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Research Article

DETERMINATION OF LEVELS OF GLUCOSAMINE HYDROCHLORIDE AND CHONDROITIN SULFATE IN MIXTURES IN TABLET AND CREAM FORMS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE

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ABSTRACT

Objective: Glucosamine hydrochloride (HCl) and chondroitin sulfate are glycosaminoglycan compounds and major structural components of bones in the form of proteoglycans. These compounds maintain bone structure by stimulating the synthesis of synovial fluid and inhibiting the degradation of joint cartilage, and they can be used for the treatment of osteoarthritis. The aim of this study was to identify a selective analytical method for determining glucosamine HCl and chondroitin sulfate levels in tablet and cream forms.

Methods: After derivatization using ortho-phthalaldehyde and 2-mercaptoethanol, the samples were analyzed using high-performance liquid chromatography (HPLC) with a fluorescence detector at an excitation wavelength of 335 nm and an emission wavelength of 445 nm. Deacetylation using sodium hydroxide was required to break the acetyl group bond. The mobile phase used tetrahydrofuran 0.25% in water-acetonitrile (87:13) with a flow rate of 1.5 ml/minute.

Results: The average levels of glucosamine HCl and chondroitin sulfate were 92.76% and 96.11% in tablets and 101.15% and 100.33% in creams, which fulfilled the acceptance criteria.

Conclusions: Our validation method for glucosamine HCl and chondroitin sulfate met the acceptance criteria of accuracy, precision, selectivity, and linearity.

Keywords: Chondroitin sulfate, Derivatization, Glucosamine hydrochloride, Fluorescence, High-performance liquid chromatography, Validation.

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INTRODUCTION

Glucosamine (2-amino-2-deoxy-D-glucose) is an amino monosaccharide, a component of glycoproteins in connective tissues and mucous membranes, involved in the formation of glycosaminoglycans. For two decades, the salt forms of glucosamine (hydrochloride [HCl] and sulfate) have been used independently or together with other active ingredients such as chondroitin sulfate in capsules, tablets, and solutions for oral administration. At present, topical forms of glucosamine, such as creams, gels, and patches, are being developed by the pharmaceutical industry [1]. Glucosamine HCl and chondroitin sulfate are used to treat osteoarthritis (OA). Glucosamine HCl stimulates improved joint function, and various studies have shown that it reduces the pain of OA, rehabilitates cartilage, improves synovial fluid formation, and improves joint damage caused by OA. Chondroitin sulfate supports cartilage health by absorbing liquids (especially water) into the connective tissue, and it can reduce the pain of OA and act as an anti-inflammatory medication [2].

The prevalence of OA in Indonesians older than 65 years is 60.5% in men and 70.5% in women, with approximately 2 million people suffering from a disability due to OA [3]. The increased prevalence of OA has led to a growing need for pharmaceuticals containing glucosamine and chondroitin. Therefore, a selective analysis method is required to ensure the quality of different pharmaceutical delivery forms [4].

Based on a search of literature, analysis of glucosamine HCl can be performed using high-performance liquid chromatography (HPLC) with a fluorescence detector. Glucosamine HCl does not have chromophore groups in the ultraviolet/visible (UV/Vis) spectra, but it can form fluorescence compounds after being derivatized with a fluorogenic reagent. Commonly used reagents for analyzing the derivatization of glucosamine HCl include ortho-phthalaldehyde (OPA), phenylisothiocyanate, and 9-fluorenylmethoxycarbonyl chloride [4]. Several methods are used to analyze chondroitin sulfate, such as titration with cetylpyridinium chloride, which enables the analysis of compounds with large molecules such as proteins but not specifically chondroitin sulfate [5]. Another technique for analyzing chondroitin sulfate is ion exchange chromatography with a fluorescence detector, commonly involving enzymatic digestion [6].

