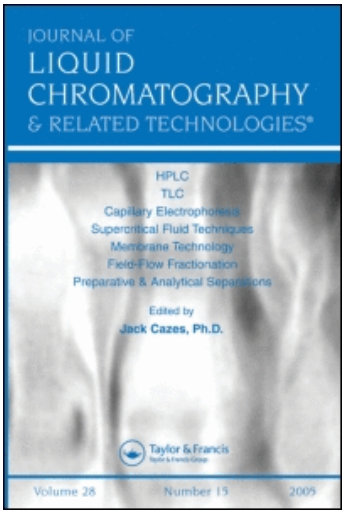

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RP-HPLC ANALYSIS OF HYDROPHOBIC ALENDRONATE AMIDATED DERIVATIVES

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RP-HPLC ANALYSIS OF HYDROPHOBIC ALENDRONATE AMIDATED DERIVATIVES

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□ *The reversed phase high performance liquid chromatography (RP-HPLC) was evaluated for its application in the analysis of the quantiation of the alendronate derivatives. The anti-phase Agilent CLC-ODS C_{18} column was used at the temperature of 50°C. The RP-HPLC trace was monitored with a differential refractive index detector for the lack of ultraviolet (UV) absorbing groups in the derivative molecule. The mobile phase was an aqueous solution mixture of MeOH and H_2O at pH 7.2, containing small amounts of dodecoulde tributyl bromide and disodium ethylente diamine tetraacetic acid. The chromatographic conditions, such as the concentration of ion-pairing agent, pH, and methanol content in mobile phase, were studied. This RP-HPLC method provides high separation and specificity to derivative analysis. In quantitative analysis, the method showed satisfactory precision (less than 0.62%) and recovery (higher than 98.3%), good linearity ($r^2 = 0.9994$) with the linear range of $0.5 \sim 250\,\mu g \cdot mL^{-1}$. The limit of quantification ($0.5\,\mu g \cdot mL^{-1}$) and limit of detection ($0.2\,\mu g \cdot mL^{-1}$) were also achieved.*

Keywords alendronate sodium, amidate derivatives, bisphosphate, differential refractive index detector, hydrophobic, RP-HPLC

INTRODUCTION

Bisphosphonates (BPs) had a similar chemical structure to that of inorganic pyrophosphate (PPi), an endogenous regulator of bone mineralization.[1,2] From a chemical point of view, BPs can be categorized as the non-N-containing BPs and N-containing BPs. The research showed that the N-containing BPs had higher pharmacological functions than those of non-N-containing ones,[3] and their pharmacological functions were determined by the group of lateral chain R_2, including its chemical

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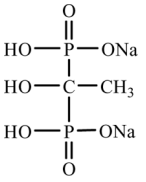
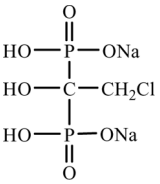
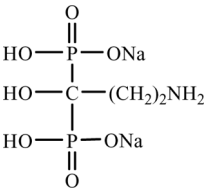
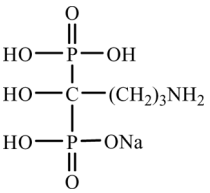
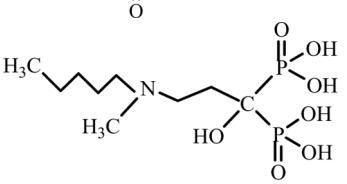
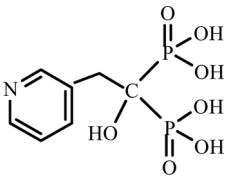
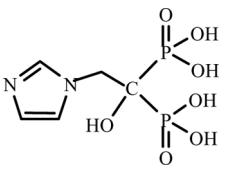
TABLE 1 The Chemical Structure and Relative Potency of Bisphosphonates

	BPs	Chemical Structure	R_2 Structural Feature	Relative Potency[a]
Non-N-containing BPs	Etidronate		$R_2 = CH_3$	1
	Clodronate		Introduced chlorine into R_2 chain	10
N-containing BPs	Pamidronate		Introduced nitrogen into R_2 chain	100
	Alendronate		Increased the length of R_2 chain	500
	Ibandronate		R-NH_2 methylation, from primary amine to secondary amine	1,000
	Risedronate		Nitrogen in the ring	2,000
	Zoledronate		Two nitrogens in one ring	10,000

[a]Potency relative to that of etidronate.

