Drug Development and Industrial Pharmacy, 1:91–97, 2005

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1081/DDC-200044181

Taylor & Francis
Taylor & Francis Group

Evaluation of the Physicochemical Stability and Skin Permeation of Glucosamine Sulfate

Marion Kanwischer, Seok-Yong Kim and Jung Sun Kim
Department of Biotechnology, Dongseo University, Busan, South Korea

Shengjie Bian
College of Pharmacy, Pusan National University, Busan, South Korea

Kyoung Ae Kwon
Pharm. Develop. Res. Team, Handok Pharmaceuticals Co. Ltd., Chungbuk, South Korea

Dae-Duk Kim
College of Pharmacy, Seoul National University, Seoul, South Korea

ABSTRACT Glucosamine sulfate (GS) is known to stop the degenerative process of osteoarthritis. Because most of the GS formulation on the market is in the oral form, an alternative formulation such as a transdermal delivery system (TDS) is necessary in order to increase patient compliance. As the initial step to develop a TDS of GS, the physicochemical stability and permeation study in rat skin were examined. Evaluation of the stability of GS at different pHs showed the compound to be most stable at pH 5.0. The degradation rate constant at 25°C was estimated to be 5.93×10^{-6} hr^{-1} ($t_{90} \sim 2.03$ years) in a pH 5 buffer solution. Due to its hydrophilic characteristic, low skin permeability was expected of GS. However, the skin permeation rate was determined to be 13.27 $\mu g/cm^2/hr$ at 5% concentration. Results of this study suggest the possibility of developing GS into a transdermal delivery system.

KEYWORDS Glucosamine sulfate, TDS, Stability

INTRODUCTION

Osteoarthritis, a degenerative disease of the joints, is caused by decreased production of the cartilage matrix, which then leads to roughening and fissuring of the cartilage, followed by the change of the underlying bones. Common treatments include physical therapies such as exercises and weight reduction and use of pain relievers including nonsteroidal anti-inflammatory drugs (NSAIDs). However, NSAIDs can lead to gastrointestinal distress as well as inhibition of glycosaminoglycan formation while long-term application of cortisones is harmful to the tissue and bones (Barclay et al., 1998; Cooper et al., 2002; Laine, 2003). Results of recent studies show that exogenously applied glucosamines can act as precursors for the formation of glycosaminoglycan, which can help regenerate the cartilage matrix. These results have prompted the use of glucosamines for osteoarthritis.

Glucosamine sulfate (GS) is an amino monosaccharide that is currently classified as a dietary supplement by the Food and Drug Administration in the U.S. (Fig. 1). However, in Europe, it has been approved as a prescription drug (Häuselmann, 2001; Hochberg & Dougados, 2001). When orally ingested,

Address correspondence to Jung Sun Kim, Department of Biotechnology, Dongseo University, Busan 617-716, South Korea; Fax: +82-51-313-0432; E-mail: jsk@gdsu.dongseo.ac.kr or Dae-duk Kim, College of Pharmacy, Seoul National University, Seoul, South Korea; E-mail: ddKim@snv.ac.kr

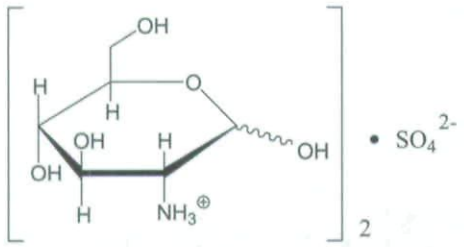

FIGURE 1 Chemical Structure of Glucosamine Sulfate.

GS is incorporated into the plasma compartment as plasma proteins and from there transported to the tissue (Calatroni et al., 1992). It is found to not only stop the degenerative process of osteoarthritis but also to reverse the process without affecting cyclooxygenase. The oral bioavailability is known to be only 26% (Ilic et al., 2003). However, currently most of the GS formulations are in the oral form and like other NSAIDs, an increasing demand for alternative formulation such as a transdermal delivery system (TDS) is called for in order to increase patient compliance.