Several studies have analyzed mixtures of glucosamine HCl and chondroitin sulfate, including isotachophoresis and ionic analyte separation techniques using electrophoresis with UV detection at 254 nm [7]. Ion exchange chromatography is performed using a CarboPac PA20 column with potassium hydroxide as the eluent, with a flow rate of 0.5 ml/minute at 30°C [8]. Previous studies have also used reverse-phase HPLC with a fluorescence detector to analyze a mixture of glucosamine HCl and chondroitin sulfate in the Laboratory Quality Testing Center of Drugs, Food and Cosmetics Faculty of Pharmacy, Universitas Indonesia. The present study aimed to conduct further research on the optimization, validation, and determination of glucosamine HCl and chondroitin sulfate levels in tablet and cream forms using HPLC with a fluorescence detector. The expected advantage of this technique was that the results would be more selective than with a UV detector.

MATERIALS AND METHODS

Chemical and reagents

Standard glucosamine HCl (Sigma-Aldrich, US), standard chondroitin sulfate (PT. Dua Lima Farma, Indonesia), Aqua Bidest (PT. Ikapharmindo PUTRAMAS, Indonesia), Acetonitrile Pro HPLC (Merck, US/Canada), tetrahydrofuran (THF, Mallinckrodt chromAR® HPLC, US), OPA (Bio

Basic Inc., Canada), 2-mercaptoethanol (Merck, US/Canada), boric acid (Merck, US Canada), and NaOH (Merck, US Canada); Methanol Pro HPLC (Merck, US Canada), hydrochloric acid (Merck, US Canada), and dichloromethane (Merck, US/Canada).

Samples

Caplet Viostin DS® (Pharos, Indonesia) and Flexamine Cream® (Novell Pharmaceutical, Indonesia) were used.

Instruments

LC-20AT (Shimadzu, Japan) equipped with a pump, YMC-Triart® C18 column (250 × 4.6 mm, 5µm), 20A RF fluorescence detector (Shimadzu, Japan), manual injector, data processor, HPLC syringes (SGE, Australia), centrifuge (Kubota, Japan), vortex (Thermo Scientific, US), micropipettes (Eppendorf, Germany), 0.45 µm filter membranes, analytical balance, ultrasonic cleaner (Elma Elmasonic S40H, Germany), and glass tools.

Chromatography system

This study implemented HPLC equipped with a pump, a C18 column, and a fluorescence detector at λ_{ex}=335 nm and λ_{em}=445 nm. Mobile phase THF in water-acetonitrile (87:13) was used at a flow rate of 1.5 ml/minute.

Preparation of standard solutions

Standard solution of glucosamine HCl

Standard glucosamine HCl was weighed at 50 mg, then diluted with 10 ml of methanol:water (2:1), and Aqua Bidest was added up to 50 ml. This was diluted to obtain a concentration of 10 µg/ml.

Standard solution of chondroitin sulfate

The chondroitin sulfate standard was weighed at 50 mg, then 10 ml of NaOH 6N was added to a 100 ml glass beaker. This was heated at 60°C for 30 minutes with stirring until homogenized, cooled, and then neutralized to pH 7 with HCl 2N. It was then diluted with 10 mL of methanol:water (2:1), Aqua Bidest was added up to 50 mL, and the mixture was diluted to obtain a concentration of 100 µg/ml.

Preparation of tablet sample solution

Ten tablets were obtained and weighed to calculate the average mass; then the tablets were crushed into a homogeneous powder. The tablets were equivalent to ±100 mg.

Sample preparation of glucosamine HCl

Tablet samples were weighed to ±216 mg and diluted with 10 ml of methanol:water (2:1). The solution was centrifuged at 3500 rpm for 10 minutes, 5 ml of dichloromethane was added, and the solution was shaken for 5 minutes, then allowed to separate. The water layer was filtered with 0.45 µm filter membranes. Aqua Bidest was added and diluted to a certain concentration.

Sample preparation of chondroitin sulfate

Tablet samples were weighed to ±265 mg, then placed with 10 mL of NaOH 6N in a 100 ml glass beaker. This was heated at 60°C for 30 minutes with stirring, cooled, neutralized to pH 7 with HCl 2N, then diluted with 10 ml of methanol:water (2:1). The solution was centrifuged at 3500 rpm for 10 minutes, and 5 mL dichloromethane was added. The solution was shaken for 5 minutes, then allowed to separate. The water layer was filtered with a 0.45 µm filter membrane, then Aqua Bidest was added and diluted to a certain concentration.

