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Short communication

High-performance liquid chromatography method for determining alendronate sodium in human plasma by detecting fluorescence: Application to a pharmacokinetic study in humans

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed using diethylamine (DEA) solid-phase extraction (SPE), 9-fluorenylmethyl derivative (FMOC) and fluorescence detection for quantifying alendronate in human plasma. Sample preparation involved a manual protein precipitation with trichloroacetic acid, a manual coprecipitation of the bisphosphonate with calcium phosphate and derivatization with 9-fluorenylmethyl chloroformate in citrate buffer at pH 11.9. Liquid chromatography was performed on a Capcell Pak C_{18} column (4.6 mm × 150 mm, 5 μm particles), using a gradient method starting with mobile phase acetonitrile/methanol-citrate/pyrophosphate buffer (32:68, v/v). The total run time was 25 min. The fluorometric detector was operated at 260 nm (excitation) and 310 nm (emission). Pamidronate was used as the internal standard. The limit of quantification was 1 ng/ml using 3 ml of plasma. The intra- and inter-day precision expressed as the relative standard deviation was less than 15%. The assay was applied to the analysis of samples from a pharmacokinetic study. Following the oral administration of 70 mg of alendronate sodium to volunteers, the maximum plasma concentration (C_{max}) and elimination half-life were 40.94 ± 19.60 ng/ml and 1.67 ± 0.50 h, respectively. The method was demonstrated to be highly feasible and reproducible for pharmacokinetic studies including bioequivalence test of alendronate sodium in humans.

Keywords: Alendronate; HPLC; FD; FMOC

1. Introduction

Alendronate [(4-amino-1-hydroxybutylidene)bisphosphonate] (Fig. 1) is an important representative of the bisphosphonates used to treat metabolic disorders of calcium, like Paget's disease of bone, hypercalcemia, and osteoporosis [1,2]. The physicochemical effects of bisphosphonates are similar to those of pyrophosphate and polyphosphates [2]; Alendronate is selectively accumulated in the skeleton, and its oral absorption is approximately 1% of the administered dose [1]. To obtain more detailed clinical pharmacological data, a reliable, sensitive bioanalytical assay is required.

In the last decade, several analytical methods for quantifying different bisphosphonates have been reported. However, the number of methods for use with serum or plasma is limited, due to the extremely low plasma concentrations of alendronate [3]. Consequently, many pharmacokinetic studies rely on determining alendronate concentrations in urine, rather than plasma [4,5].

The published methods for determining bisphosphonates in urine use similar strategies. All the published procedures separate bisphosphonates from the biological matrix by repeated coprecipitation with calcium phosphate under alkaline conditions, and then remove the calcium ions using various SPE columns [6–14]. Since bisphosphonates lack chromophores, with rare exceptions [6], derivatization of the analyte is necessary (alendronate with 2,3-naphthalene dicarboxylaldehyde [4,7], pamidronate with 1-naphtylisothiocyanate[8], fluorescamine [9,10], or olpadronate with 9-fluorenyl-methylchloroformate [11]). Alternatively, an electrochemical detector can be used to

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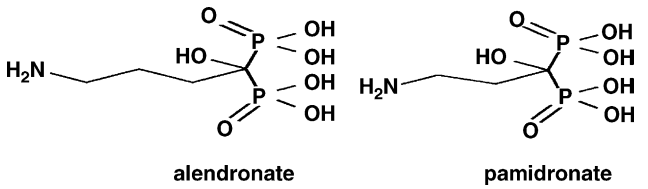

Fig. 1. Structures of alendronate and the internal standard pamidronate.

detect either bisphosphonates directly [14] or their derivatives [4]. Various other methods of detection requiring expensive or complicated instruments in combination with HPLC have also been used, including negative ion electrospray ionization mass spectroscopy [15], an evaporative light-scattering detector [16] and gas chromatography mass spectroscopy [17].

