Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 1229–1236
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

# A size-exclusion HPLC method for the determination of sodium chondroitin sulfate in pharmaceutical preparations

Don Woong Choi *, Mi Jung Kim, Hee Sung Kim, Soo Hyun Chang, Gi Sook Jung, Kyung Yong Shin, Seung Yeup Chang

*Department of Drug Evaluation, Korea Food and Drug Administration, 5 Nokbun-Dong, Eunpyung-Gu, Seoul 122-704, Republic of Korea*

Received 25 September 2002; received in revised form 13 December 2002; accepted 20 December 2002

**Abstract**

A size-exclusion HPLC method for the determination of sodium chondroitin sulfate (SCS) in pharmaceutical preparations has been developed and validated. The most important feature of this method compared with the previously reported assay methods was improved economical and determinative applications through direct analysis of SCS from pharmaceuticals. The linearity, precision, specificity, and accuracy of the method were established and validated. The intra- and inter-day precision was satisfactory with relative standard deviation lower than 1.0%. The recovery of SCS from multi-components pharmaceutical preparations were from 93.38 to 100.46%. Comparing our HPLC assay results with classical spectrophotometric methods, the developed method was considerably easy, simple and reproducible. As a result, the present method was supposed to be successfully applied to the assay of SCS for the routine quality control in pharmaceutical preparations.


*Keywords:* Size-exclusion HPLC; Sodium chondroitin sulfate; Determination; Pharmaceutical preparation

## 1. Introduction

Sodium chondroitin sulfate (SCS), a homopolymeric glycosaminoglycan having disaccharide repeating unit formed by a hexuronic acid and a hexosamine residue [1], has been used as therapeutic medicines for the chronic inflammatory diseases such as rheumatoid arthritis, cirrhosis, and chronic photo damage [2–4]. The eminent feature of molecular structure of SCS is the presence of a sulfate ester group in the hexosamine residue (Fig. 1), which makes the SCS molecule have highly negative ion charge. Until now, the determination of SCS from various types of matrix materials have been performed by either chemical reaction of the carbohydrate constituents or the chromogenic reactions of the anionic groups. In addition, for the analysis of SCS in biological resources, complex techniques such as high performance capillary electrophoresis method [5,6], enzymatic digestion method using chondroitinase ABC enzyme [7–9] have been established.

* Corresponding author. Tel.: +82-2-380-1710; fax: +82-2-387-7857.
*E-mail address:* cdwkje@hanmail.net (D.W. Choi).

0731-7085/03/$ - see front matter 
doi:10.1016/S0731-7085(02)00731-8

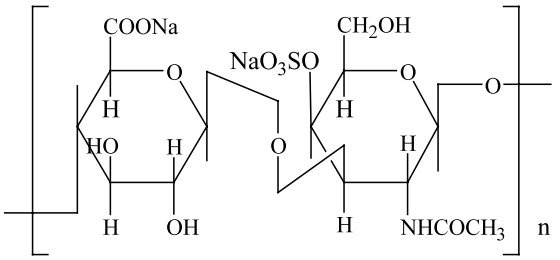


Fig. 1. Molecular structure of disaccharide unit of SCS.

The most common assay method for the analysis of SCS from pharmaceutical preparations was spectrophotometric assay, which was based on the chromogenic reaction between hexuronic acid produced by acid hydrolysis of SCS and the color forming reagents including carbazole [10,11]. Recently, a photometric titration assay method for SCS in chewable tablet using cetylpyridium chloride has been reported [12].

But, it has been known that these determining methods for SCS were dependent on the analysis of alternating disaccharide sequence, which required especially time-consuming experimental procedures and high-skilled techniques. Moreover, the interferences of other constituents co-formulated with SCS in drugs have been reported at the spectrophotometric analysis [13]. Therefore, the purpose of present study was to develop and validate a rapid and convenient HPLC method for the determination of SCS in pharmaceutical preparations.

