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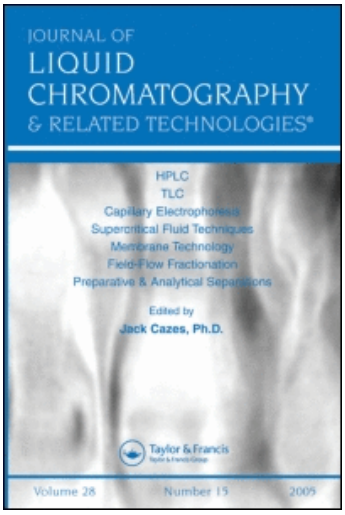

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HPLC Method Validation and Pharmacokinetic Study of Alendronate Sodium in Human Urine with Fluorescence Detection

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Abstract: The purpose of this study was to validate a reliable analytical method for the pharmacokinetic study of alendronate sodium in human urine by a high performance liquid chromatography (HPLC) system with fluorescence detection. Alendronate sodium was extracted by using diethylamine (DEA) solid phase extraction (SPE) and derivatized with 9-fluorenylmethyl chloroformate (FMOC). Pamidronate was used as the internal standard. The sample was precipitated with sodium hydroxide and derivatized with FMOC in sodium carbonate buffer at pH 11.9. Separation was performed on a Capcell Pak UG C_{18} column (4.6 mm × 150 mm, 5 μm particles), using a gradient method starting with mobile phase acetonitrile/methanol-citrate/pyrophosphate buffer (28 : 72, v/v). The fluorometric detector was operated at 260 nm (excitation) and 310 nm (emission). The intra- and inter-day precision expressed as the relative standard deviation was less than 15%. The limit of quantification was 25 ng/mL of alendronate sodium using 5 mL of urine. The calibration curve was linear in the concentration range of 25–5000 ng/mL ($r^2 = 0.9999$). Following the oral administration of 70 mg alendronate to volunteers, the cumulative amount of alendronate excreted (A_{et}) and peak excretion rate (U_{max}) were 198.39 ± 81.16 μg and 65.67 ± 20.83 μg/mL, respectively. The method was demonstrated to be highly

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feasible and reproducible for pharmacokinetic studies of alendronate sodium in seven volunteers after oral administration (70 mg as alendronate).

Keywords: Alendronate sodium, HPLC, Human urine, Fluorescence detection

INTRODUCTION

Alendronate [(4-amino-1-hydroxybutylidene) biosphosphonate] (Figure 1) is used for the treatment of a variety of bone diseases including osteoporosis, Paget's disease, and metastatic bone disease.[1,2] The physicochemical effects of bisphosphonates are similar to those of pyrophosphate and polyphosphates;[3] alendronate is selectively accumulated in the skeleton, and its oral absorption is approximately 1% of the administered dose.[4] To obtain more detailed clinical pharmacological data, a reliable pharmacokinetics, as well as, analytical assay is required.

In recent years, several HPLC methods have been reported for its determination, many of which relied on derivatization of alendronate, using either pre-column[5] or post-column techniques.[6] Especially, HPLC methods are routinely used for analysis of alendronate sodium in biological fluid. Direct HPLC analysis of alendronate using a refractive index detector,[7] ion chromatography with conductivity detection,[8] or indirect UV detection have also been reported. Ion chromatography-MS techniques have been employed in the characterization of alendronate sodium.[9] Alendronate sodium was determined in pharmaceutical dosage forms by HPLC after derivatization with 9-fluorenylmethyl chloroformate (FMOC). Excess reagent had to be

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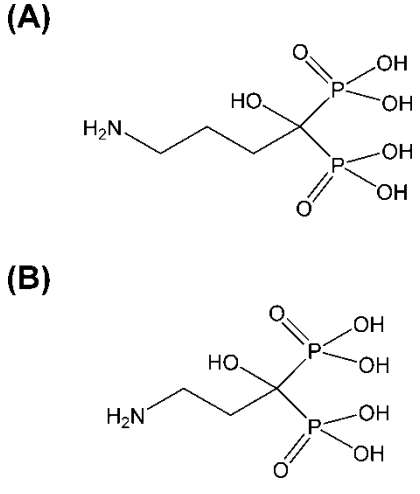

Figure 1. Chemical structures of (a) alendronate and (b) pamidronate.

extracted with methylene chloride and an aliquot of the aqueous portion was assayed on RP HPLC.[10]

Moreover, due to the extremely low plasma concentrations of alendronate,[11] many pharmacokinetic studies relied on the determination of alendronate concentrations in urine rather than plasma.[5,12] A recent report demonstrated the HPLC method for determining alendronate sodium in human plasma by detecting fluorescence.[13] When FMOC was used to derivatize alendronate, a LOQ of 1 ng/mL was achieved using 3 mL of plasma with fluorescence detection.[13] In that method, a gradient system was required and total run time for one sample was 25 min. However, it is still desirable to routinely validate a method and establish pharmacokinetic parameters using urine samples.