A prerequisite for developing new formulations is an effective analytical procedure. Methods reported include anion exchange chromatography coupled with pulsed amperometric detection, normal phase chromatography (HPLC) in combination with an RI-detector or colorimetric methods (Ander et al., 2001; Campo et al., 2001; Flannery et al., 2001; Liang et al., 1999; Scotti et al., 2001; Yuan and Chen, 1999). For high throughput quantitation, a chromatographic analysis with spectrophotometry is desirable. Thus, the precolumn derivatization method using HPLC/UV by Liang et al. (1999) has been modified and used in this study. Herein, we report the application of this analytical procedure in obtaining results of the physicochemical stability of and rat skin permeation study of GS as the initial steps to develop a transdermal delivery system of glucosamine.

MATERIALS AND METHODS

Reagents

D-glucosamine sulfate was provided by Handok Pharmaceutical Company (Seoul, Korea). Phenylisothiocyanate (99%) (PITC) was purchased from Aldrich Chem. Co. (Milwaukee, WI) and dissolved in methanol from Hayman (Essex, England) to prepare a 5% PITC solution. Phosphate buffers were made with potassium dihydrogen phosphate from Sigma Co. (Saint Louis, MO). Phosphoric acid (85%) was received from Kanto Chemical (Kanto, Japan) and potassium hydroxide (85%) from TEDIA (Fairfield, OH). Water used in this study was purified (18 MΩ) with the "Millipore" system and degassed and filtered with a 0.2 μm filter (Sartorius, Germany) before use. All samples were filtered before injection with cellulose membrane filters (Minisart® RC 4) purchased from Sartorius. The HPLC-grade solvents were purchased from Merck (Darmstadt, Germany). Other reagents were analytical grade or better and used without further purification.

Preparation of Glucosamine Sulfate Standard Solutions

A modified procedure by Liang et al. (1999) was used. The GS stock solution (1.0 mg/mL) was prepared in 4% (v/v) methanol/water. Aliquots to make the appropriate standard concentrations were taken from the stock solution and added to 250 μL of phosphate buffer (0.3 M, pH 8.1) to make the standard solutions of 20 ~ 60 μg/mL. This solution was allowed to react for 25 minutes at room temperature (Liang et al., 1999), after which 250 μL of 5% PITC solution was added and gently mixed. The reaction was continued at 60°C for 25 minutes, after which the samples were evaporated under a nitrogen stream at 75°C until dryness. The residues were redissolved with 250 μL of mobile phase, and then these solutions were filtered through membrane filters (syringe filters, Minisart®-RC4, 17822k, Sartorius, Germany) before injected into the equilibrated HPLC system with a 20 μL injection loop.

HPLC Analysis

Concentrations of GS in samples were determined using a HPLC system (Waters 2690) coupled with a UV detector (Waters 2487 dual λ absorbance detector). A Merck RP-18 Lichro-cart® Lichrospher column (5 μL, 125 × 4 mm, Merck, Darmstadt, Germany) was used at ambient temperature. The mobile phase consisted of methanol/phosphate buffer (0.3M, pH 8.1) (10:90, v/v), which was filtered through a membrane filter ("Pall corporation" supor® 450, 47 mm, 0.45 μm) and was thoroughly degassed in an ultrasonic bath before use. The flow rate was set at 1.2 mL/min and the wavelength of UV detector was

set at 254 nm. The retention time of GS was about 3.5 minutes.

To study the accuracy and precision of this method, samples of three different concentrations, 16, 20, and 28 μg/mL, were injected three times a day (Intra-day) as well as once on three different days (Inter-day). The precision was expressed as the relative standard deviation (RSD) and accuracy as relative error (RE).

Stability of Glucosamine Sulfate

Effect of pH

To study the influence of pH on the stability of GS, 1000 μg/mL solutions were prepared with the appropriate buffer solutions of pH 2.97 (0.05 M acetate buffer), pH 5.00 (0.05 M acetate buffer), pH 6.99 (0.3 M phosphate buffer), and pH 8.59 (0.3 M phosphate buffer). The solution aliquots were withdrawn and sealed in 5 mL amber glass ampules (Type I, Wheaton Scientific, Millville, NJ). For the kinetic studies, these samples were placed in a dark mechanical convection oven (EYELA NDO-600ND, Tokyo Rikakikai Co. Ltd., Japan) maintained at 60°C up to 5 days. At appropriate time intervals, samples were removed from the oven and equilibrated to room temperature and derivatized as mentioned in standard preparation. The pH values were checked (Micro processor pH meter HI 9321, HANNA Instruments, Portugal) for each sample to detect any significant change of pH at each designated time. Concentrations of GS were determined in triplicate by HPLC. The initial concentration was considered as 100%, and the remaining concentrations were expressed as a percentage of the initial.