This report describes the development of an HPLC method for determining alendronate in plasma, based on the method previously described for urine samples [11]. The aim of this study was to develop a robust HPLC method for alendronate determination in plasma, with a limit of quantification (LOQ) of at least 1 ng/ml, to enable pharmacokinetic studies based on plasma data. Our validated HPLC method would be applied to a pharmacokinetic study in humans.

2. Experimental

2.1. Chemicals

Alendronate sodium was obtained from Hanmi Pharm. Co. Ltd., Korea. Acetonitrile (for liquid chromatography) was from Duksan (Seoul, Korea). Methanol (for liquid chromatography) and potassium dihydrogen phosphate (analytical grade) were manufactured by Merck (Darmstadt, Germany). The 9-fluorenylmethyl chloroformate (Purris p.a.) and other chemicals (analytical grade) were Aldrich products (Seoul, Korea). Injectable pamidronate disodium (Panorin®, the internal standard (I.S.)) was obtained from a local pharmacy (Seoul Hanlim Pharm. Co. Ltd., Korea). The DEA SPE cartridges were purchased from Varian (Bond Elut-DEA 100 mg/1 ml, Seoul, Korea).

2.2. Apparatus

The HPLC analysis was performed using a Shimadzu Class-VP HPLC system consisting of a SIL-10ADVP autosampler, an LC-10ADVP pump, a DGE-14A degasser, an SCL-10AVP controller, and a RF-10AXL fluorescence detector. The separation was performed on a 150 mm × 4.6 mm i.d. column (Shiseido, Japan) filled with Capcell Pak C_{18} stationary phase, with 5 μm particles. The mobile phase was a series of steps in a gradient consisting of a mixed organic solution (acetonitrile:methanol = 1:1, solvent A) and buffer (25 mM citric acid and 25 mM sodium pyrophosphate without pH adjustment, solvent B) range: 0–9 min 32:68 (v/v); 9–16 min 60:40 (v/v); and 16–24 min 32:68 (v/v). The column was equilibrated for 1 min before injecting each subsequent sample. The total run time was 25 min. The flow-rate was 1.5 ml/min at 35 °C. The excitation and emission wavelengths were 260 and 310 nm, respectively, and the time constant was set to 2 s.

2.3. Standards

Stock solutions of alendronate sodium were made by dissolving approximately 10,000,000 ng in 10 ml of distilled water. Separate solutions were prepared for the calibration standards and quality control samples. These solutions were diluted immediately before use with purified water, to obtain working solutions of 1000, 500, 200, 100, 50, 20, and 10 ng/ml for each analyte. The Panorin® ampoule contained a lyophilized mixture of 15,000,000 ng/ml pamidronate disodium. An aqueous solution containing approximately 12,500 ng/ml was prepared, and 0.03 ml of this solution were added to a 3 ml plasma sample as the internal standard. The identical internal standard solution was used throughout the entire study. All solutions were stored at 4 °C and protected from light.

2.4. Calibration curves and quality control samples

A calibration curve was constructed for the range of 1–100 ng/ml to encompass the usual concentrations measured in samples from pharmacokinetic studies, using free human plasma from heparinized blood. The calibrating samples were prepared immediately before use by spiking 2.7 ml of free human plasma with 0.3 ml of a convenient working solution in purified water, in order to add the following amounts of alendronate sodium: 1, 2, 5, 10, 20, 50, and 100 ng/ml. Two samples (free plasma and free plasma spiked with the internal standard alone) were also analyzed (but not used to calculate the calibration equation) with each calibration curve to check for the absence of the interfering peak caused by the biological matrix. Standard curves were constructed by plotting the peak area ratio of the analyte to the internal standard as a function of the concentration added. The weighting procedure giving the best-weighted least-squares linear regression was chosen. Finally, the calibration equation was validated if the relative difference between the theoretical and back-calculated concentrations of each sample of the calibration set did not exceed 20% at the lowest concentration and 15% at the other concentrations.

Quality control samples (four different levels: 1, 2, 50, and 100 ng/ml) were used to determine the intra- and inter-assay precision and accuracy of the method.