Here, the aim of the present study was to validate a reliable and accurate HPLC method with fluorescence detection for the determination of alendronate sodium in human urine. This assay method was applied to determine the urine levels after oral administration of alendronate sodium to humans.

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EXPERIMENTAL

Materials

Alendronate sodium and pamidronate disodium (internal standard, IS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetonitrile were HPLC grade and purchased from J. T. Baker (Phillipsburg, NJ, USA). The 9-fluorenylmethyl chloroformate (FMOC) was obtained from Fluka Chemie (Germany). All other chemicals were analytical grade and used without further purification.

Preparation of Standards

Stock solutions of alendronate sodium (10 mg/mL) were made by dissolving in 0.2 M sodium citrate and diluted to concentration of 12.5, 25, 100, 250, 500, and 2500 μg/mL. Standard solutions of alendronate sodium in human urine were prepared by spiking the appropriate volume (less than 10 μL per mL) of various diluted stock solutions giving trial concentrations of 25, 50, 200, 500, 1,000, and 5,000 ng/mL. Internal standard, pamidronate, structurally similar to alendronate, as shown in Figure 1, was dissolved in distilled water to make a stock solution at a final concentration of 1,000 μg/mL.

Preparation of Samples

The samples were stored in the freezer at −18°C and allowed to thaw at room temperature before processing. Each 5 mL of urine was pipetted into a glass tube and 40 μL of 6 N HCl was added to prevent the precipitation of

alendronate sodium in urine. Then, the tube was briefly shaken by vortex-mixing for 10 sec with 10 μL of internal standard (1,000 μg/mL of pamidronate disodium). One hundred μL of 0.1 M KH_2PO_4 and the same amount of 0.1 M $CaCl_2$ were added and the sample was made alkaline with 450 μL of 1 M NaOH. The sample was shaken for 30 sec and centrifuged at 3,000 rpm for 10 min, and the supernatant was removed. The precipitate was completely dissolved in 0.5 mL of 0.2 M acetic acid and 5 mL of distilled water was added. Consequently, the precipitation with sodium hydroxide was repeated twice. The resulting precipitate was finally dissolved in 1 mL of 0.2 M acetate buffer (pH 4.5) and diluted with 2 mL of distilled water. The sample was then loaded on a DEA SPE cartridge (Bond Elut®-DEA, Varian, USA) pre-washed with water. After washing the cartridge with 2 × 0.5 mL water, the drug eluted 1.6 mL of 0.2 M sodium citrate and an aliquot of the eluate was taken for the derivatization.

The derivatization procedure involved addition of 100 μL of 1 M sodium carbonate buffer (pH 11.9) to 270 μL of the sample and subsequent addition of 100 μL of FMOC solution (1 mg in 4 mL of acetonitrile). After 3 min, 100 μL of 1 M citric acid was added to adjust pH and 100 μL of the sample was injected into the chromatographic system.

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Apparatus

The determination of alendronate sodium was carried out using an HPLC system composed of a Shimadzu Class-VP HPLC system (Shimadzu, Tokyo, Japan), equipped with two LC-10ADVP pumps, a RF-10AXL fluorescence detector, an SIL-10ADVP autosampler, an SCL-10AVP controller, and a DGE-14A degassing unit. The separation was performed on a Capcell Pak UG C_{18} column (4.6 mm × 150 mm I.D., 5 μm, Shiseido, Tokyo, Japan).

Chromatographic Conditions

The mobile phase was a series of steps in a gradient consisting of a mixed organic solution (acetonitrile:methanol = 17 : 10, v/v, solvent A) and buffer (25 mM citric buffer and 25 mM sodium pyrophosphate buffer without pH adjustment, solvent B), range: 0–24 min 28 : 72 (v/v); 24–30 min 70 : 30 (v/v); and 30–40 min 28 : 72 (v/v). The column was equilibrated for 2 min before injecting each subsequent sample. The flow-rate was 1.0 mL/min at 40°C. The excitation and emission wavelengths were 260 and 310 nm, respectively, and the time constant was set to 2 sec.

