INTRODUCTION

Glucosamine (2-amino-2-deoxy-D-glucose) is a 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 isochromophores 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. Bakarjaya PUTRAMAS, Indonesia), Acetonitrile Pro HPLC (Merck, US/Canada), tetrahydrofuran (THF, Mallinkrodt 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, 5 μm), 20A RF fluorescence detector (Shimadzu, Japan), manual injector, data processor; HPLC syringes (SGE, Australia), centrifuge (Kubota, Japan), vortex (ThermoScientific, US), micro pipettes (Eppendorf, Germany), 0.45 μm filter membranes, analytical balance, ultrasonic cleaner (Elma Elasonic S40H, Germany), and glass tools.

Chromatography system
This study implemented HPLC equipped with a pump, a C18 column, and a fluorescence detector at λ_em = 335 nm and λ_ex = 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 Bisted 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 Bisted 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 then allowed to separate. The water layer was filtered with a 0.45 μm filter membrane, and then Aqua Bisted 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 of dichloromethane was added. The solution was then allowed to separate. The water layer was filtered with a 0.45 μm filter membrane, then Aqua Bisted was added and diluted to 100 μg/ml.

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 then allowed to separate. The water layer was filtered with a 0.45 μm filter membrane, and then Aqua Bisted 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, then allowed to separate. The water layer was filtered with a 0.45 μm filter membrane, and then Aqua Bisted was added and diluted to a certain concentration.

Derivatization
Pipelets 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 of UV light (chromophores) or fluorescence groups (fluorophores). Glucosamine has primary amine groups (NH₂) 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 (COCH₃) and the nitrogen atom to form primary amine groups [NH₂]₉ [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 | 448830 |
| 50 | 41932818 | 2261195 |
| 100 | 27697397 | 1407211 |
| 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
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 chondroitin sulfate.
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

### 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)                                     | 18.440 28.456            | 1.020 1.024 | 2.33×10⁻³ 1.96×10⁻³ | 10740 12743 | 11.646 |
| (80:20)                                     | 18.026 27.674            | 1.156 1.173 | 2.78×10⁻³ 2.68×10⁻³ | 9001 9317 | 10.120 |
| (85:15)                                     | 19.088 21.181            | 1.421 1.273 | 3.03×10⁻³ 3.76×10⁻³ | 8238 6650 | 2.164 |
| (87:13)                                     | 17.235 22.247            | 1.687 1.277 | 2.96×10⁻³ 4.43×10⁻³ | 8441 5641 | 5.180 |
| (89:11)                                     | 16.603 21.642            | 1.535 1.133 | 3.17×10⁻³ 5.54×10⁻³ | 7884 4511 | 4.948 |
| (95:5)                                      | 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 |
|                                             | 7.2764 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 to theoretical 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   | 0.26 | 1.01   | 98.93        |
| 25.84   | 25.65   | 0.06 | 0.20   | 100.26       |
| 31.98   | 32.09   | 0.10 | 0.27   | 98.81        |
| 37.81   | 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
calculated using the one-point 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  | 7.34 | 1.14    | 101.08       |
| 652.25  | 657.75  | 99.65|         |              |
| 806.41  | 800.15  | 6.01 | 0.75    | 100.02       |
| 812.17  | 814.08  | 96.03|         |              |
| 970.69  | 973.52  | 17.95| 1.87    | 101.10       |

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   | 0.13 | 0.40    | 101.84       |
| 32.33   | 32.45   | 101.50| 101.42  |
| 40      | 40.60   | 40.43| 0.15    | 101.07       |
| 48      | 48.31   | 48.59| 0.13    | 101.22       |

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  | 0.92 | 0.28    | 100.82       |
| 322.89  | 321.19  | 98.03|         |              |
| 392.10  | 393.58  | 2.47 | 0.63    | 98.40        |
| 396.93  | 478.65  | 99.72|         |              |

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

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
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.

REFERENCES

1. Tjahjono DH, Slamet IS, Sasanti TD. Analysis of glucosamine in cream dosage form and diffusion liquid by high performance liquid chromatography. J Ion Exch 2007;18(4):1420-1.
2. Nagarajan P, Sathis KD, Somsubhra G, Harani A, Maheswaran N, David B. Method development and validation for glucosamine and chondroitin sulphate in softgel form by RP-HPLC: Res Rev J Pharm Anal 2013;2:6-10.
3. Pratiwi AI. Diagnosis and Treatment Osteoarthritis, Thesis. Indonesia: Universitas Lampung; 2015.
4. Andriani S. Optimasi derivatisasi glukosamin hidroklorida dengan 9-fluorenilmetoksikarbonil klorida (FMOC-Cl) secara kromatografi cair kinerja tinggi-fluoresensi. Depok: Universitas Indonesia; 2012.
5. Topping J. A review of methods available for the determination of chondroitin sulphates in supplements. United Kingdom: Laboratory of the Government Chemist Queens Road Teddington Middlesex; 2012.
6. David JI, Roman M, Zhou J, Hildreth J. Determination of chondroitin sulphate content in raw materials and dietary supplements by high performance liquid chromatography with ultraviolet detection after enzymatic hydrolysis. J AOAC Int 2007;90(3):659-69.
7. Vaclavikova E, Frantisek K. Isotachophoretic determination of glucosamine and chondroitin sulphate in dietary supplements. Czech J Food Sci 2013;31:55-65.
8. Dionex Corporation. Determination of Glucosamine in Dietary Supplements Using HPAE-PAD; Application Note 197, LPN 2001. Sunnyvale, CA: Dionex Corporation; 2008.
9. Lee KS, Drescher DG. Derivatization of cysteine and cystine for fluorescence amino acid analysis with the o-phthalaldehyde/2-mercaptoethanol reagent. J Biol Chem 1979;254(14):6248-51.
10. Rhee WM, Richard AB. Glysaminoglycan-Synthetic Polymer Conjugates. European Patent Application, Report; 1995.
11. Association of Official Analytical Chemist (AOAC). Guidelines for Single Laboratory Validation of Chemical Methods for Dietary. Available from: https://www.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/SLV_Guidelines_Dietary_Supplements.pdf.
12. Harmita H. Buku Ajar Analisis Fisikokimia. Depok: Universitas Indonesia; 2006.

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                       | 328650587                      | 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