## 2. Experimental

### 2.1. Materials

SCS standard raw materials were purchased from following companies: Sigma (Chondroitin sulfate C, Lot. 110K1497, USA); Seikagaku (Lot. ND-3633, Japan). Sodium phosphate (dibasic), Ambelite® IRC-50, acriflavine (neutral), carbazole, *p*-nitrobenzenediazonium tetrafluoroborate, potassium acetate, potassium hydroxide, and sodium borate were purchased from Sigma Chemical Co. (St Louis, MO). HPLC grade acetonitrile and water were obtained from Merck (Darmstadt, Germany). Hydrochloric acid, acetic acid and nitric acid were purchased from Wako Pure Chemical Industries, Ltd (Japan). Condro® capsule, Condrin® tablet, and Tobrin® capsule were manufactured by local pharmaceutical company as robot-scaled production. All eluants were filtered through 0.45-μm filters (Millipore) on use.

### 2.2. Instruments

HPLC analysis was carried out using a Waters Alliance® system (Milford, MA) consisting of pump control separation module (Model 2690), a programmable photodiode array detector (Model 996), multiwavelength UV absorbance detector (Model 490), an autosampler (Model 717). Samples were introduced via autoinjector with a 20 μl and all chromatographic separations were carried out at ambient temperature. Data acquisition and analysis were performed on a computer using the Millenium 32® chromatography software (Waters Association), which communicated with the HPLC equipment. Spectrophotometer UV2101-PC (Schimadzu, Japan) and centrifuge 5417 (Eppendorf) were used for the analyses.

### 2.3. Size-exclusion HPLC conditions

SCS was directly separated by HPLC on a TSK gel HW-40F® column (250 mm × 9.4 mm I.D., Tosoh, Japan) with UV detector attachments. Aliquot (20 μl) of the samples were eluted with acetonitrile–phosphate buffer (pH 6.0; 10 mM) (2:98, v/v) at a flow rate of 1.0 ml/min. The detection wavelength was set at 210 nm.

### 2.4. Standard and sample preparation

Stock standard solution of SCS raw materials were prepared in water at a concentration of 1.0 mg/ml. Further dilutions were prepared in water to give concentration in the range of 0.05–0.5 mg/ml for standard calibration (Fig. 2). More than 20 tablets or capsules were weighed individually for recording the average weight and ground to fine powder. The accurately weighed quantity of powder, equivalent to about 100 mg of SCS was transferred to 100-ml volumetric flask, and dis-