Validation of the Method

Evaluation of the reversed-phase HPLC method was based on proportionality (linearity assay), precision, and accuracy.

Specificity

Drug free blank human urine was tested for interference using the proposed HPLC method, and the result was compared with those obtained from alendronate sodium and the internal standard.

Linearity

The calibration curve consisted of the six concentrations; 25, 50, 200, 500, 1,000, and 5,000 ng/mL for alendronate sodium. The calibration curves were obtained by linear regression; the ratio of alendronate sodium peak area to internal standard peak area was plotted vs. alendronate sodium concentration in ng/mL

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Precision and Accuracy

The intra- and inter-day precision (coefficients of variation, CV%) and inter-day accuracy (bias%) of the assay procedure were determined by the analysis of six samples of each concentration in the same day and one sample of each concentrations in 5 different days, respectively.

Sensitivity

The limit of quantification (LOQ) was defined as the lowest concentration at which the precision expressed by CV% was lower than 20%, the accuracy expressed by bias% was within 80–120%, and ratio of signal to noise was better than 10.

Matrix Effect

To show signal suppression caused by matrix components, matrix effects were determined by comparing the peak area of the analytes at the concentrations of 200 and 1,000 ng/mL in the samples after extraction and derivatization to that of each analyte obtained in neat solution.

Stability

Freeze and Thaw Stability

Human urine samples containing 200 and 1,000 ng/mL of alendronate sodium were prepared. The samples were stored at -70°C for 24 hrs, subjected to three thaw and freeze cycles, and analyzed by HPLC.

Short Term Stability

Human urine samples containing 200 and 1,000 ng/mL of alendronate sodium were exposed to room temperature for 12 hrs and analyzed by HPLC.

Long Term Stability

Human urine samples containing 200 and 1,000 ng/mL of alendronate sodium were stored in the deep freezer at −70°C for 30 days and analyzed by HPLC.

Standard Solution Stability

The stock solution of alendronate sodium was left at room temperature for 12 hrs.

Processed Sample Stability

Human urine samples containing 200 and 1,000 ng/mL of alendronate sodium were left in the autosampler at ambient temperature (ca. 20°C) for 24 hrs and analyzed by HPLC.

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Preparation of Biological Samples

The validated method was applied to evaluate the bioavailability of alendronate sodium. Seven (7 males) healthy volunteers were selected for this study according to medical history, physical examination, and standard laboratory test results (blood cell count, biochemical profile, and urinalysis). The demographic data of these volunteers were; mean age 23.3 years, mean height 174.1 cm, and mean weight 64.4 kg.

After an overnight fast, a pre-dosing urine sample was collected. Each volunteer was then orally administered one tablet (70 mg as alendronate), namely Fosamax®, 70 mg (MSD Korea Ltd., Seoul, Korea) with 240 mL of water. The volunteers continued to fast for 4 hrs, after which a standard lunch was served. Urine samples were collected before administration and at designated time intervals, i.e., 0.25, 1, 2, 3, 4, 6, 8, 12, 24, and 30 hrs post-dosing. The collected urine was stored at −70°C until assayed for the alendronate sodium content. The pharmacokinetic parameters were calculated by the bioavailability analytical program, BA Calc 2002.[14]

RESULTS AND DISCUSSION

High Performance Liquid Chromatography

Specificity

Drug free human urine was screened and no endogenous interference was observed at the retention time of the alendronate sodium and internal standard (Figure 2). The Capcell Pak UG C_{18} column has been used as the analytical column since it has 80 Å pore size, which limits the access of large molecules such as proteins, retain drug molecules longer, and improved the sensitivity.[15] A chromatogram of extracted blank human