structure and the chain's length. The chemical structure and the potency of bisphosphates relative to that of etidronate were showed in Table 1.[4,5]

Many separation analytical methods[6–8] to dedicate the analysis of bisphosphonates were well established with extensive application in bioanalysis and pharmaceutical quality control, especially alendronate, which was approved by the Food and Drug Administrate (FDA). Liquid chromatography (LC),[9] including reversed phase liquid chromatography (RP-HPLC),[10] ion-pair HPLC,[11] and ion chromatography (IC),[12] offer reliable methods characterized by sensitivity, ruggedness, and accuracy[13] in determining the quantitation of alendronate. For the lack of ultraviolet (UV) absorbing groups in bisphosphonate, pre-[14] or post-column[15] derivatization reactions were employed in order to enhance both chromatographic separation and the detection limits. For example, the precolumn derivatizations of alendronate with the 9-fluorenylmethyl derivative (FMOC)[16] to determine the quantitation of alendronate in human urine were described. FMOC could react with amines and the resulting derivatives were determined using the fluorimetric detector, which was operated at wavelengths of 260 nm (excitation) and 310 nm (emission), so this analysis could not applied for the bisphosphate which had no amine groups.

In our previous work, we synthesized the alendronate amidate derivatives (ALN-Cn, n was the carbon number introduced) through introducing a long alkyl into its lateral chain R_2 in order to increase the lipophilicity of alendronate.[17] In this paper, we established the reversed phase high performance liquid chromatography (RP-HPLC) method (with ion pairing agent added) to determine the quantitation of these derivatives.

EXPERIMENTAL

Chemicals and Solutions

Alendronate (4-Amino-1-hydroxybuty-lidene)bis-phosphonate) sodium bisphosphate, was supplied by Shenyang Dongrui Technology. The alendronate amidate derivative was prepared in our lab with the purity of 90% and its chemical structure was shown in Figure 1. The ammonium dihydrogen phosphate, dodecoulde tributyl bromide, disodium ethylente diamine tetraacetic acid were all supplied by Xi'an Chemical Agent Company, China. Methanol used was of HPLC grade and purchased from Sigma Corp. The distilled water was prepared from deionized water by distilling it twice. Other agents were used without further treatment.

Chromatographic Conditions

The reversed phase high performance liquid chromatography (RP-HPLC) was performed using Water ALLIANCE 2695 series HPLC

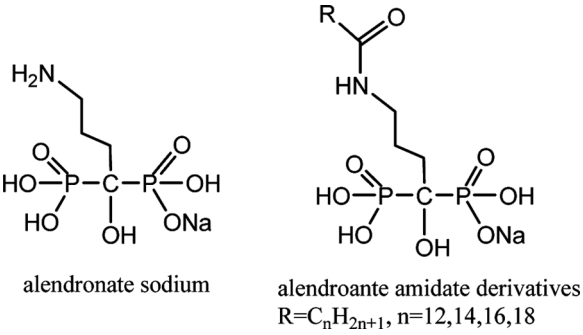


FIGURE 1 The chemical structure of alendronate and its amidate derivative.

system. The anti-phase Agilent CLC-ODS C_{18} (4.6 mm × 250 mm, 5 μm, Agilent Technologies Inc., California, USA) column was used at the temperature of 50°C. The mobile phase was an aqueous solution mixture of MeOH and H_2O at pH7.2, which contain 5 mmol · L ammonium dihydrogen phosphate, 2 mmol · L dodecoulde tributyl bromination, and 1.5 mmol · L disodium ethylente diamine tetraacetic acid. The flow rate was 1.0 mL · min^{-1} and 20 uL of samples were injected. The RP-HPLC trace was monitored with a differential refractive index detector (Water 2414). The chromatographic control system, data acquisition, and analysis were performed using Water Empower software.

Sample Preparation

Stock solutions of alendronate amidate derivative were prepared by dissolving the equivalent of 100 mg of derivative in 100 mL of mobile phase liquid. Then, different sets of working standards at different concentrations were prepared by appropriate dilution of the stock solution, obtaining the sample concentration with $0.1 \sim 250\,\mu g \cdot mL$. A portion of the resulting solution was filtered through a Millipore $0.22 \sim$ pm tilter unit and transferred to an HPLC vial for analysis. All solutions were stored at 4°C and protected from light. All the samples must be degassed before being injected into the HPLC system.