Preparation of cream sample solution

Sample preparation of glucosamine HCl

The cream was weighed out in equivalents of ±250 mg, then dissolved in 10 ml of methanol:water (2:1) and the solution was centrifuged at 3500 rpm for 10 minutes. Next, 5 ml of dichloromethane was added and the solution was shaken for 5 minutes, then allowed to separate. The water layer was filtered with a 0.45 µm filter membrane, and then Aqua Bidest was added and diluted to a certain concentration.

Sample preparation of chondroitin sulfate

The cream was weighed in equivalents of ±5 g and then added to 10 ml of NaOH 6N in a 100 ml glass beaker. This was heated at 60°C for 30 minutes with stirring, then cooled and neutralized to pH 7 with 2N HCl. The solution was then diluted with 10 ml of methanol:water (2:1) and centrifuged at 3500 rpm for 10 minutes. Next, 5 ml of dichloromethane was added and the solution was shaken for 5 minutes, and then allowed to separate. The water layer was filtered with a 0.45 µm filter membrane, and then Aqua Bidest was added and diluted to a certain concentration.

Derivatization

Pipettes of 100 µl of standard solutions of glucosamine HCl and chondroitin sulfate were placed in a vial at a concentration of 100 µg/ml, 50 µl of OPA/2-ME reagent was added, and the mixture was homogenized with a vortex for 20 seconds. This was allowed to react for 2 minutes and was then analyzed with the HPLC system (Figs. 1 and 2).

RESULTS AND DISCUSSION

Analyses of glucosamine HCl and chondroitin sulfate levels were performed with pre-column derivatization using OPA with the addition of 2-mercaptoethanol (2-ME). Both of these form fluorescent compounds that can be detected. OPA/2-ME is commonly used as a reagent to improve the detection of amino acids. It reacts with the primary amine group substrate to form isoindole derivatives with high molar absorptivity of UV light (chromophores) or fluorescence groups (fluorophores). Glucosamine has primary amine groups (NH_2) that can be derivatized with OPA/2-ME, while chondroitin has an acetyl group on the amine. Thus, NaOH is used to break the bond between the acetyl group ($COCH_3$) and the nitrogen atom to form primary amine groups (NH_2) [9,10].

Optimization of derivatization reagent volume and incubation time

Experiments were conducted to determine the volume of OPA/2-ME reagents required to produce optimum and stable derivatives (Table 1). Volumes of 25 µl, 50 µl, and 100 µl were analyzed, and the 50 µl volume showed larger peak areas and was more stable in both compounds. Derivatization produces perfect derivative compounds, so the analyte must be incubated after derivatization at a specific time (Table 2).

Table 1: Optimization of derivatization reagent volume

Volume (µl)	Area (µv/s)	
	Glucosamine HCl	Chondroitin sulfate
25	40172900	1448830
	45389489	2034847
	40131451	2013597
50	41932818	2261195
	41751503	2120182
	40008905	1920195
100	27697397	1407211
	27726704	1117807
	29130675	993686

HCl: Hydrochloride

Table 2: Incubation times

Time (minutes)	Area	
	Glucosamine HCl	Chondroitin sulfate
2	36067148	1169841
5	33926297	1225551
10	34540488	1329426

HCl: Hydrochloride

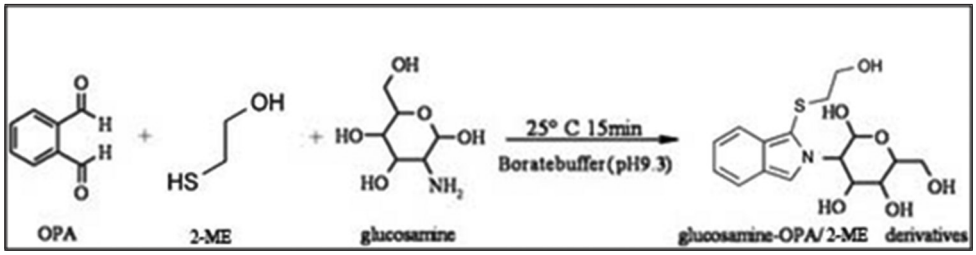

Fig. 1: Derivatization reaction of glucosamine hydrochloride [11,12]