2.5. Sample preparation

The samples were stored in the freezer at −20 °C and allowed to thaw at room temperature before process-

ing. Thirty microliters of the internal standard solution (12,500 ng/ml pamidronate disodium) were added to 3 ml of plasma and the tube was shaken briefly. The procedure started with a protein precipitation by adding 3 ml of 6% trichloroacetic acid (TCA) and the precipitate was removed by centrifugation at 4000 rpm for 10 min. The clear supernatant was transferred into a 10 ml conical glass tube, 0.2 ml 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 0.4 ml of 1 M NaOH. The sample was centrifuged for 5 min at 3000 rpm and the supernatant was discarded. After removal of the liquid phase, the precipitate was dissolved completely in 0.5 ml of 0.2 M acetic acid and 3 ml of water were added. The precipitation with NaOH was repeated twice. The resulting precipitate was dissolved in 1 ml of 0.2 M acetate buffer (pH 6.0) and 0.04 ml of acetic acid, diluted with 2 ml of water. The sample was then loaded on a DEA SPE cartridge pre-washed with water. After washing the cartridge twice with 0.5 ml of water, the drug was eluted with 1 ml of 0.2 M sodium citrate and an aliquot of the eluate was taken for derivatization. The derivatization procedure involved adding 0.2 ml of 1 M sodium carbonate buffer (pH 11.9) to 0.54 ml of the sample, and then adding 0.2 ml of FMOC solution (1 mg in 4 ml of acetonitrile). After 5 min, 0.2 ml of 1 M citric acid were added to adjust the pH, and 0.05 ml of the sample were injected into the chromatographic system.

2.6. Application of the method

Our method was applied to a pharmacokinetic study in which the alendronate concentration was measured in four human volunteers. In this study, four male volunteers were given a 70 mg tablet of an alendronate formulation developed by Merck (Posamax 70 mg®) as a single oral dose. The medication was taken with 250 ml of water after fasting. Blood samples were collected 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, and 7 h after drug administration. After each blood sampling, plasma was separated by centrifugation at 3,000 rpm for 10 min and stored at −20 °C until it was assayed.

The pharmacokinetic parameters of alendronate was preformed using non-compartmental pharmacokinetic methods with the WinNonlin software package (Pharsight Corporation, California). The non-compartmental analysis was performed, using standard methods, for each subject. The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule and extrapolated to infinity. Total clearance (CL_{total}) was divided by AUC_{inf}. The elimination half-life ($t_{1/2}$) was calculated using the relationship:

$$t_{1/2} = \frac{0.693}{k_e}$$

All data were expressed as means ± standard deviation.

3. Results and discussion

3.1. The influence of pH on sample preparation

FMOC was chosen as the derivative reagent because of its superior properties, as compared to the other reagents. It reacts under mild conditions with amines and the resulting derivatives are stable. The reaction mixture can be injected on a standard silica-based analytical column, directly after simple pH adjustment, and no preceding removal of unreacted reagent is required. The yield of the derivatization reaction depends on the pH, reagent concentration, reaction mixture composition, and reaction time. It had to be optimized, as only data on alendronate FMOC derivatives are available [5,20]. The presence of citrate ions in the reaction mixture was necessary to prevent alendronate adsorption losses. The amount of organic solvent in the derivatization mixture is also important, since the phases occasionally separate and the FMOC derivatives have limited solubility in water. The derivative yield is strongly pH dependent, gradually increasing to pH 11.5, and rapidly falling above pH 12.5. The derivatization is fast; prolonged reaction times had no effect on the reaction yield.

3.2. Specificity

Alendronate adsorption is reported to be a major problem [1–5]. Various additives have been used to avoid this phenomenon, including citrate buffer. We used a combination of a structurally similar pyrophosphate anion and chelating citrate ions in the mobile phase. The gradient step was incorporated to speed up the elution of late derivatization product peaks, resulting in a total run time of 25 min.

