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Gas chromatographic analysis of 3-amino-1-hydroxypropylidene-1,1-bisphosphonate and related bisphosphonate as their N-isobutoxycarbonyl methyl ester derivatives

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Abstract

A selective and sensitive method for the determination of 3-amino-1-hydroxypropylidene-1,1-bisphosphonate and related bisphosphonate by gas chromatography (GC) has been developed. These compounds were converted into their N-isobutoxycarbonyl methyl ester derivatives and measured by GC with flame photometric detection (FPD) using a HP-1 capillary column. The derivative preparation and GC analysis were accomplished within 30 min. The derivatives were sufficiently volatile and stable, gave single and symmetrical peaks, and provided excellent FPD responses. The detection limits of these bisphosphonates, at a signal-to-noise ratio of 3, were ca. 100 pg injected, and the calibration curves for these compounds in the range 20–1000 ng were linear and sufficiently reproducible for quantitative determination. This method could be applied to the analysis of plasma sample.

Keywords: Derivatization, GC; Bisphosphonate; 3-Amino-1-hydroxypropylidene-1,1-bisphosphonate

1. Introduction

3-Amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPrBP) and 6-amino-1-hydroxyhexylidene-1,1-bisphosphonate (AHHxBP) are second generation bisphosphonates that inhibit bone resorption in vivo. These compounds are used in the treatment of patients with bone diseases, such as Paget's disease and hypercalcemia of malignancy and osteoporosis [1–3].

These bisphosphonates are highly water-soluble and have plural ionizable groups. But it is necessary to convert these compounds into suitable derivatives before and after chromatography, because these compounds do not have ultraviolet absorption or fluorescence and are not readily detectable. In general, the determination of these bisphosphonates has been carried out by high-performance liquid chromatography (HPLC) based on the pre-column derivatization with fluorescamine [4,5] and 2,3-naphthalene dicarboxyaldehyde-cyanide reagent [6] followed by fluorescence detection. Furthermore, a HPLC method based on the post-column oxidation of bisphosphonate to orthophosphate has been reported [7]. On the other hand, these bisphosphonates cannot be analysed readily with gas chromatography (GC) because of their low volatility and polar nature. For GC analysis of aminoalkylphosphonates, per-tri-

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methylsilyl (TMS) [8–12], N-acetyl TMS ester [9], acetone Schiff-base TMS [9], isothiocyanate TMS [9], *tert*-butyldimethylsilyl [13,14] and N-acyl alkyl ester [15–21] derivatives have been used, but these methods require a time-consuming procedure under anhydrous conditions.

Recently, we have developed a convenient and reliable method for the determination of amino-alkylphosphonates by GC with flame photometric detection (FPD), in which these compounds were analysed as their N-isobutoxycarbonyl (isoBOC) methyl ester derivatives [22–24]. But this technique is not yet applied to the determination of bisphosphonate compounds. In this paper, we investigated a selective and sensitive method for the determination of AHPrBP and AHHxBP by the use of this FPD–GC technique.

2. Experimental

2.1. Reagents

AHPrBP disodium pentahydrate ($C_3H_9NO_7P_2Na_2 \cdot 5H_2O$, M_r 369.11), AHHxBP ($C_6H_{17}NO_7P_2$, M_r 277.15) and 5-amino-1-hydroxy-pentilidene-1,1-bisphosphonate (AHPeBP, $C_5H_{15}NO_7P_2$, M_r 263.12) as an internal standard (I.S.) were supplied from Ciba-Geigy Japan (Takarazuka, Japan). Each compound was dissolved in distilled water at a concentration of 1 μg/ml and stored at 4°C. Triphenyl phosphine (TPP, Tokyo Kasei Kogyo, Tokyo, Japan) was dissolved in ethyl acetate at 2 μg/ml and used as an IS to examine optimum derivatization conditions. Isobutylchloroformate (iso-BCF) was obtained from Tokyo Kasei Kogyo and used without further purification. N-Methyl-N-nitroso-*p*-toluenesulphonamide and diethyleneglycol mono-methyl ether for the generation of diazomethane [25] were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical-reagent grade.