Evaluation of Method Performance

Standard Curve and Linear Range

Linear relationship was assessed by putting the derivative samples about 1000 μg into the volumetric flasks with different volumes, adding mobile phase liquid to the bottle scale, and obtaining the solutions with the concentrations between $0.5 \sim 250\,\mu g \cdot mL^{-1}$. Of the above solutions, 20 μL

with eight different concentrations between $0.5 \sim 250\,\mu g \cdot mL^{-1}$ were injected into the HPLC. Each concentration was carried out in triplicate during the HPLC procedure. The peak area ratios were obtained from HPLC chromatography, which were plotted against the corresponding concentrations of the derivative and the calibration curves set up by means of the least square method. The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated as the derivative concentrations, which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

Robustness

The experimental conditions, such as mobile phase pH (±0.5 pH units), mobile phase composition (±5% to the methanol percentage) and flow rate ($\pm 0.1\,mL \cdot min^{-1}$), were varied to test their influence on the method performance.

Recovery

The recovery was assessed by injecting the derivative samples of $1.0 \sim 50\,\mu g \cdot mL^{-1}$ for six times during the same day to obtain average recovery and R.S.D. data.

Precision

The precision was assessed by continuously injecting the samples of the derivative 6 times within the same day to obtain intraday precision, and 6 times over 6 different days to obtain interday precision, both expressed as R.S.D.% values.

Stability

The samples were thawed at room temperature and kept at this temperature for 8 h, then analyzed.

RESULTS AND DISCUSSION

Chromatographic Conditions

There were more hydrophilic groups in alendronate than that in its amidate derivative molecule (the structure showed in Figure 1), so the derivatives would have the longer retention time that of alendronate in the anti-phase column. Furthermore, the two phosphate radicals would dissociation and formed free state and dissociation state in aqueous solution, so the some ion pairs must be added into the anti-phase column and the ion pairs agents offered cation could be used. During our experiment, we choose dodecoulde tributyl bromination (DDTB) as the ion-pairing

agent and added small amounts of disodium ethylene diamine tetraacetic acid (DEDTA) in mobile phase in order to cut down the complexation of derivatives with trace quantity of metal ion in stable phase. Moreover, there were no violet or fluorescent groups in derivatives molecule, so the differential refractive index detector was applied.

Concentration of Ion-Pairing Agent DDTB

We discussed the influence of the concentration of DDTB in the retention behavior of derivatives. The detailed chromatography were shown in Figure 2. When the concentration of DDTB was about 1 mmol $\cdot$ L^{-1} (Figure 2a) and the sample injection was 20 μL per 10 minutes, we could see that the chromatography was unstable, the decreasing peak area and the peak's appearance time, which indicated the low concentration of ion-pairing agent. When the concentration of DDTB was about 2 mmol $\cdot$ L^{-1} (Figure 2b), the

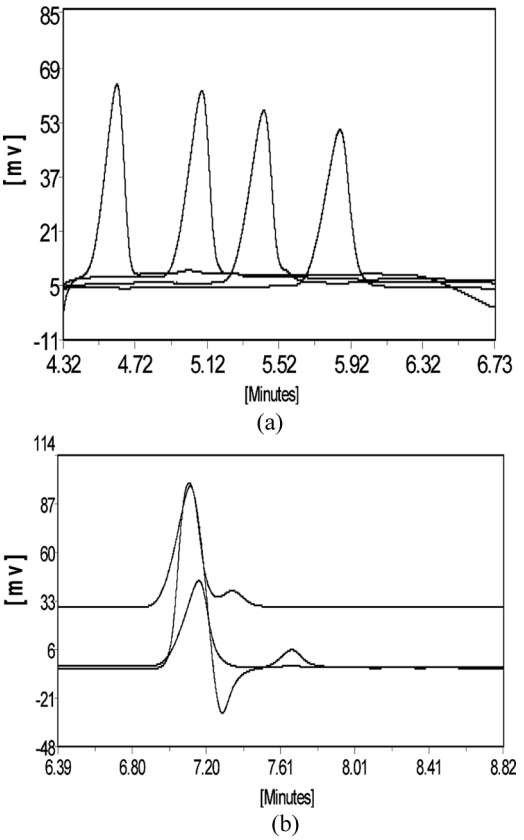


FIGURE 2 The effect of ion-pairing agent concentration on the appearance time.

chromatographic shape and retention time was stable and the chromatograph had the good peak overlapping per sample injection. The shape did not change with DDTB concentration increasing as high as $6\,mmol \cdot L^{-1}$, which suggested the saturation of the ion-pairing agent with the derivatives. So we chose DDTB concentration of $2\,mmol \cdot L^{-1}$.