The method selectivity was demonstrated on four blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks. Alendronate and the internal standard were well resolved, with retention times of 7.5 and 6.4 min, respectively. A typical chromatogram of blank plasma and the chromatogram of a plasma sample are shown in Fig. 2. The alendronate concentration was 44.26 ng/ml.

3.3. Linearity and limit of quantitation

The calibration curves were linear in the range studied. The calibration curve equation took the form $y = bx + c$, where y is the ratio of the alendronate to pamidronate peak areas and x the concentration of the alendronate. The mean equation (curve coefficients ± standard deviation) for the calibration curve ($n = 6$), obtained from eight points, was $y = 0.0099(\pm 0.0004)x - 0.0092(\pm 0.0052)$ (correlation coefficient, $r = 0.9995$). The limit of quantitation was 1 ng/ml ($n = 6$). This level was selected with respect to the expected concentrations of the samples from the pharmacokinetic study. The precision, characterized by the relative standard deviation (R.S.D.), was 14.66% and the accuracy, defined

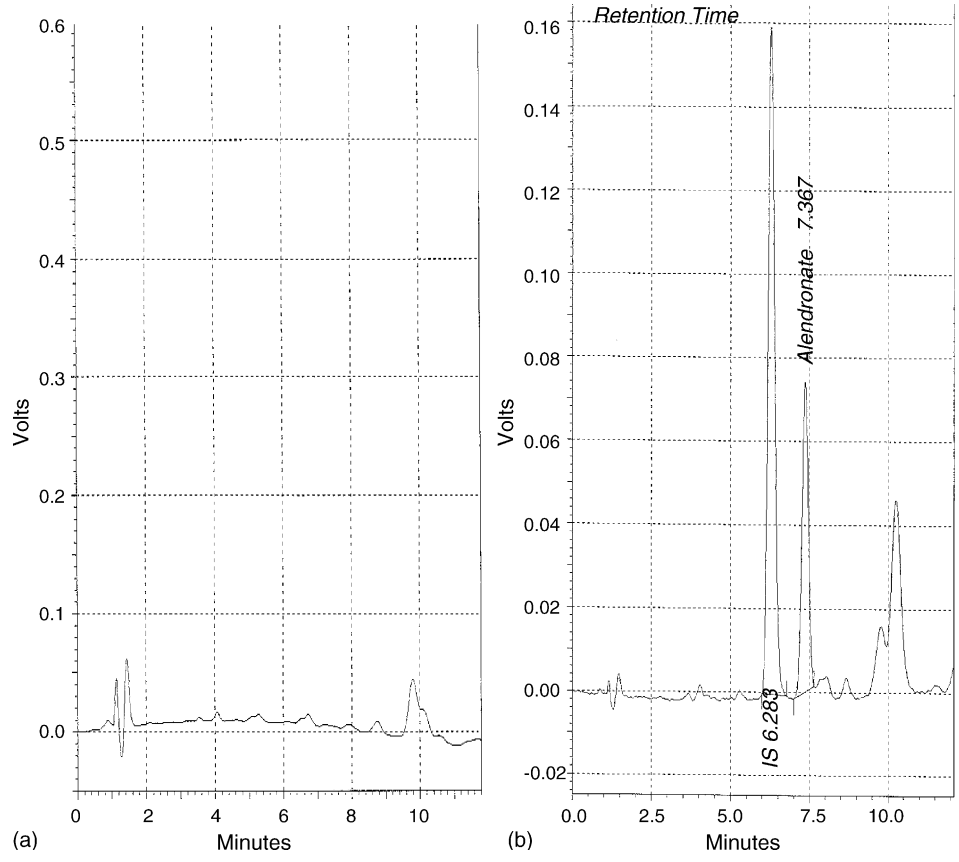

Fig. 2. (a) Typical chromatogram of drug-free human plasma. (b) Chromatogram of the plasma sample collected from volunteer no. 1 1.5 h after administering 70 mg of alendronate. The measured alendronate concentration was 44.26 ng/ml.

as the deviation between the true and measured values, expressed as a percentage, was 8.89% at this concentration ($n = 6$).