2.2. Derivatization procedure

An aliquot of the sample solution containing 20–1000 ng of AHPrBP and AHHxBP was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. After addition of 0.2 ml of 1 μg/ml AHPeBP (I.S.) solution, the solution was adjusted to pH>10 with 2 *M* sodium hydroxide solution and the total reaction volume was made up to 1 ml with distilled water, if necessary. Immediately after addition of 0.05 ml of isoBCF, the mixture was shaken by hand for 10–30 s at room temperature. The reaction mixture was extracted with 3 ml of diethyl ether in order to remove the excess reagent, the ethereal extracts being discarded. The aqueous layer was acidified to pH 1–2 with 2 *M* hydrochloric acid and saturated with sodium chloride, and then extracted with 3 ml of diethyl ether containing 30% isopropanol. To the ethereal extracts, 6 ml of diethyl ether was added and the solution was methylated by bubbling diazomethane, which had been generated according to the micro-scale procedure of Schlenk and Gellerman [25], through this solution until a yellow tinge became visible. After standing at room temperature for more than 5 min, the solvents were evaporated to dryness at 80°C under a stream of dry air. The residue was dissolved in 0.05–0.1 ml of ethyl acetate, and 1 μl of this solution was injected into the FPD–GC system. The derivatization process is summarized in Fig. 1.

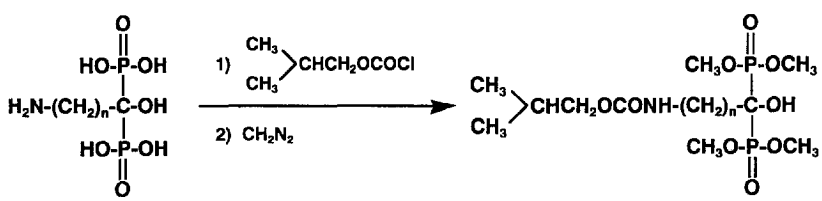

Fig. 1. Derivatization process of bisphosphonates containing an amino group.

2.3. Gas chromatography

GC analysis was carried out with a Shimadzu 14A gas chromatograph equipped with a flame photometric detector (P-filter). A fused-silica capillary column (5 m × 0.53 mm I.D., 2.65-μm film thickness) of cross-linked HP-1 (Hewlett-Packard, Avondale, PA, USA) was used. The operating conditions were as follows: column temperature, programmed from 180°C to 280°C at 10°C/min; injection and detector temperature, 290°C; nitrogen flow-rate, 10 ml/min. The peak heights of AHPrBP, AHHxBP and AHPeBP (I.S.) were measured and the peak height ratios against the I.S. were calculated to construct a calibration curve.

2.4. Gas chromatography–mass spectrometry

A Hewlett-Packard Model 5890A gas chromatograph was operated in conjunction with a VG Analytical Model 70-SE mass spectrometer and a VG 11-250J mass data system. A fused-silica capillary column containing cross-linked OV-1 (Quadrex, New Haven, CT, USA, 12 m × 0.25 mm I.D., 0.25-μm film thickness) was used. Column temperature: programmed from 180 to 250°C at 5°C/min; injection temperature, 250°C; ion-source temperature, 250°C; ionizing voltage, 40 eV; helium flow-rate, 1 ml/min.

3. Results and discussion

AHPrBP and AHHxBP could be successfully converted into their N-isoBOC methyl ester derivatives by essentially the same procedure that was described in a previous report [22]. The derivatization process for these compounds is shown in Fig. 1. The optimum derivatization conditions were examined using 400 ng of standard and 200 ng of TPP (I.S.) which was added to residue after derivatization. As shown in Fig. 2, the isobutoxy-carbonylation of the amino function of these compounds was accomplished within 10 s in aqueous alkaline media using 0.05 ml of isoBCF by shaking at room temperature. Although N-isoBOC derivatives of AHPrBP and AHHxBP were not extracted into diethyl ether, the addition of isopropanol to diethyl ether was effective to the extraction of these derivatives. As shown in Fig. 3, the optimum concentration of isopropanol for the extraction of bisphosphonate was found to be 30%, although that for monophosphonates was in the range 5–15% [22]. The extraction yield of N-isoBOC AHPrBP by the extraction with 3 ml of diethyl ether containing 30% isopropanol was above 92%. Because the subsequent methylation with diazomethane was insufficient in this solvent, diethyl ether was added to the extract in order to dilute isopropanol. The methylation of the phosphonic acid group could be successfully carried

