1 show that the RSD% of the retention time and the peak area are both less than 2%. The chromatographic parameters are within the acceptable range: from 0.8 to 1.5 for asymmetry factor (As) and higher than 1.5 for resolution (Rs). Consequently, instrumentation quality is acceptable for research.

Table 1. System suitability testing

		t_R	S	As	Rs	k'
Glucosamine	**Mean**	2.24	17437.5	1.45	3.8	1.49
	RSD%	0.4	0.21	1.23	1.2	0.86
Chondroitin	**Mean**	0.69	1400.2	1.39	2.5	0.75
	RSD%	1.58	0.32	1.45	0.87	0.67

3.3. Selectivity

Figure 3-5 shows that sample chromatograms had peaks of glucosamine-FMOC-Cl and chondroitin with retention times equivalent to the prominent peaks in the standard chromatograms. No significant interference from mobile phase, solvent, underivatized glucosamine, and reaction solvent was observed at the retention time of analytes. The procedure for quantifying glucosamine and chondroitin meets the requirements for selectivity.

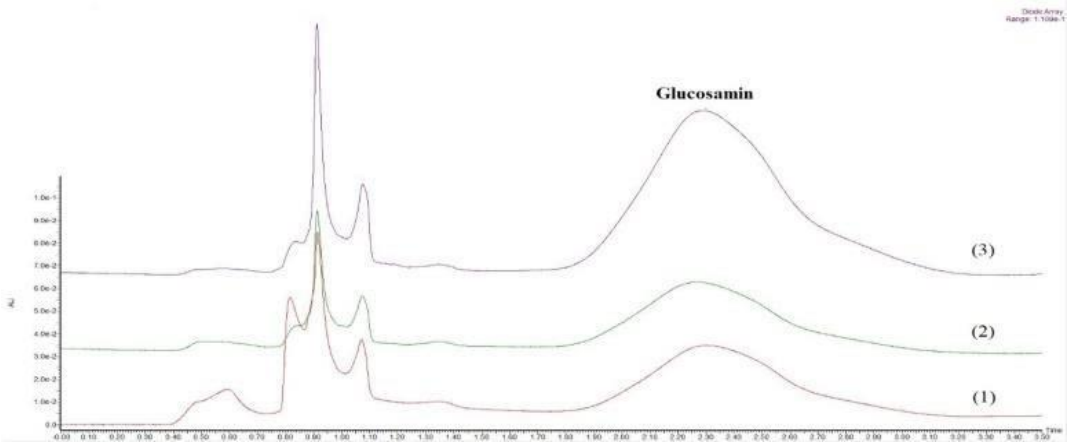

Figure 4. Chromatogram of glucosamine-FMOC-Cl in the standard sample (1), testing sample (2), and spiked sample (3)

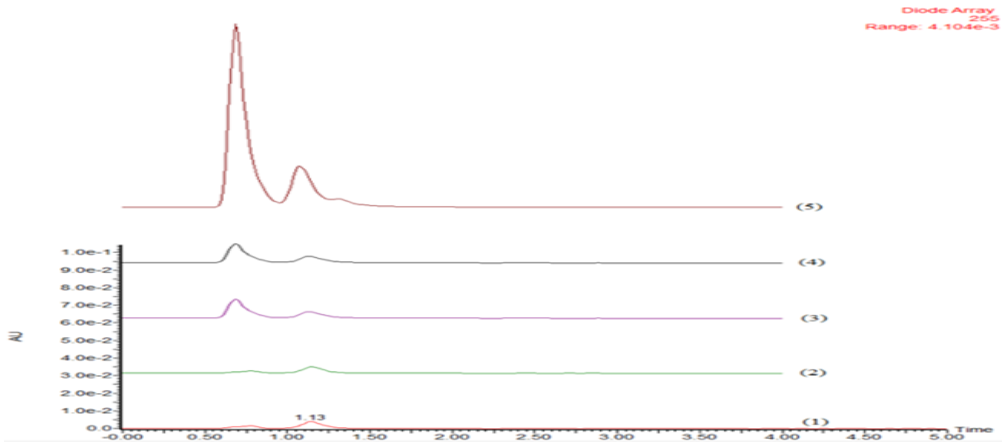

Figure 5. Chromatogram of mobile phase (1), solvent (2), testing sample (3), chondroitin standard sample (4), spiked sample (5)

3.4. Linearity

The investigation of linearity is over the concentration range of 10-100 µg/mL for Glucosamine- FMOC-Cl and 50-1000 µg/mL for chondroitin. The correlation coefficients during the validation were 0.999 and 0.998, respectively.