Effect of pH on the Retention Time

The retention time of the derivative was reflected by the capacity factor. The higher the capacity factor, the longer the retention time. The effect of pH on the capacity factor of derivatives was shown in Figure 3. The capacity factor was increased with the pH increasing of mobile phase, which indicated the derivative would act as an anion and had the strong retention in the reserved column at the high pH value of mobile phase. The anionic derivatives would form neutrality ion pairs with the cation in DDTB, and eventually had the improved retention in the C_{-18} column. Furthermore, the high pH would be favorable to the ionization of derivatives, so we chose the pH of mobile phase keeping at 7.2.

Methanol Content in Mobile Phase

The effect of methanol content in the retention time of derivatives was shown in Figure 4. The retention time was about 1.5 when the methanol content was 5%, the appearance time and the peak shape were not stable. The longer retention time of derivative in the reserve column would result in the longer time consumed during the HPLC processing, which was not acceptable. With the methanol content increasing the polarity of mobile phase decreased, so the retention time of the derivative decreased. When the methanol content increased to 40%, the capacity factor was about 0.1, which indicated the low retention time of derivative in the column,

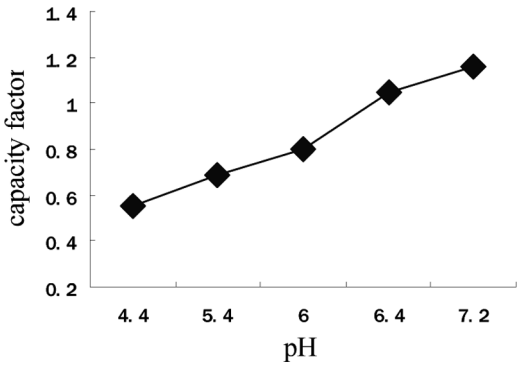


FIGURE 3 The effect of pH on the capacity factor.

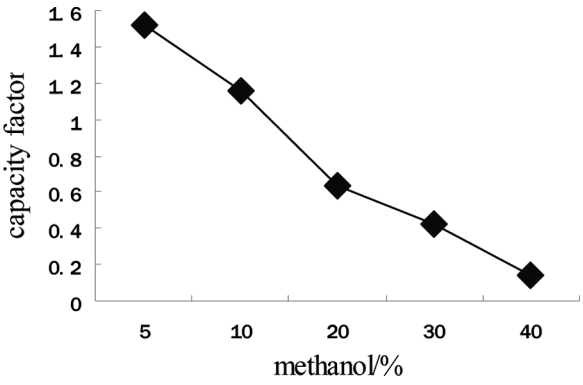


FIGURE 4 The effect of methanol content on retention time.

which was not advantageous to HPLC processing. So we chose the methanol content of about 10%.

RP-HPLC Analysis of Derivatives

Results of Chromatograms

Figure 5 was the HPLC chromatogram of alendronate sodium, fatty acid, and ALN-C_{12} derivative. The peak in the chromatogram was alendronate sodium (1), ALN-C_{12} derivative (2), and fatty acid (3). The appearance time of alendronate sodium was 6.08 min, while it was 7.12 min for the

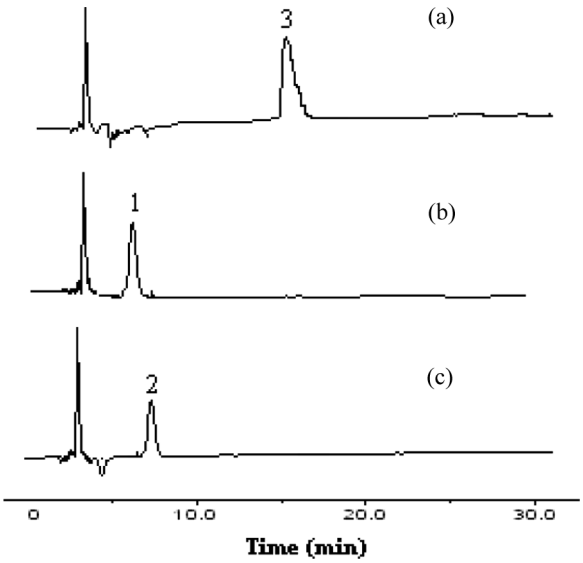


FIGURE 5 HPLC chromatogram of alendronate sodium, fatty acid and derivatives.