3.3.1. Intra-assay accuracy and precision

The intra-assay accuracy and precision of the method are illustrated in Table 1. The precision of the assay was measured by the percent coefficient of variation over the concentration range of LOQ, low (*L*), medium (*M*1, *M*2) and high (*H*) quality control samples six times in the same analytical run. The accuracy of the assay was defined as the absolute value of the ratio of the back calculated mean values of the quality control samples to their respective nominal values, expressed as percentage. The accuracy and precision did not exceed 9% at any level.

3.3.2. Inter-assay accuracy and precision

The inter-assay accuracy was evaluated by processing a set of quality control samples (low (*L*), medium (*M*1, *M*2) and high (*H*) four levels analyzed) in five separate runs. The samples were prepared in advance and stored at −20 °C. The respective data are given in Table 1. The accuracy did not exceed 15% at any level.

3.4. Application to pharmacokinetic study

Based on our validation parameters, we used our method to determine the plasma concentrations of alendronate in an open, balanced, randomized, pharmacokinetic study of four healthy volunteers to assess the bioavailability of 70 mg of alendronate sodium (Posamax 70 mg®) after a single

Table 1
Intra-day accuracy and precision and inter-day precision for alendronate sodium analyzed using our HPLC–FD method

Plasma alendronate concentration (ng/ml)	Intra-day ($n = 5$)[a]		Inter-day ($n = 5$)[a]	
	Accuracy	Precision	Accuracy	Precision
1	4.86	1.04	6.15	14.66
2	8.89	1.98	9.56	9.37
50	7.75	7.61	8.35	5.59
100	3.81	3.78	3.92	1.95

[a] R.S.D. (%): relative standard deviation.

Table 2
Pharmacokinetic parameters of alendronate after oral administration of a 70 mg capsule

	Alendronate (mean ± standard deviation, $n = 4$)
$AUC_{0-7\,h}$ (ng/ml/h)	118.55 ± 27.55
AUC_{inf} (ng/ml/h)	129.37 ± 25.67
C_{max} (ng/ml)	40.94 ± 19.60
T_{max} (h)	1.00 ± 0.41
CL (L/h)	0.56 ± 0.11
V_d (L)	1.38 ± 0.65
$t_{1/2}$ (h)	1.67 ± 0.50

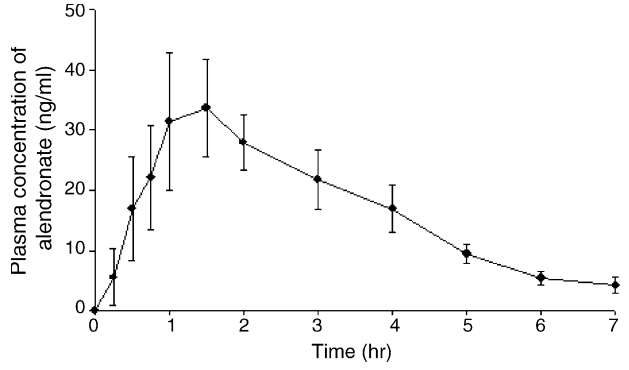

Fig. 3. The mean plasma alendronate concentration–time profiles of healthy subjects following oral administration of 70 mg alendronate. Vertical bars represent the standard deviation ($n = 4$).

oral dose. The limit of quantification of alendronate sodium allowed the plasma concentration to be followed for up to 7 h after drug administration. The pharmacokinetic parameters were calculated using WinNonLin 2.1 Pro software (Pharsight Inc.).

The obtained parameters are given in Table 2. Fig. 3 shows the mean and S.D. of the plasma concentration–time curve following oral administration of 70 mg of alendronate sodium.

4. Conclusion

We have described an analytical method for determining alendronate in plasma. We also validated the quality of the results. The method was demonstrated to be highly feasible and reproducible. The applicability of this method was evaluated in the analysis of unknown samples from volunteers in a pharmacokinetic study.

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