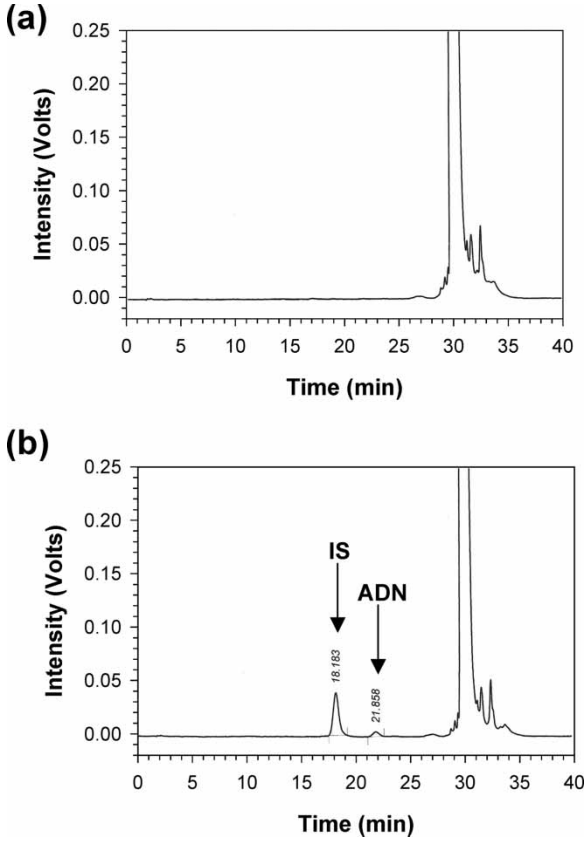

Figure 2. Representative chromatograms of (a) blank urine and (b) blank urine spiked with alendronate sodium (25 ng/mL) and pamidronate as internal standard (2000 ng/mL). ADN and IS in chromatograms represent alendronate sodium and internal standard, respectively.

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urine sample, a representative chromatogram of extracted urine sample containing 25 ng/mL of alendronate sodium, a representative chromatogram of an extracted urine sample containing 25 ng/mL alendronate sodium, and 2000 ng/mL internal standard, as well as alendronate sodium in urine collected at 2 hr after oral administration of 70 mg to human subjects, are shown in Figure 3.

Linearity

The calibration curves were linear in the studied range. The mean equation of the calibration curve consisting of six points was: $y = 0.0031 (\pm 0.0001)x + 0.0677(\pm 0.0352)$ with correlation coefficient $r^2 = 0.9999$

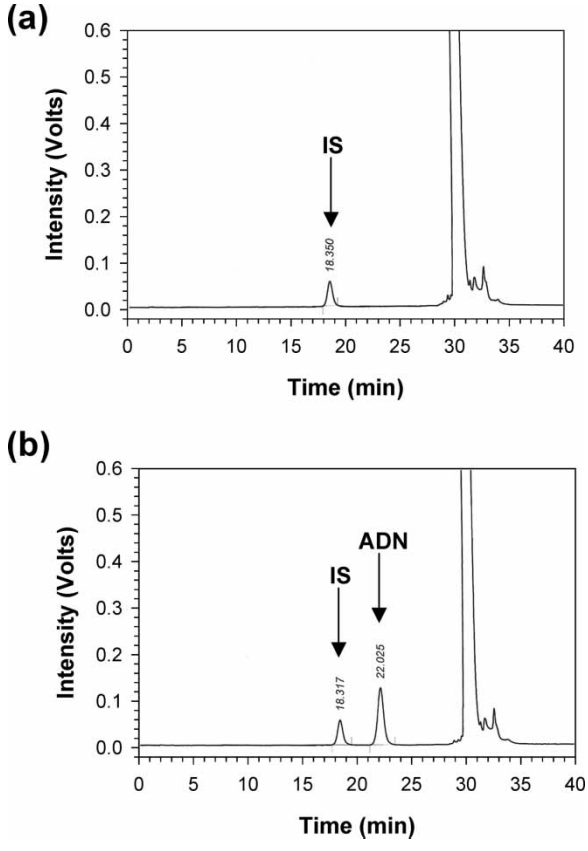

Figure 3. Representative chromatograms of (a) pre-dose urine and (b) urine sample from a human subject at 2 hr after an oral administration of 70 mg alendronate (874.86 ng/mL). ADN and IS in chromatograms represent alendronate sodium and internal standard, respectively.

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Table 1. Reproducibility of alendronate sodium determination in human urine (n = 5)

Alendronate sodium concentration (ng/mL)	Precision (CV%)		Accuracy
	Intra-day	Inter-day	
25 (LOQ)	15.4	14.2	81.3
50	6.55	12.4	85.6
200	14.2	10.3	111.0
500	1.96	3.52	100.7
1000	1.83	3.79	99.4
5000	2.46	4.38	100.6

(± 0.0001), where y represents the ratio of alendronate sodium peak area and the internal standard one, and x represents the alendronate sodium concentration in ng/mL.