Effect of Temperature

An accelerated stability study of GS (1000 μg/mL) was conducted at temperatures of 30°C, 60°C, 70°C, and 90°C over a 5 day period. Solutions were stored in a 0.05 M acetate buffer solution (pH 5.00), which was determined from the pH stability study mentioned above. Samples were also prepared in triplicate and in ampules. Sampling and derivatization procedures were identical to those indicated for the standard preparation.

In Vitro Rat Skin Permeation Study

Preparation of Rat Skin

The animals used for the preparation of skin were male Sprague–Dawley (220–250 g) rats obtained from Dae-Han Laboratory Animal Research Center Co. (Taejon, Korea). The animals had free access to food and water until used for experiments, and were sacrificed in a CO_2 chamber right before the experiments. The dorsal hairs were removed with a clipper and full-thickness skin (about 16 cm^2) was surgically removed from each rat. The skin specimen was cut into appropriate sizes after carefully removing subcutaneous fat and washing with normal saline.

In Vitro Permeation Study

In vitro skin permeation across rat skin was conducted with Keshary-Chien diffusion cells at 37°C. Freshly excised rat skin was mounted between the donor and receptor cell (stratum corneum side facing the donor). The area of diffusion for all in vitro

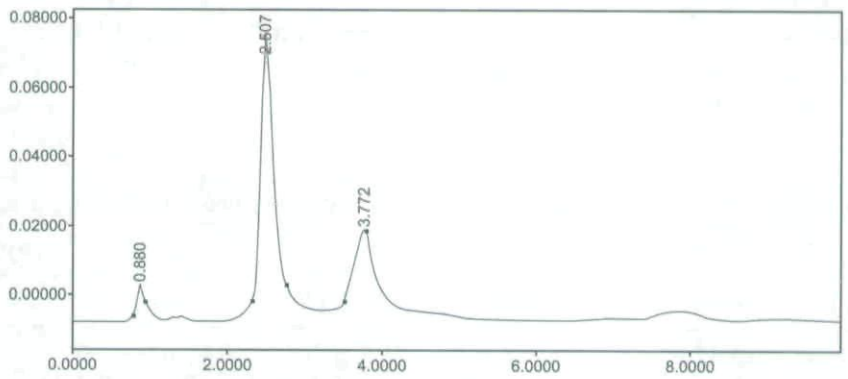

FIGURE 2 Chromatogram of Glucosamine Sulfate After Elution with Methanol/Phosphate Buffer (0.01 M, pH 8.1) as Mobile Phase on an RP-18 Column. Glucosamine was Detected at 3.77 min.

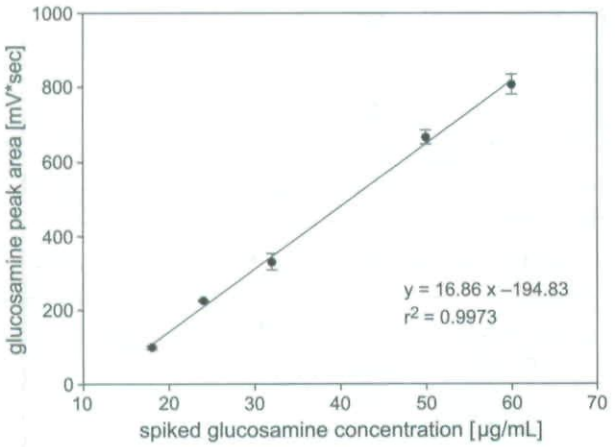

FIGURE 3 Calibration Curve of Glucosamine Sulfate Standard.

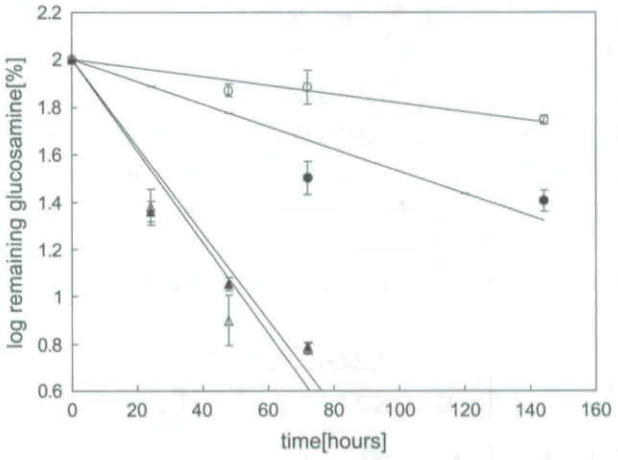

FIGURE 4 Glucosamine Sulfate Stored in Acetate Buffer pH 2.97 (●), Acetate Buffer pH 5.00 (○), Phosphate Buffer pH 6.99 (▲), and Phosphate Buffer pH 8.59 (△) at 60°C.