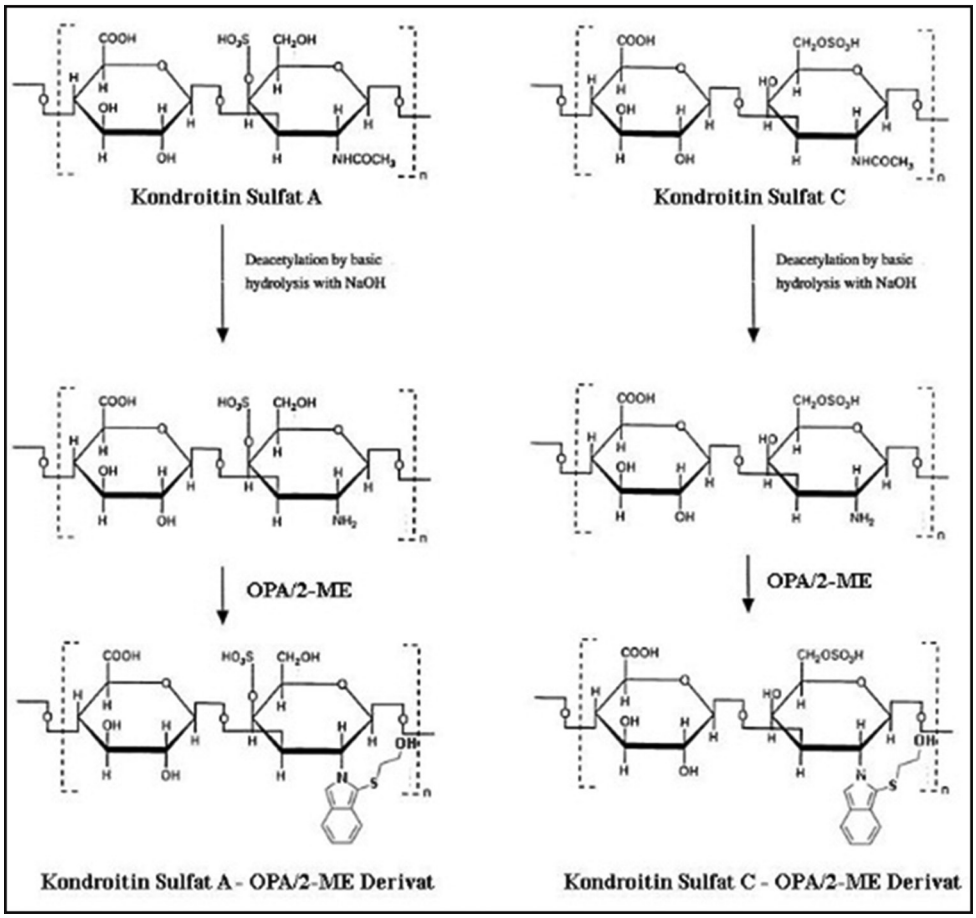

Fig. 2: Derivatization reaction of chondroitin sulfate [10]

Derivatization was compared at 2, 5, and 10 minutes, and the results showed that the optimum derivatization time was 2 minutes.

Chromatography optimization

The following parameters were optimized, among others: Column type, mobile phase composition, flow rate, injection volume, and injection method. The selected column was the YMC-Triart® C18 (250 × 4.6 mm, 5 µm). The injection method was manual, and the injection volume was 20 µl. Optimization of the mobile phase was performed by isocratic elution by changing the ratio of the mobile phase (THF 0.25% in water-acetonitrile) with the composition ratio (70:30, 80:20, 85:15, 87:13, 89:11, and 95:5), at a flow rate of 1.5 ml/minute. Two injections were performed. The results for optimization of mobile phase and flow rate are shown in Tables 3 and 4.

Validation

Linearity and range

The calibration curve consisted of a minimum six standard solutions in a linear range of 5-80 µg/mL for glucosamine HCl and 100-1000 µg/ml for chondroitin sulfate. Linearity was used to observe whether the results were directly or mathematically proportional to the analyte concentration of a sample in a given range. Linearity met the acceptance criteria if the correlation (r) was ≥0.99 [11]. The results are shown in Table 5.

Limits of detection (LOD) and limits quantitation (LOQ)

The LOD and LOQ are important for determining the lower concentration limit of a substance that can still be determined accurately and precisely. The LOD and LOQ for glucosamine HCl and chondroitin sulfate were calculated statistically through the line of linear regression from the calibration curve. LOD and LOQ values for glucosamine HCl and chondroitin sulfate are shown in Table 6.