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Precision and Accuracy

The intra- and inter-day precision and accuracy results are shown in the Table 1. The values obtained were lower than the limits required for biological samples, $\pm 20\%$ for the precision and inaccuracy of the lower limit of quantification (25 ng/mL), and $\pm 15\%$ for both of the other concentrations.

Sensitivity

The LOQ of alendronate sodium was 25 ng/mL. This method was sufficiently sensitive, with a quantification limit lower than the minimum concentration recommended for plasma samples obtained after the administration of 70 mg alendronate. The sensitivity of alendronate sodium is shown in Figure 2.

Table 2. Matrix effect and stability of alendronate sodium determination in human urine (n = 3)

Alendronate sodium concentration (ng/mL)	Matrix effect (%)	Freeze-thaw stability (%)	Short term stability (%)	Long term stability (%)	Standard solution stability (%)	Processed sample stability (%)
200	95.83	103.29	106.55	100.50	98.61	102.41
1000	101.12	100.35	99.31	99.98	101.44	99.35

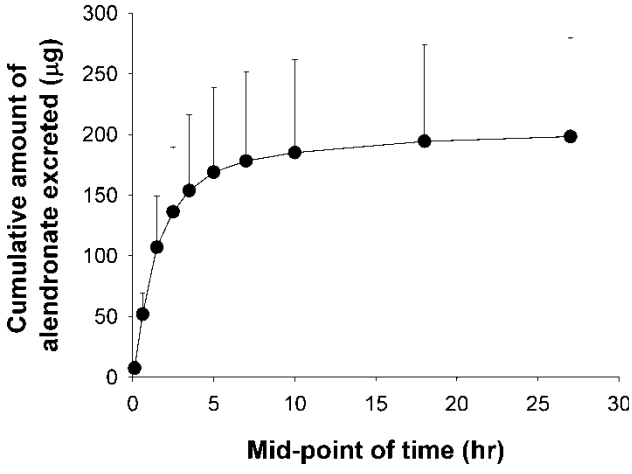

Figure 4. Mean cumulative amount of alendronate excreted versus mid-point of time plots after administration of alendronate sodium tablet formulations to seven healthy male volunteers. The results represent the mean ± s.d. (n = 7).

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Matrix Effect and Stability

The results are shown in Table 2. Thus, this assay method for the determination of alendronate sodium in human urine has sufficient recovery of extraction and stability in human urine for the bioavailability assessment of alendronate sodium.

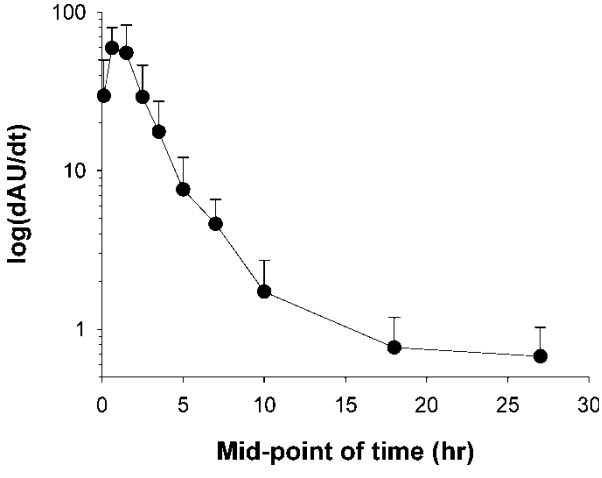

Figure 5. Mean log excretion rate (log(dAU/dt)) versus mid-point of time plots after administration of alendronate sodium tablet formulations to seven healthy male volunteers. The results represent the mean ± s.d. (n = 7).

Application to the Bioavailability of Alendronate Sodium

The cumulative excreted amount and the log excretion rate of alendronate versus mid-point of time plots after a dose of Fosamax® tablet, 70 mg, are shown in Figures 4 and 5, respectively. The pharmacokinetic parameters of alendronate sodium were: $198.39 \pm 81.16\,\mu g$ of A_{et}, $65.67 \pm 20.83\,\mu g/mL$ of U_{max}, and 0.93 ± 0.59 hr of T_{max}, which were very similar to the previous reports.[5,16]

The present method offers practical advantages over the immunological, HPLC-MS and GC-MS in the viewpoint of speed and sample throughput. Moreover, compared with previously reported liquid chromatographic determination of alendronate sodium, this method improved sample throughput and presented the pharmacokinetic data of 70 mg of alendronate.