experiments was 2.14 cm^2. The donor cells, which faced the stratum corneum surface, contained 5% GS aqueous solution (3.0 mL). The receptor cells, which faced the dermis side, were filled with normal saline solution (12 mL). At predetermined time intervals, 0.5 mL of the receptor solution was withdrawn and refilled with the same volume of fresh receptor solution. Samples were kept in a freezer (−20°C) until derivatized and analyzed by HPLC as described above.

RESULTS

HPLC Analysis of Glucosamine Sulfate

Figure 2 shows a typical chromatogram for the elution process where the peak area was plotted vs. the retention time. The peak of GS (25 µg/mL) was detected at around 3.8 min, while the remaining PITC reagent was detected at 2.5 minutes. No discernible peaks were observed within the time frame in which GS was detected and was appropriately separated from PITC. The calibration curve (y = 16.86x–194.83) was linear up to 60 µg/mL as shown in Fig. 3 with a correlation coefficient (r^2) of 0.9973.

The reproducibility of the method was determined by both intra- and inter-day variability as relative standard deviation (RSD). For the intra-day validation study, the GS concentrations of 16, 20, and 28 µg/mL were injected, while for the inter-day study, these three concentrations were evaluated for 3 days. In both cases, accuracy as well as precision were determined as shown in Table 1. For all concentrations tested, both inter-day and intra-day precisions were within 8.91% RSD, while inter-day and intra-day accuracy were within 17.64% RE. The accuracy and precision data show that this method is reproducible and reliable.

Stability Studies

As shown in Fig. 4, the degradation of GS in various buffer solutions followed the typical first-order kinetics at 60°C. The degradation rate constants were calculated by the regression analysis method (pH 2.97: $y = -0.0047x + 2.0000$, $r^2 = 0.8432$, pH 5.00: $y = -0.0018x + 2.0000$, $r^2 = 0.9389$, pH 6.99: $y = -0.0184x + 2.0000$, $r^2 = 0.9299$, pH 8.59: $y = -0.0193x + 2.0000$ $r^2 = 0.9095$), and were

TABLE 1 Intra-Day and Inter-Day Precision (RSD) and Accuracy (RE) for Glucosamine Sulfate at 16 µg/mL, 20 µg/mL, and 28 µg/mL

	Intra-day			Inter-day		
Concentration (µg/mL)	conc ± sd	RSD [%]	RE [%]	conc ± sd	RSD [%]	RE [%]
16	18.82 ± 1.68	8.91	17.64	18.61 ± 1.01	5.44	16.32
20	23.24 ± 1.14	4.91	16.18	22.27 ± 0.25	1.19	11.34
28	30.01 ± 1.16	3.87	7.19	26.96 ± 0.67	2.48	− 3.72

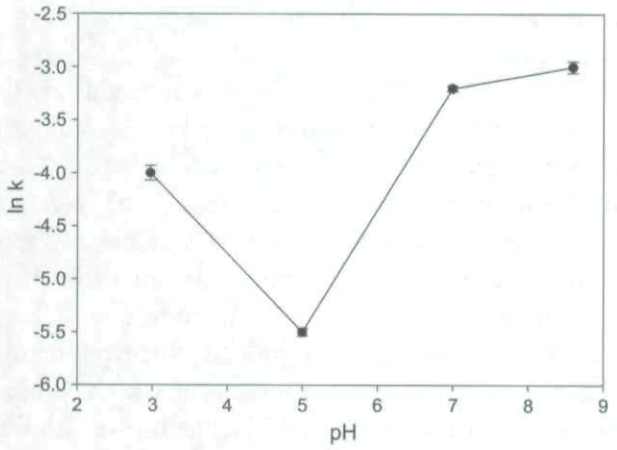

FIGURE 5 pH Rate Profile for the Degradation of Glucosamine Sulfate at 60°C.