Selectivity

The results of 20 µl injections of a placebo solution (matrix tablets and creams) were analyzed under the selected optimum conditions. There was no interference in compound's retention times, which proves that the analytical methods were selective for the glucosamine HCl and

Table 3: Optimization of mobile phase composition

Mobile phase THF 0.25% in water-acetonitrile	Retention time (minutes)		Tf		HETP		Number of theoretical plates (N)		R
	G	K	G	K	G	K	G	K	
(70:30)	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
(80:20)	18.440	28.456	1.020	1.024	2.33×10^{-3}	1.96×10^{-3}	10740	12743	11.646
	18.026	27.674	1.156	1.173	2.78×10^{-3}	2.68×10^{-3}	9001	9317	10.120
(85:15)	15.922	17.614	1.480	1.271	3.03×10^{-3}	3.76×10^{-3}	8238	6650	2.161
	19.088	21.181	1.421	1.273	3.12×10^{-3}	4.52×10^{-3}	8007	5537	2.101
(87:13)	17.235	22.247	1.687	1.277	2.96×10^{-3}	4.43×10^{-3}	8441	5641	5.180
	16.603	21.642	1.535	1.133	3.17×10^{-3}	5.54×10^{-3}	7884	4511	4.948
(89:11)	23.379	35.332	1.639	1.296	2.93×10^{-3}	5.50×10^{-3}	8531	4543	7.689
	23.628	35.573	1.643	1.191	2.85×10^{-3}	5.54×10^{-3}	8782	4513	7.641
(95:5)	72.764		1.093	-	2.57×10^{-3}	-	9730	-	-
	71.313		1.122	-	2.15×10^{-3}	-	11648	-	-

G: Glucosamine HCl, K: Chondroitin sulfate, R: Resolution, THF: Tetrahydrofuran, HETP: Height equivalent totheoretical plate, Tf: Tailing factor, HCl: Hydrochloride

Table 4: Optimization of flow rate

Flow rate (ml/minutes)	Retention time (minutes)		Tailing factor (Tf)		HETP		Number of theoretical plates (N)		R
	G	K	G	K	G	K	G	K	
1/2	23.695	32.111	1.116	1.073	2.14×10^{-3}	2.44×10^{-3}	11696	10255	7.846
1/5	18.837	25.755	1.072	1.013	2.62×10^{-3}	2.66×10^{-3}	9540	9402	7.545

G: Glucosamine HCl, K: Chondroitin sulfate, R: Resolution, HCl: Hydrochloride

Table 5: Linearity of glucosamine HCl and chondroitin sulfate

Solution	a (intercept)	b (slope)	R
Glucosamine HCl	−9714206	3418646	0.9989
Chondroitin sulfate	−1457766	21894	0.9988

HCL: Hydrochloride

Table 6: LOD and LOQ values

Solution	LOD (µg/ml)	LOQ (µg/ml)
Glucosamine HCl	5.51	18.38
Chondroitin sulfate	154.81	516.02

LOD: Limits of detection, LOQ: Limits quantitation

Table 7: Accuracy and precision of glucosamine HCl detection in tablet form

C (ppm)	X (ppm)	SD	CV (%)	Recovery (%)
25.6	25.33			98.93
	25.84	0.26	1.01	100.94
	25.65			100.18
32	31.98			99.93
	32.08	0.06	0.20	100.26
	32.09			100.28
38.4	37.81			98.48
	37.94	0.10	0.27	98.81
	37.74			98.27

C: Concentration, X: Measurable concentration, SD: Standard deviation; CV: Coefficient of variation, HCL: Hydrochloride