Table 2. Linearity of glucosamine-FMOC-Cl and chondroitin

	Linearity range (µg/mL)	**Regression equations, r^2**
Glucosamin- FMOC-Cl	10-100	y = 668.46x , r^2 = 0.999
Chondroitin	50-1000	y = 6.42x, r^2 = 0.998

3.5. Precision

The method's precision was verified by evaluating the intra-day and inter-day precisions. The relative standard deviation (%RSD) was selected to measure precision. The intra-day precision was examined by analyzing six samples in a single day, while the inter-day precision was determined by analyzing six samples each day for three days. The precision results were shown in Table 3, indicating that the overall intra- and inter-day variations (%RSD) were less than 2% (1.50% and 0,79%, respectively).

Table 3. Intra-day and Inter-day precision of glucosamine-FMOC-Cl (n = 6)								
Intra-day			**Inter-day**					
Sample	**Peak area (mAu.s)**	**Content (mg)**	**Peak area (mAu.s)**			**Content (mg)**		
			Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
1	11948	491.653	11948	11651	11472	491.65	477.94	469.75
2	11657	478.256	11657	11439	11401	478.25	468.25	466.51
3	11586	475.140	11586	11526	11484	475.14	472.24	470.31
4	11429	467.662	11429	11541	11462	467.66	472.93	469.28
5	11634	477.321	11634	11554	11446	477.32	473.52	468.57
6	11539	472.959	11539	11521	11479	472.96	471.99	470.06
Mean	11632.17	477.165	11542.72			473.01		
RSD%	1.50	1.685	0.79			0.88		

3.6. Accuracy

Spiked samples evaluated the accuracy at three concentrations of 80, 100, 120 g/mL for glucosamine-FMOC-Cl and chondroitin. Each concentration was analyzed in duplicate three times. The mean recoveries of glucosamine-FMOC-Cl and chondroitin are 101.5% (RSD = 1.07%) and 100.59% (RSD = 0.34%), respectively, which meet the requirement of ICH guidelines. The results are shown in Table 4. The validation parameters followed the ICH (2005) criteria, making this study potentially a widely used procedure for determining glucosamine and chondroitin.

Table 4. Accuracy of glucosamine-FMOC-Cl and chondroitin (n = 6)			
	Concentration µg/mL	**Recovery (%)**	**RSD %**
Glucosamine- FMOC-Cl	80	100.67	2.7
	100	101.78	0.25
	120	102.05	0.19
Chondroitin	80	100.57	0.32
	100	100.05	0.59
	120	101.15	0.11

3.7. Application

The quantitative procedure after good validation was applied to quantify glucosamine and chondroitin in two tablets containing glucosamine and chondroitin simultaneously. The quantitative results are presented in Table 5, which shows that two samples do not meet the content requirement. This result brings back a reminder of the necessity to have the quantification process for the quality control of the functional foods sold in the market.

Table 5. Application study		
Producer	**Content of analytes (%)***	
	Glucosamine	**Chondroitin**
Company A	99.7 ± 0.88	99.5 ± 1.18
Company B	31.67 ± 0.28	79.9 ± 0.87
*: The mean values ± SD (n = 3)		

4. Conclusion

A procedure for glucosamine quantification by pre-column derivatization with FMOC-Cl reagent on UPLC Acquity H-Class Waters system - TUV probe, with C18 (2.1 × 100 mm, 1.7 µm), mobile phase consisting of ACN – water with gradient elution program and a process to quantify chondroitin by UPLC/TUV system, with BEH HILIC column (2.1 × 100 mm, 1.7 µm), mobile phase consisting 50:50 ACN – 5 mM ammonium acetate (pH 8) were researched. Both procedures were validated according to

ICH guidelines to fully meet the requirements of a quantitative analytical procedure, including selectivity, linearity, accuracy, and precision. Experiment for quantifying glucosamine and chondroitin in dietary supplements was proved to be a potential application and pharmaceuticals.

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Conflict of interest. The authors declare that there are no conflicts of interest regarding the publication of this paper.

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