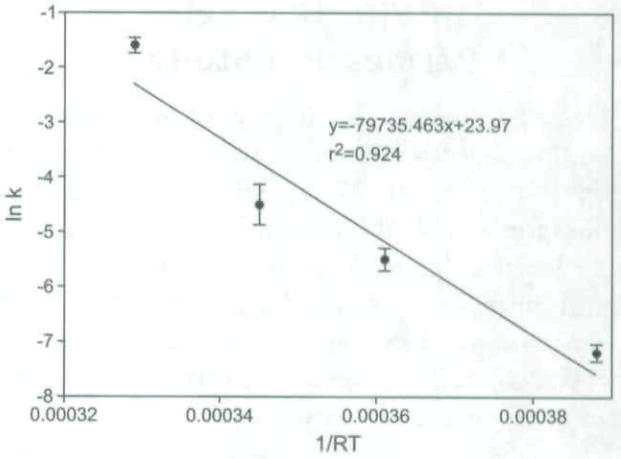

FIGURE 7 Arrhenius Plot for the Degradation Process of Glucosamine Sulfate at Different Temperatures.

plotted as function of pH of solutions (Fig. 5). GS was most stable at pH 5.00, and the stability decreased in the following order:

$$\text{pH } 5.00 > \text{pH } 2.97 > \text{pH } 6.99 > \text{pH } 8.59$$

The stability of glucosamine at pH 5.00 was reflected in the lowest degradation constant k (4.3×10^3 hr^{-1}) and the highest values for $t_{1/2}$ (161.2 hr) and t_{90} (24.4 hr), whereas at pH 8.59, the highest k (51.2×10^3 hr^{-1}) value and the lowest $t_{1/2}$ (13.5 hr) and t_{90} (2.1 hr) values were observed at 60°C.

Figure 6 represents the degradation profile of GS at different temperatures (30–92°C). The degradation rate of GS (20 μg/mL) as a function of temperature in pH 5 buffer was demonstrated by plotting the logarithm of the degradation rate constant vs. 1/ temperature as shown in Fig. 7. According to the Arrhenius equation, $k = A \exp(-E_A/RT)$, where E_A is the activation energy, R is the gas constant, T the absolute temperature and A the frequency factor that determines the probability of collisions of the molecules, a plot in the form of $\ln k = -E_A/RT + \ln A$ is possible. The Arrhenius plot was linear ($y = -79735.463x + 23.97$, $r^2 = 0.924$), indicating that the degradation mechanism was not altered with a change in temperature inside the temperature range of study. The energy of activation in this solution was calculated from the slope and was determined to be 18.98 kcal/mole for pH 5.00 buffer solution. Then, the degradation rate constant of GS at 25°C was estimated to be 5.93×10^{-6} hr^{-1} ($t_{90} \sim 2.03$ year) in a pH 5.00 buffer solution.

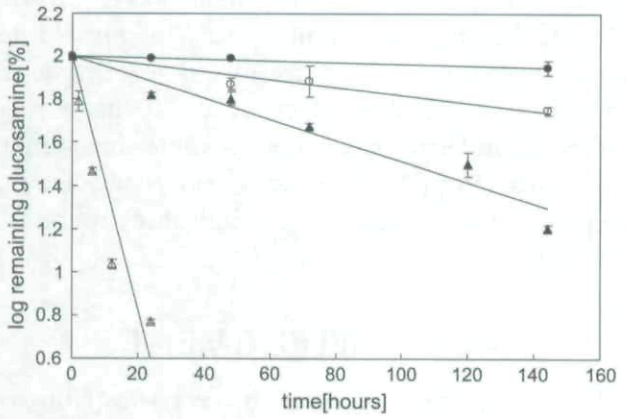

FIGURE 6 Degradation Profile of Glucosamine Sulfate Stored at 30°C (●), 60°C (○), 70°C (▲), and 92°C (△).