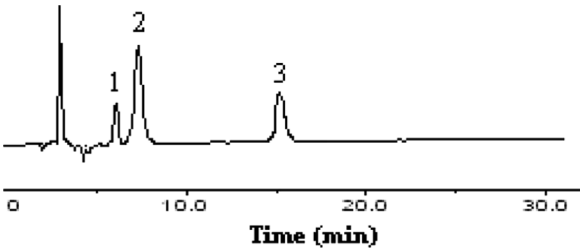


FIGURE 6 Chromatogram of three different materials at the same HPLC condition.

ALN-C_{12} derivative, which was later than the time of alendronate sodium. The late appearance time in the anti-phase chromato-spectrum indicated the lower polarity of derivative than that of alendronate sodium. In order to investigate the HPLC system suitability of alendronate amide derivatives, we used the mixture of alendraonate(1), deriveative (2), and fatty acid(3) and injected this mixture of the sample under the same HPLC condition as above described.

Figure 6 was the HPLC chromatograph result and it suggested that the three different materials could be well separated under the same RP-HPLC condition.

Figure 7 was the HPLC chromatogram of three derivatives with different alkyls introduced (ALN-C_{12}, ALN-C_{14}, ALN-C_{16}). The appearance times

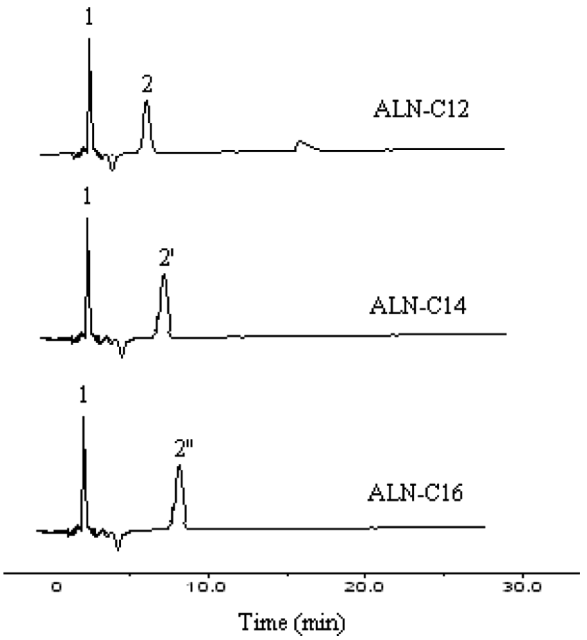


FIGURE 7 HPLC chromatogram of BPs derivatives with different alkyl introduced.

for ALN-C_{12}, ALN-C_{14}, and ALN-C_{16} were 7.12 min, 7.42 min, and 8.79 min, respectively. The appearance time suggested the different polarity of the derivatives with different alkyls introduced. The longer the alkyl chain was introduced the weaker of its polarity. ALN-C_{16} was the derivative with the longest alkyl chain (containing 16 carbon atoms) introduced, so it had the weakest polarity among the three derivatives.

The Relationship of Retention Time with Alkyl Chain Length

The retention time of derivatives in the reversed phase column related to the alkyl chain length which was introduced into the molecules. The longer the alkyl chain introduced, the weaker the polarity and the longer the retention time. The carbon number in the alkyl chain was plotted against the retention time and the calibration curves set up by means of the least square method, and the result was shown in Figure 8.

Based on the above result, we could establish a semiempirical formula, the retention time t of derivatives in the reverse phase column had the relationship with the carbon number N introduced in the molecule:

$$t = 0.415N + 1.965$$

The formula showed that the derivative's retention time was in line with the carbon number introduced into the molecule. The retention time in the reverse phase column could reflect the polarity of the derivative. The more the carbon number was introduced, the longer of the alkyl chain, and as a result the more hydrophilic of the derivatives.

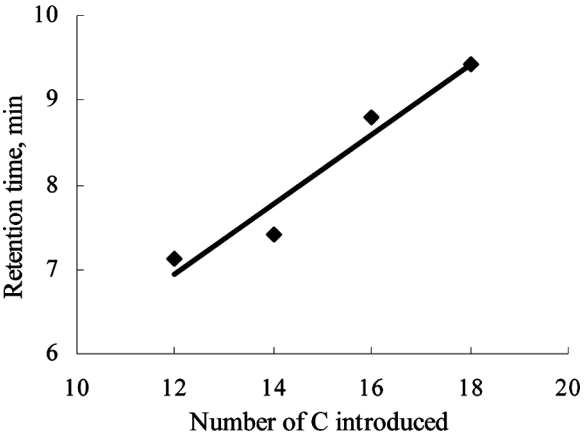


FIGURE 8 Relationship of retention time and number of C introduced.