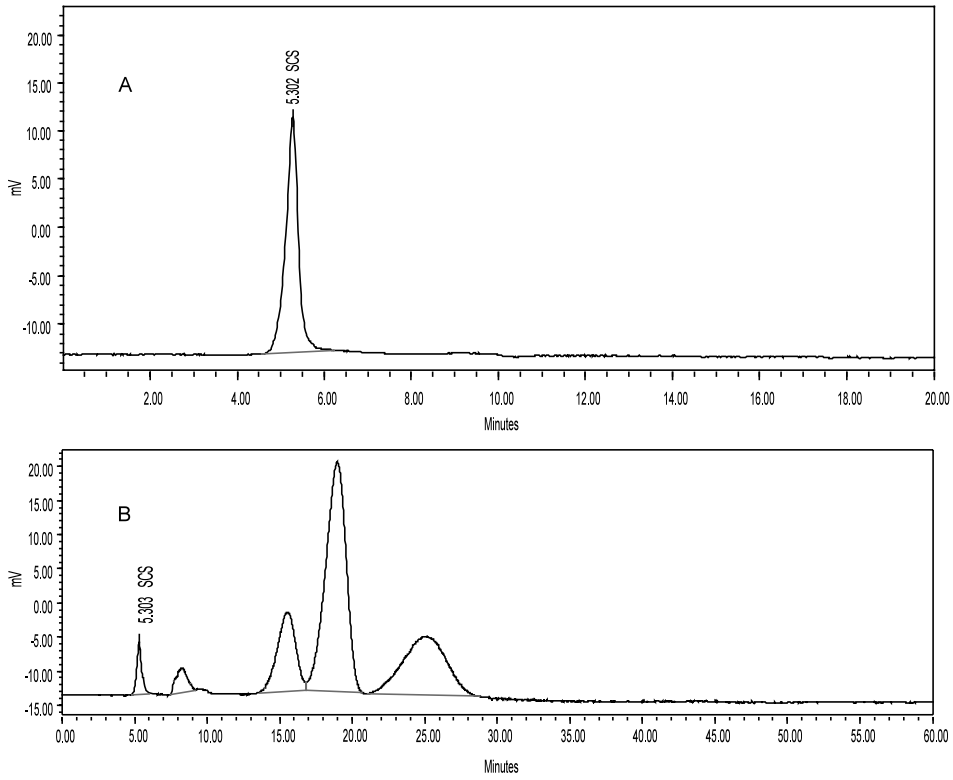


Fig. 2. Typical chromatograms of SCS obtained from standard raw material (A) and multi-components pharmaceutical preparation (B): A, SCS conc. 0.5 mg/ml; B, SCS conc. 0.1 mg/ml.

solved in 100 ml water. After shaking and extraction, filtered a portion of the solution through a membrane filter (pore size 0.45 μm), and diluted with water to the concentration of SCS 0.1 mg/ml.

### 2.5. Validation of the analytical method

The HPLC method validation was established according to International Conference on Harmonization guideline [14], including specificity, linearity, detection and quantitation limit (LOQ), precision, accuracy, recovery, and robustness test. The specificity of the HPLC method was evaluated with peak retention time (RT) and peak area (PA) through the assay precision analysis. Calibration curves were constructed from PA versus concentrations of SCS. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient ($r^2$) of each calibration curve. A new standard curve was generated for each HPLC run and the calibration was checked again at the end of each run by running one standard of known concentration. The precision of the HPLC method was assessed by determination of SCS with five replicates ($n = 5$) of five different concentrations (0.05, 0.2, 0.3, 0.4, 0.5 mg/ml) of standard solutions. Intra- and inter-day precision were estimated by percent of relative standard deviation (RSD%) from the analysis of freshly prepared solutions on 3 separate days. Accuracy of the HPLC method was established by percentage relative error (RE%). The standard SCS, concentration ranged 0.05, 0.3, 0.5 mg/ml was spiked with three different runs ($n = 3$), and performed over 7 days. The mean of three runs was calculated and compared with the spiked value, individually. The percentage ER (ER%) was determined by dividing concentration of mean minus spiked, [mean-spiked], into concentration of spiked, [spiked]. For recovery of SCS from pharmaceuticals, stan-

dard SCS was added into single- and multi-component pharmaceuticals in the following amount 50, 100, 150 mg, and then analyzed by HPLC. The percent of recovery ratio (%) and their RSD% on the assay results were calculated. Robustness was assessed by analysis the SCS standard solution at different analytical temperatures, flow rates, and influence of variations of pH and buffer compositions.

### 2.6. Analysis of pharmaceuticals

An accurately counted number of capsules and tablet, equivalent to about 100 mg of SCS was transferred to a 100-ml volumetric flask, added about 50-ml of water, and stirred using a magnetic bar for about 20 min followed by shaking with vortex mixer. And then, this solution was diluted with water to make up the volume and stirred for an additional 5 min, a portion of the solution ( ~ 5-ml) was filtered with 0.45-μm filter. An aliquot of the 5 ml of this solution was transferred to a 50-ml volumetric flask, diluted with water to volume, and mixed. The percent assay (%) of the labeled claim for pharmaceuticals was calculated by dividing the determined amount (mg) into labeled amount (mg).

### 2.7. Spectrophotometric absorbance test

For showing up the applicability of the developed HPLC method to pharmaceuticals, HPLC assay results were compared with representative three classical spectrophotometric methods. Up to now, the spectrophotometric methods have been used generally as assay method for quality control of pharmaceuticals.