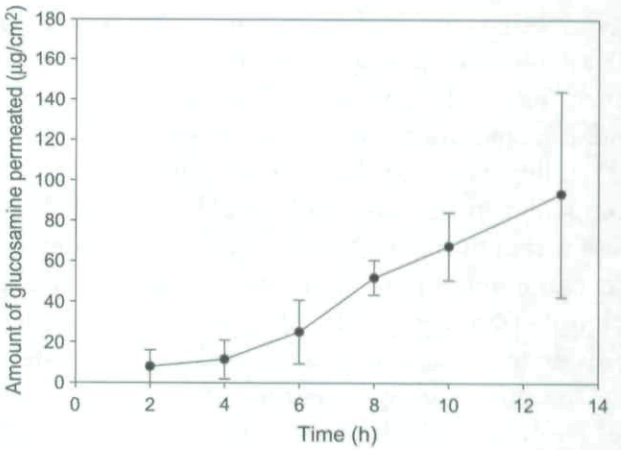

FIGURE 8 Skin Permeation of 5% Glucosamine Sulfate Aqueous Solution.

In Vitro Rat Skin Permeation Study

Figure 8 shows the rat skin permeation profile of GS (5%) in aqueous solution, using Keshary-Chien permeation cells at 37°C. After a time lag, the permeation of GS through the skin followed zero-order kinetics up to 13 hours. The skin permeation rate at steady-state obtained from the slope of the linear portions of the plot (between 6 hr and 13 hr, $r^2 = 0.9886$) was 13.27 $\mu g/cm^2/hr$, while the lag time from the intercept of x-axis was 2.83 hr.

DISCUSSION

In order to develop a transdermal formulation for GS, the physicochemical properties of the drug was evaluated. As the first step, an analytical method for in vitro assays needed to be established. The analytical method used in the present study was a modification of the work by Liang et al. (1999). Because GS itself does not have a chromophore, derivatization with phenylisothiocyanate (PITC) was necessary to make detection possible at 254 nm. Since the reaction time and results varied depending on the reaction temperature and solvents used, the derivatization process was carefully optimized (see methods for details) to obtain reproducible results in a short time period.

As shown in the chromatogram in Fig. 2, the glucosamine derivative peak does not overlap with other peaks. It was also possible to get a linear relationship between injected glucosamine concentration and yielded peak area as indicated by the correlation coefficient, $r^2 = 0.9973$. The relative standard deviations (precision) obtained from the intra-day as well as inter-day validation studies were below 10%. Upon establishing an analytical method that was quick (retention time of 3.7 minutes), reproducible, and accurate, the following studies were conducted.

Stability of GS at different pH values showed the compound to be most stable at pH 5.0. Degradation was faster as the pH increased. It has been reported that glucosammonium ion is quite unstable in alkaline solutions (Zimmerman, 1958). Its weak acidic character leads to degradation accompanied by browning of the solution. During this study these aspects could be confirmed. It is supposed that the degradation behavior of GS is very different from their structurally related hydroxysugars because of the attached amino group.

On the other hand, stability decreased as the temperature increased as shown in Fig. 6. From the Arrhenius equation, shelf-life of GS in pH 5.0 was calculated to be about 2 years ($t_{90} \sim 2.03$ years) at 25°C, indicating that GS is relatively stable and may be suitable for a transdermal formulation if it maintains a pH near 5 at low temperature.

During the derivatization process, the experimental solutions became brown colored when high temperature (over 90°C) was used. When the brown solution was analyzed by HPLC, a decrease in GS peak with an additional unknown peak at a higher retention time was observed (data not shown). It is not clear what this by-product was, nor what caused the brown colour. However, it is speculated that a Maillard product was probably formed, which is known to occur when high amounts of sugars and amino acids are exposed to high temperature (Bruins et al., 2003) .The products are often determined by certain smell and brown color. Because the glucosamine molecule has the backbone of a sugar molecule attached to an amino group, it is highly possible that the carbohydrate ring of one molecule reacts with the amino moiety of the other. Because this reaction leads to a conformational change, the appearance of an additional peak may be explained.

Due to the inherent lipophilic nature of skin, drug candidates for transdermal delivery should have an optimal lipophilicity. In the beginning of this study, we expected very low skin permeability of GS because of its relatively hydrophilic characteristic with the calculated log P value of -2.375. However, the skin permeation rate was determined to be 13.27 $\mu g/cm^2/hr$ at a 5% concentration, which was unusually high considering the optimum log P of most drugs are known to be in the range of $2 \sim 4$ (Diez et al., 1991; Doh et al., 2003; Kim et al., 2000). Further studies are underway to examine possible glucosamine transdermal formulations taking into consideration the present physicochemical stability study results.

ACKNOWLEDGMENT

This study was supported by a grant (Dongseo Frontier Project) from Dongseo University.