CONCLUSION

A determination method of alendronate sodium from urine samples has been developed using HPLC. This analytical method showed sensitivity, speed, specificity, and reproducibility using the plasma sample. This method could be successfully applied to evaluate the bioavailability of alendronate sodium in healthy volunteers.

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REFERENCES

1. Russell, R.G.; Rogers, M.J. Bisphosphonates: From the laboratory to the clinic and back again. Bone **1999**, *25* (1), 97–106.
2. Hosking, D.; Chilvers, C.E.; Christiansen, C.; Ravn, P.; Wasnich, R.; Ross, P.; McClung, M.; Balske, A.; Thompson, D.; Daley, M.; Yates, A.J. Prevention of bone loss with alendronate in postmenopausal women under 60 years of age. Early Postmenopausal Intervention Cohort Study Group. N. Engl. J. Med. **1998**, *338* (8), 485–492.
3. Fleisch, H. Bisphosphonates. Pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. Drugs **1991**, *42* (6), 919–944.
4. Fitton, A.; McTavish, D. Pamidronate. A review of its pharmacological properties and therapeutic efficacy in resorptive bone disease. Drugs **1991**, *41* (2), 289–318.
5. Ptáček, P.; Klíma, J.; Macek, J. Determination of alendronate in human urine as 9-fluorenylmethyl derivative by high-performance liquid chromatography. J. Chromatogr. B **2002**, *767* (1), 111–116.

6. Lovdahl, M.J.; Pietrzyk, D.J. Anion-exchange separation and determination of bisphosphonates and related analytes by post-column indirect fluorescence detection. J. Chromatogr. A **1999**, *850* (1–2), 143–152.
7. Han, Y.H.R.; Qin, X.Z. Determination of alendronate sodium by ion chromatography with refractive index detection. J. Chromatogr. A **1996**, *719* (2), 345–352.
8. Tsai, E.W.; Ip, D.P.; Brooks, M.A. Determination of alendronate in pharmaceutical dosage formulations by ion chromatography with conductivity detection. J. Chromatogr. A **1992**, *596* (2), 217–224.
9. Qin, X.Z.; Tsai, E.W.; Sakuma, T.; Ip, D.P. Pharmaceutical application of liquid chromatography-mass spectrometry: II. Ion chromatography-ion spray mass spectrometric characterization of alendronate. J. Chromatogr. A **1994**, *686* (2), 205–212.
10. De Marco, J.D.; Biffar, S.E.; Reed, D.G.; Brooks, M.A. The determination of 4-amino-1-hydroxybutane-1,1-diphosphonic acid monosodium salt trihydrate in pharmaceutical dosage forms by high-performance liquid chromatography. J. Pharm. Biomed. Anal. **1989**, *7* (12), 1719–1727.
11. Lin, J.H. Bisphosphonates: a review of their pharmacokinetic properties. Bone **1996**, *18* (2), 75–85.
12. Al Deeb, S.K.; Hamdan, I.I.; Al Najjar, S.M. Spectroscopic and HPLC methods for the determination of alendronate in tablets and urine. Talanta **2004**, *64* (3), 695–702.
13. Yun, M.H.; Kwon, K.I. High-performance liquid chromatography method for determining alendronate sodium in human plasma by detecting fluorescence: Application to a pharmacokinetic study in humans. J. Pharm. Biomed. Anal. **2006**, *40* (1), 168–172.
14. Lee, Y.J.; Kim, Y.G.; Lee, M.G.; Chung, S.J.; Lee, M.H.; Shim, C.K. Analysis of bioequivalence study using log-transformed model. Yakhakhoeji. **2000**, *44* (4), 308–314.
15. Park, J.S.; Park, K.M.; Kim, C.K. Determination of triflusal in human plasma by high performance liquid chromatography with automated column switching system. J. Liq. Chromatogr. & Rel. Technol. **2000**, *23* (16), 2513–2524.
16. Jeal, W.; Barradell, L.B.; McTavish, D. Alendronate. A review of its pharmacological properties and therapeutic efficacy in postmenopausal osteoporosis. Drugs **1997**, *53* (3), 415–434.

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