The first method was described in Korean Pharmaceutical Codex [15], which was used acriflavine reagent. An accurately weighed portion of the fine powder, equivalent to about 4 mg of SCS was transferred to a 50-ml volumetric flask, and added 50 ml of potassium acetate buffer (pH 4.0). And this solution was mixed by mechanical shaker for 15 min and centrifuged at 3000 × *g* for 5 min, a portion of this supernatants (5 ml) was added to 5 ml of 0.05% acriflavine solution, and then filtered with millipore (0.45 μm) membrane filter. An aliquot of 3 ml of filtrate was diluted with methanol to 50 ml, and measured the absorbance of this solution at 460 nm immediately using potassium acetate buffer prepared by the same procedures as blank solution.

The second method was based on carbazole reagent according to the Bitter and Muir [10]. An equivalent to about 4 mg of SCS were dissolved in 50 ml of water, and centrifuged at 3000 × *g* for 5 min. An aliquot of 1 ml of supernatant was added to 5 ml of 0.2% sulfuric borate solution, and boiled for 10 min in 100 °C water bath. And then, 0.2 ml of 0.125% aqueous carbazole solution was added and boiled in water bath for 15 min. The absorbance of this solution was measured at 530 nm using water prepared by the same procedures as blank solution.

The third method was described in Japanese Pharmaceutical Codex [16], which used a simple glass column packed with Ambelite IRC-50® anion exchange resin. An aliquot of 5 ml of aqueous supernatant described above the second method was loaded onto column, washed sufficiently with 0.01 N-HCl for elimination of other impurities, and then eluted the adsorbed SCS using 20 ml of N-HCl. The eluate was hydrolyzed by some addition of 3 N-HCl, then boiled for 1 h in water bath. After neutralizing this solution with N-KOH, 5 ml of phosphate buffer (pH 8.0; 0.05 M) and 2 ml of 0.4% *p*-nitrobenzenediazonium tetrafluoroborate solution were added. After standing for 50 min at room temperature, 3 ml of 3 N-KOH was added. The absorbance of this solution was measured immediately at 516 nm.

## 3. Results and discussion

### 3.1. Specificity and linearity

The developed size-exclusion HPLC method showed suitable specificity for SCS identification. Chromatographic specificity was investigated by peak RT and PA of SCS using standard solutions. The intra- and inter-day variations (RSD%) of RT were between 0.22 and 0.60, and PA were between 0.49 and 0.48, respectively (Fig. 1). Also, the HPLC method showed good linearity for the

Table 1
Summary of the size-exclusion HPLC performance

| Concentration range (mg/ml) | Correlation coefficient ($r^2$) | LOD[a] (mg/ml) | LOQ[b] (mg/ml) | Specificity (RSD%) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Intra-day | | Inter-day[c] | |
| | | | | RT[d] | PA[d] | RT | PA |
| 0.05–0.50 | 0.9998 | 0.005 | 0.015 | 0.22 | 0.49 | 0.60 | 0.48 |

[a] Limit of detection.
[b] Limit of quantitation.
[c] For 3 days ($n = 10$).
[d] Peak retention time (RT) and peak area (PA) of SCS.

examined concentration range (0.05–0.5 mg/ml). The minimum correlation coefficient ($r^2$) from the calibration curves was not less than 0.999. The limit of detection (LOD) of the HPLC method, defined as signal-to-noise $\geqslant 3$, and the LOQ was 5.0 and 15.0 μg/ml, respectively. Those results were considered favourable specificity, linearity and sensitivity for assay of SCS from pharmaceuticals. The HPLC performances were summarized in Table 1.

### 3.2. Precision

Method precision was assessed by repeatability and reproducibility of the HPLC method with five concentrations of SCS standard solution and five replicates for 3 days. The intra- and inter-day variations expressed by percent RSD% were 0.269 and 0.215%, respectively (Table 2).