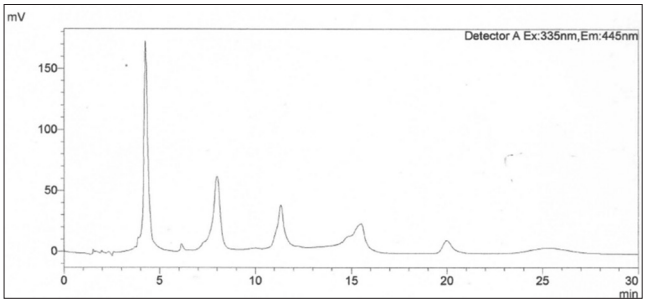

Fig. 3: Chromatogram of selectivity for tablet placebo

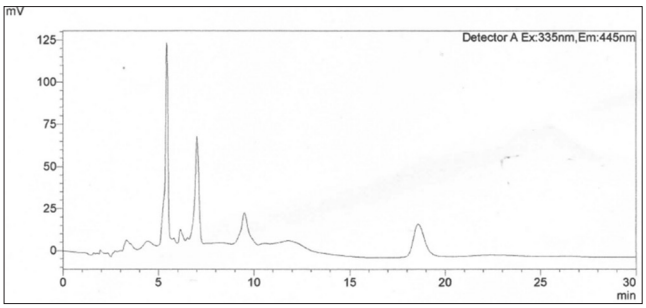

Fig. 4: Chromatogram of selectivity for cream placebo

chondroitin sulfate derivatives. The chromatograms of selectivity can be seen in Figs. 3 and 4, and the chromatogram of the standard solution is shown in Fig. 5.

Accuracy and precision

Accuracy is a measure of the closeness of the test result or the average value of the set of data against the true value. In this study, we used the spiked placebo recovery method with a range of concentrations at 80%, 100%, and 120% (three replicas per concentration). The accuracy met the acceptance criteria if the recovery value was 98-100%. Precision or repetition tests were measured by calculating the coefficient of variation (%CV) data of three replicas for each concentration. The acceptance criteria of CV were 2% (≤2%) [12]. The results are shown in Tables 7-10.

Assay

The validated method was used to analyze two samples on the market containing glucosamine HCl and chondroitin sulfate. Levels were

calculated using the onepoint measurement method. The average levels of glucosamine HCl and chondroitin sulfate in tablet form were 92.76% and 96.11%, respectively. In cream form, the average levels of glucosamine HCl and chondroitin sulfate were 101.15% and 100.33%, respectively. The results are shown in Tables 11-14. Chromatogram samples are shown in Figs. 6 and 7.

Table 8: Accuracy and precision of chondroitin sulfate detection in tablet form

C (ppm)	X (ppm)	SD	CV (%)	Recovery (%)
640	646.90			101.08
	652.25	7.34	1.14	101.91
	637.75			99.65
800	806.41			100.80
	800.15	6.01	0.75	100.02
	812.17			101.52
960	841.08			98.03
	970.69	17.95	1.87	101.10
	973.52			101.41

C: Concentration, X: Measurable concentration, SD: Standard deviation, CV: Coefficient of variation

Table 9: Accuracy and precision of glucosamine HCl detection in cream form

C (ppm)	X (ppm)	SD	CV (%)	Recovery (%)
32	32.59			101.84
	32.33	0.13	0.40	101.02
	32.45			101.42
40	40.60			101.50
	40.43	0.15	0.36	101.07
	40.31			100.77
48	48.51			101.07
	48.59	0.13	0.27	101.22
	48.34			100.70

C: Concentration; X: Measurable concentration; SD: Standard deviation; CV: Coefficient of variation, HCL: Hydrochloride

Table 10: Accuracy and precision of chondroitin sulfate detection in cream form

C (ppm)	X (ppm)	SD	CV (%)	Recovery (%)
320	322.63			100.82
	322.89	0.92	0.28	100.90
	321.19			100.37
400	392.10			98.03
	393.58	2.47	0.63	98.40
	396.93			99.23
480	478.65			99.72
	480.00	0.95	0.20	100.00
	480.49			100.10

C: Concentration; X: Measurable concentration; SD: Standard deviation; CV: Coefficient of variation

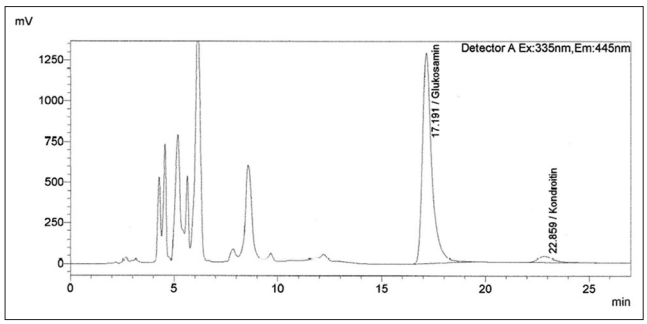

Fig. 5: Chromatogram of standard solutions of glucosamine hydrochloride and chondroitin sulfate