Evaluation of Method Performance

Standard Curve and Linear Range

Eight point calibration curves were set up over $0.5 \sim 250\,\mu g \cdot mL^{-1}$ range. The peak area ratios A were plotted against the corresponding concentrations C ($\mu g \cdot mL^{-1}$) and the standard curve was obtained. The regression equation and a linear response function were obtained as follows:

$$A = 1.8608C + 4.5666 \quad r^2 = 0.9994$$

This result showed that the method had good linear range within $0.5 \sim 250\,\mu g \cdot mL^{-1}$ and the values of LOQ and LOD were about $0.5\,\mu g \cdot mL^{-1}$ and $0.2\,\mu g \cdot mL^{-1}$, respectively.

Robustness

Three chromatographic parameters, such as mobile phase pH, methanol content, and flow rate, were varied in order to assess their influence on the analysis. The results showed that the change of pH had almost no significant influence on resolution and applicability of the method and only slightly longer retention time (increasing pH) and a shorter one (decreasing pH) were observed. The methanol content, which changed between $5 \sim 15\%$ also had no significant effect on the method performance, but the high increase of methanol content, for example 30%, would cause a reduction of retention time, which was not suitable for the method processing. Changes to the flow rate (by $\pm 0.1\,mL \cdot min^{-1}$) caused inverse changes in run times, without generating problems with respect to resolution, efficiency, or peak shape.

Recovery

The recovery was assessed by injecting the derivative samples of $1.0 \sim 50\,\mu g \cdot mL^{-1}$ for 6 times and the results are shown in Table 2. The average recovery was about 98.3% and R. S. D. was about 0.4% ($n = 6$).

TABLE 2 Recovery Results of Alendronate Amidate Derivatives

Added Amount (μg)	Measured Amount (μg)	Recovery (%)	Average Recovery (%)	R.S.D. (%)
50	48.75	97.5	98.3	0.4
37.6	37.07	98.6		
30.1	29.62	98.4		
24.9	24.50	98.4		
20.1	19.84	98.7		
9.9	9.72	98.2		

Precision

The precision was assessed by continuously injecting the samples of $1.5\,\mu g \cdot mL^{-1}$ derivatives solution for 6 times, recording the chromatograms, and determining R.S.D. for intraday precision and interday precision, which was about 0.35% and 0.62%, respectively.

Stability

Stability was an indication of analytical method reliability. It could be determined by obtaining the chromatograms of $1.5\,\mu g \cdot mL^{-1}$ derivatives samples at different time of 0, 1, 2, 4, 6, and 8 h. The result shows that the R.S.D. was about 0.62%, which indicates the stability of the methods within 8 h.

CONCLUSIONS

The reversed phase high performance liquid chromatography (RP-HPLC) was evaluated for its application in analyzing the quantiation of the alendronate amidate derivatives, which had the longer alkyl chain introduced to its lateral chain R_2 through the amide bond. The chromatographic conditions, such as the concentration of ion pair agent, pH, and the methanol content in mobile phase, were investigated and the chromatographic conditions were determined as: the ion pairing agent concentration $2\,mmol \cdot L^{-1}$, pH 7.2, and MeOH content 10%. The chromatogram results show that the derivatives have a later appearance time than that of alendronate, which indicates the lower polarity of the derivative in the anti-phase chromatospectrum. The retention time of derivatives in the reversed phase column was related to the alkyl chain length introduced into the molecules. The longer the alkyl chain introduced, the weaker the polarity and the longer the retention time. Standard curve and linear range, robustness, precision, recovery, and stability were assessed to evaluate the RP-HPLC method performance. The results showed that the method had good linear range within $0.5 \sim 250\,\mu g \cdot mL^{-1}$ and the values of LOQ and LOD were about $0.5\,\mu g \cdot mL^{-1}$ and $0.2\,\mu g \cdot mL^{-1}$, respectively. The results of the precision showed R.S.D for intraday and interday precision was about 0.35% and 0.62%, respectively. The average recovery was about 98.3% and the method had the stability within the experiment hours. All of the above results showed that this RP-HPLC method was suitable to analysis alendronate amidate derivative products.

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