### 3.3. Accuracy

The accuracy was estimated by application of the developed HPLC analytical procedure to mixture of drug product to which known amounts of SCS standard raw material had been added. The intra- and inter-day accuracy through spiked SCS standard solution, as indicated by RE%, were ranged from 0.031 to 0.887% (Table 2). The recovery of SCS using the HPLC analysis was estimated by addition with known concentration of standard raw material (50, 100, and 150 mg) into pharmaceuticals. The percent recoveries (RSD%) were ranged from 93.38 (1.265%) to 100.46% (1.418%) (Table 3). Those results were similar to that of Zhongming et al. (RSD 0.69%) [12].

### 3.4. Application to pharmaceuticals

The HPLC method was applicated for determination of pharmaceuticals including single-component capsule (Condro®), multi-component capsule (Tobrin®) and multi-component tablet (Condrin®)[1]. The HPLC assay results were 96.7, 100.5, and 99.4%, respectively (Table 4). All of the excipients contained in pharmaceutical capsules and tablet were analyzed by HPLC method to identify the interferences to SCS determination. The excipients showed no interferences when analyzed the SCS by developed size-exclusion HPLC (data not shown). These results evaluated that the HPLC method might be usefully applicable assay method for quality control of single- and multi-component pharmaceuticals.

### 3.5. Robustness

The robustness of the HPLC method was estimated by analytical HPLC conditions includ-

[1] Condro® single-components capsule contains SCS, corn starch, magnesium stearate, colloidal silicone dioxide, lactose. Tobrin® multi-components capsule contains SCS, riboflavin, retinol palmitate, thiamin hydrochloride, choline tartrate, magnesium stearate, lactose. Condrin® multi-components tablet contains SCS, fursultiamine, inositol, nicotinamide, pyridoxine hydrochloride, riboflavin tetrabutyrate, γ-oryzanol, cyanocobalamin, calcium pantothate, magnesium stearate, lactose.

Table 2
HPLC method precision and accuracy for SCS

| Concentration (mg/ml) | Precision | | | | Accuracy | |
|---|---|---|---|---|---|---|
| | Intra-day | | Inter-day[a] | | Intra-day | Inter-day |
| | M (%)[b] | RSD (%) | M (%) | RSD (%) | RE (%) | RE (%)[c] |
| 0.05 | 99.77 ± 0.28 | | 99.83 ± 0.29 | | −0.140 | −0.031 |
| 0.20 | 100.36 ± 0.29 | | 99.95 ± 0.56 | | | |
| 0.30 | 99.83 ± 0.19 | 0.269 | 99.63 ± 0.36 | 0.215 | −0.423 | −0.887 |
| 0.40 | 100.29 ± 0.24 | | 100.13 ± 0.34 | | | |
| 0.50 | 100.18 ± 0.28 | | 100.14 ± 0.31 | | −0.378 | −0.222 |

[a] For 3 days ($n = 5$).
[b] Data were expressed as mean (M) ± S.D. ($n = 5$).
[c] Mean percent of RE for 7 days ($n = 3$).

ing influences of variations of pH 3.0, 5.0, 8.0 of mobile phase, variations of temperature at 15, 25, 35 °C, flow rate, and concentrations of phosphate buffer 0.005, 0.01, 0.05, 0.1 M in mobile phase composition. The results showed that the peak shape and response of SCS were bad at over the pH 8.0 and not more than pH 3.0. The analytical temperatures and phosphate buffer concentration of mobile phase did not affect significantly to the response of SCS at room temperature and 0.01 M (data not shown). However, it was generally accepted that the more column temperature and buffer concentration raised, the more life time of analytical gel column might be shorten in HPLC.