REFERENCES

Ander, B., Karlsson, A., & Öhrlund, A. (2001). Determination of heparin on intraocular lens surfaces by ion chromatography. *Journal of Chromatography A, 917*(1–2), 105–110.

Barclay, T. S., Tsourounis, C., & McCart, G. M. (1998). Glucosamine. *Annals of Pharmacotherapy, 32*(5), 574–579.

Bruins, M. E., Thewessen, A. J.H., Janssen, A. E.M., & Boom, R. M. (2003). Enzyme inactivation due to Maillard reactions during oligosaccharide synthesis by a hyperthermophilic glycosidase: influence of enzyme immobilisation. *Journal of Molecular Catalysis. B, Enzymatic, 21*(1–2), 31–34.

Calatroni, A., Vinci, R., & Ferlazzo, A. M. (1992). Characteristics of the interactions between acid glycosaminoglycans and proteins in normal human plasma as revealed by the behaviour of the protein-polysaccharide complexes in ultrafiltration and chromatographic procedures. *Clinica Chimica Acta, 206*(3), 167–180.

Campo, G. M., Campo, S., Ferlazzo, A. M., Vinci, R., & Calatroni, A. (2001). Improved high-performance liquid chromatographic method to estimate aminosugars and its application to glycosaminoglycan determination in plasma and serum. *Journal of Chromatography B, 765*(2), 151–160.

Cooper, M. S., Walker, E. A., Bland, R., Fraser, W. D., Hewison, M., & Stewart, P. M. (2002). Expression and functional consequences of 11β-hydroxysteroid dehydrogenase activity in human bone. *Bone, 27*(3), 375–381.

Diez, L., Colom, H., Obach, R., & Domenech, J. (1991). A comparative in vitro study of transdermal absorption of a series of calcium channel antagonists. *Journal of Pharmaceutical Sciences, 80*, 931–934.

Doh, H. J., Cho, W. J., Yong, C. S., Choi, H. G., Kim, J. S., Lee, C. H., & Kim, D. D. (2003). Synthesis and evaluation of ketorolac ester prodrugs for transdermal delivery. *Journal of Pharmaceutical Sciences, 92*, 1008–1017.

Flannery, M. B., Stott, A. W., Briggs, D. E.G., & Evershed, R. P. (2001). Chitin in the fossil record: identification and quantification of D-glucosamine. *Organic Geochemistry, 32*(5), 745–754.

Häuselmann, H. J. (2001). Nutripharmaceuticals for osteoarthritis. *Best Practice and Research. Clinical Rheumatology, 15*(4), 595–607.

Hochberg, M. C., & Dougados, M. (2001). Pharmacological therapy of osteoarthritis. *Best Practice and Research. Clinical Rheumatology, 15*(4), 583–593.

Ilic, M. Z., Martinac, B., & Handley, C. J. (2003). Effects of long-term exposure to glucosamine and mannosamine on aggrecan degradation in articular cartilage. *Osteoarthritis and Cartilage, 11*(8), 613–622.

Kim, M. K., Lee, C. H., & Kim, D. D. (2000). Rat skin permeation of testosterone and its ester derivatives. *Journal of Pharmacy and Pharmacology, 52*, 1–7.

Laine, L. (2003). Gastrointestinal effects of NSAIDs and coxibs. *Journal of Pain and Symptom Management, 25*(2S), 32–40.

Liang, Z., Leslie, J., Adebowale, A., Ashraf, M., & Eddington, N. D. (1999). Determination of the nutraceutical, glucosamine hydrochloride, in raw materials, dosage forms and plasma using pre-column derivatization with ultraviolet HPLC. *Journal of Pharmaceutical and Biomedical Analysis, 20*(5), 807–814.

Scotti, C. T., Vergoignan, C., Feron, G., & Durand, A. (2001). Glucosamine measurement as indirect method for biomass estimation of *Cunninghamella elegans* grown in solid state cultivation conditions. *Biochemical Engineering Journal, 7*(1), 1–5.

Yuan, J.-P., & Chen, F. (1999). Simultaneous separation and determination of sugars, ascorbic acid and furanic compounds by HPLC-dual detection. *Food Chemistry, 64*(3), 423–427.

Zimmerman, H. K. (1958). Studies on glucosammonium chloride. II. Protolysis constants and the stability of glucosamine in weakly alkaline aqueous solution. *Journal of Physical Chemistry, 62*, 963–965.