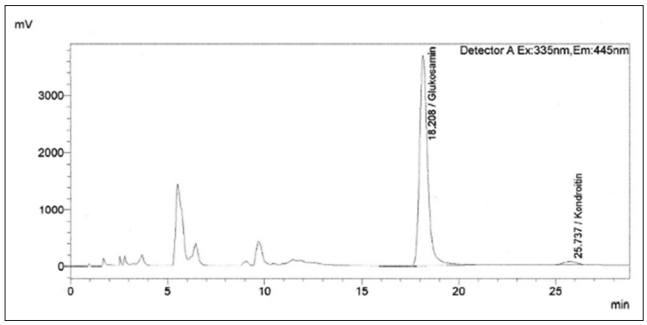

Fig. 6: Chromatogram of tablet sample

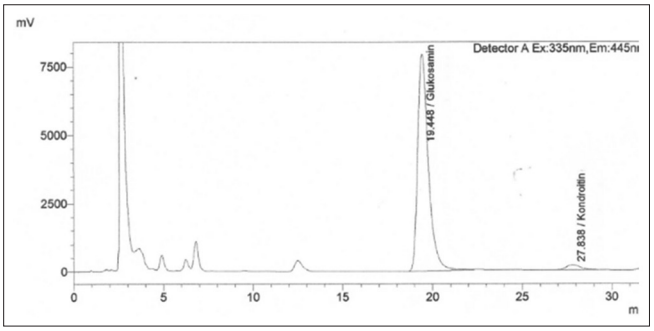

Fig. 7: Chromatogram of cream sample

Table 11: Determination of glucosamine HCl levels in tablet form

C (ppm)	Standard area of glucosamine HCl	Sample area of glucosamine HCl	Measurable concentration (ppm)	Measurable concentration (%)
20	110857856	107179978	19.34	96.68
		100539254	18.14	90.69
		100793341	18.18	90.92

C: Concentration, HCL: Hydrochloride

Table 12: Determination of chondroitin sulfate levels in tablet form

C (ppm)	Standard area of chondroitin sulfate	Sample area of chondroitin sulfate	Measurable concentration (ppm)	Measurable concentration (%)
600	2585195	2489213	577.72	96.29
		2508669	582.24	97.04
		2455966	570.01	95.00

C: Concentration

Table 13: Determination of glucosamine HCl levels in cream form

C (ppm)	Standard area of glucosamine HCl	Sample area of glucosamine HCl	Measurable concentration (ppm)	Measurable concentration (%)
80	320059616	328605087	82.14	102.67
		328510670	82.11	102.64
		328473366	82.10	102.63

C: Concentration, HCL: Hydrochloride

Table 14: Determination of chondroitin sulfate levels in cream form

C (ppm)	Standard area of chondroitin sulfate	Sample area of chondroitin sulfate	Measurable concentration (ppm)	Measurable concentration (%)
900	10382934	10431472	904.21	100.47
		10400356	901.51	100.17
		10419917	903.21	100.36

C: Concentration

CONCLUSION

The optimum conditions for determining glucosamine HCl and chondroitin sulfate in tablet and cream forms included the use of HPLC with a fluorescence detector at λex = 335 nm and λem = 445 nm, and a YMC-Triart® C18 column (length 250 mm, diameter 4.6 mm, and particle size 5 µm), a mobile phase of THF in water-acetonitrile (87:13), and a flow rate of 1.5 ml/minute. The optimum conditions for the hydrolysis of chondroitin sulfate involved NaOH and heating for 30 minutes at 60°C. A mixture of glucosamine HCl, and chondroitin sulfate was derivatized with 50 µl of OPA/2-ME reagent, and then incubated for 2 minutes, and 20.0 µl was injected into the HPLC system. The validation method for glucosamine HCl and chondroitin sulfate met the acceptance criteria of accuracy, precision, selectivity, and linearity.

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