### 3.6. Comparison with spectrophotometric methods

In order to compare the suitability for assay method, multi-components pharmaceuticals were analyzed by classical three types of spectrophotometric method using Sigma (SG) and Seikagaku (SK) as SCS standard raw materials. The achieved results showed significant differences with developed HPLC method. In acriflavine-based spectrophotometric assay method, the assay results were as follows: SG, 13.0%; SK, 11.0%. It was assumed that riboflavin co-formulated active ingredient in pharmaceuticals might interfere on the spectrophotometric absorbance of sample solution. Ribo-

Table 3
Recovery of SCS from pharmaceuticals

| Amount of SCS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Condro® capsule | | | Tobrin® capsule | | | Condrin® tablet | | |
| Added (mg) | Found (mg) | Recovery (%) | Added (mg) | Found (mg) | Recovery (%) | Added (mg) | Found (mg) | Recovery (%) |
| 50.0 | 46.5[a] | 93.03 | 50.0 | 49.8 | 99.51 | 50.0 | 49.4 | 98.78 |
| 100.0 | 92.3 | 92.27 | 100.0 | 101.7 | 101.66 | 100.0 | 100.3 | 100.31 |
| 150.0 | 140.1 | 94.83 | 150.0 | 150.3 | 100.20 | 150.0 | 150.8 | 100.51 |
| Mean | | 93.38 | | | 100.46 | | | 99.87 |
| RSD[b] | | 1.265 | | | 1.956 | | | 1.418 |

[a] Data expressed with mean of three replicates.
[b] Percent of relative standard deviation ($n = 9$).

Table 4
HPLC assay of SCS in pharmaceutical preparations

| Sample | Labeled amount | HPLC amount | Assay | | |
|---|---|---|---|---|---|
| | (mg) | (mg) | Percent (%) | Mean (%) | RSD[a] |
| Condro® | 400 | 390.16 | 97.54[b] | | |
| | 400 | 385.36 | 96.34 | 96.7 | 0.719 |
| | 400 | 384.92 | 96.23 | | |
| Tobrin® | 100 | 100.19 | 100.19 | | |
| | 100 | 101.52 | 101.52 | 100.5 | 1.259 |
| | 100 | 99.84 | 99.84 | | |
| Condrin® | 200 | 204.16 | 102.08 | | |
| | 200 | 196.32 | 98.16 | 99.4 | 2.067 |
| | 200 | 196.14 | 98.07 | | |

[a] Percent relative standard deviation ($n = 15$).
[b] Data expressed with mean of five replicates over three different lots.

flavin has spectrophometically positive absorbance at 460 nm as in itself, but the sample solution produced by acriflavine has negative absorbance at 460 nm. So that, the absorbance of sample solutions were resulted in lower value than those of normal. In case of carbazole-based spectrophotometric assay method, the assay results were as follows: SG, 48.2%; SK, 42.5%. It was assumed that glucose or lactose co-formulated inactive additives in pharmaceuticals might interfere on the spectrophotometric absorbance of sample solution. The glucose or lactose having molecular similarity to glucuronic acid produced from SCS by sulfuric borate could react with carbazole reagent. So that, the spectrophotometric absorbances of the sample were resulted in higher value than those of normal. Therefore, the classical acriflavine- and carbazole-based spectrophotometric method were not applicable for SCS assay method in the multi-components pharmaceuticals.

Meanwhile, the *p*-nitrobenzenediazonium tetrafluoroborate-based spectrophotometric method using Ambelite IRC-50® anion exchange resin showed comparatively similar assay results with the developed HPLC method. The assay results were as follows: SG, 106.0%; SK, 95.2% (Table 5).

Table 5
Comparative analysis of multi-components pharmaceuticals by the size-exclusion HPLC and classical spectrophotometric methods

| Lot | HPLC | | Spectrophotometry | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *p*-Ndtb[a] | | Acriflavine | | Carbazole | |
| | SG | SK | SG[b] | SK[b] | SG | SK | SG | SK |
| 1 | 96.2[c] | 98.4 | 100.8 | 91.1 | 13.8 | 11.3 | 50.0 | 42.5 |
| 2 | 92.8 | 93.5 | 107.4 | 97.0 | 11.7 | 10.1 | 47.4 | 39.9 |
| 3 | 92.5 | 96.8 | 109.7 | 97.4 | 13.4 | 11.6 | 46.8 | 45.1 |
| Mean | 93.8 | 96.2 | 106.0 | 95.2 | 13.0 | 11.0 | 48.2 | 42.5 |

[a] *p*-Ndtb, *p*-nitrobenzenediazonium tetrafluoroborate.
[b] Origin of standard raw material: SG, Sigma; SK, Seikagaku.
[c] Data expressed with mean of three replicates.

However, this method required time-consuming experimental procedures, high-skilled technicians and showed low reproducibility. So that, this spectrophotometric assay method was estimated inappropriate for routine quality control test in pharmaceutical industry. Consequently, the developed HPLC method was evaluated more easy, simple and reproducible method for the assay of SCS from pharmaceuticals than the three classical spectrophotometric methods.

## 4. Conclusion

This paper described the development of a rapid and convenient size-exclusion HPLC method for the determination of SCS from multi-components pharmaceutical preparations. The advantages of the suggested analytical procedure were the easiness to perform, the reproducibility and the no requirement of complicated pre-treatments before analysis. The linearity of calibration curve was good and the precision and accuracy of the analytical HPLC method were sufficient. This method offered better reproducibility and precision than the classical spectrophotometric and the enzymatic digestive methods. So that, the developed HPLC method was versatilely applicable for quality control of pharmaceutical preparations.

## References

[1] Y. Takahashi, O. Ishikawa, K. Okada, K. Ohnishi, Y. Miyachi, J. Dermatol. Sci. 10 (1995) 139–144.
[2] C. Belcher, M. Bayliss, J. Ledingham, M. Doherty, Ann. Rheum. Dis. 56 (1997) 119–122.
[3] A. Conte, N. Volpi, L. Palmieri, I. Bahous, G. Ronca, Arznei.-Forsch. 45 (1995) 918–925.
[4] A.M. Gressner, W. Koster-Eiserfunke, E. Vande Leur, H. Greiling, J. Clin. Chem. Clin. Biochem. 18 (1980) 279–285.
[5] T. Oguma, H. Toyoda, T. Toida, T. Imanari, Biomed. Chromatogr. 15 (2001) 356–362.
[6] F. Lamari, A. Theocharis, A. Hjerpe, N.K. Karamanos, J. Chromatogr.(B) Biomed. Sci. Appl. 730 (1) (1999) 129–133.
[7] C. Kodama, C. Ototani, M. Isemura, J. Aikawa, Z. Yosizawa, Clin. Chem. (Winston-Salem, NC) 32 (1986) 30.
[8] I. Koshiishi, M. Takenouchi, T. Hasegawa, T. Imanari, Anal. Biochem. 265 (1998) 49–54.
[9] J. Okazaki, A. Kamada, Y. Gonda, T. Sakaki, J. Periodontal. Res. 27 (1992) 484–488.
[10] T. Bitter, H.M. Muir, Anal. Biochem. 4 (1962) 330–334.
[11] M. Okamoto, S. Mori, M. Ishimaru, H. Tohge, Y. Nakata, H. Endo, Life Sci. 60 (20) (1997) 1811–1819.
[12] L. Zhongming, B. Corrine, S. Terrin, H. Todd, J. Pharm. Biomed. Anal. 28 (2002) 245–249.
[13] J. Okazaki, A. Kadama, F. Matsukawa, T. Sakaki, Y. Gonda, Arch. Oral Biol. 40 (8) (1995) 777–779.
[14] ICH Steering Committee validation of analytical procedures; Methodology; ICH harmonised tripartite guideline, (1996) 1–8.
[15] G.S. Kim, S.H. Lee, Korean Pharmaceutical Codex, Korean Association of Official Compendium, Seoul, 1998, pp. 359–360.
[16] S. Kamiya, T. Okada, Japanese Pharmaceutical Codex, Japanese Association of Official Compendium, Tokyo, 1997, pp. 669–672.