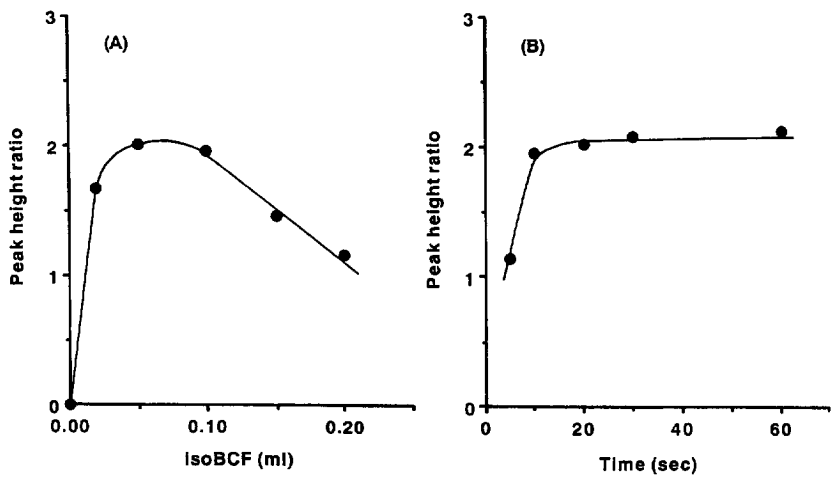

Fig. 2. Effects of (A) isoBCF amount and (B) reaction time on the isobutoxycarbonylation of AHPrBP.

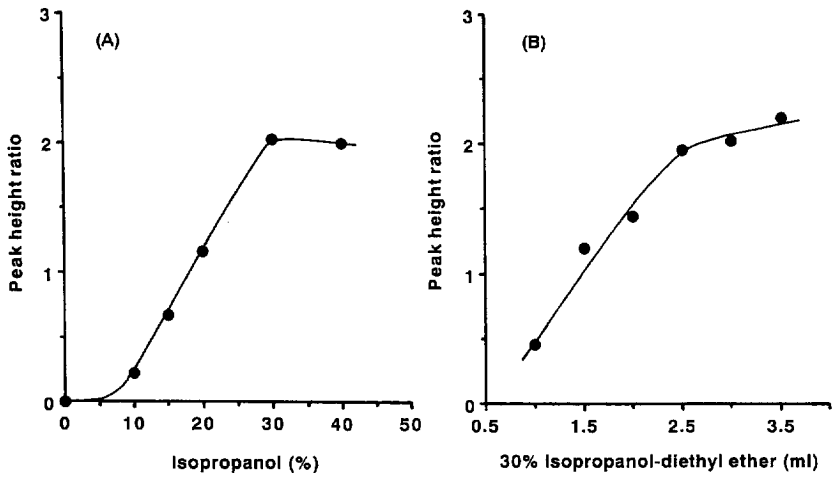

Fig. 3. Effects of (A) isopropanol concentration in diethyl ether and (B) solvent volume on the extraction of N-isoBOC AHPrBP.

out at an isopropanol concentration below 20%. The derivative preparation containing isobutoxycarbonylation, solvent extraction and methylation could be performed within 20 min.

The structures of the derivatives of AHPrBP and AHHxBP were confirmed by GC–MS analysis. As shown in Fig. 4, a molecular ion peak (M^+) was not observed, but the prominent fragment ion peaks at M^+-73 [$(CH_3)_2CHCH_2O$], m/z 261 {$CH_2C(OH)[PO(OCH_3)_2]$}, m/z 109 [$PO(OCH_3)_2$], and m/z 57 [$(CH_3)_2CHCH_2$] were observed and these peaks were useful for structure elucidation. These derivatives were stable under normal laboratory conditions, and no decomposition was observed during GC analysis.

As shown in Fig. 5A, each compound was completely separated within 8 min and provided an excellent FPD response. The minimum detectable

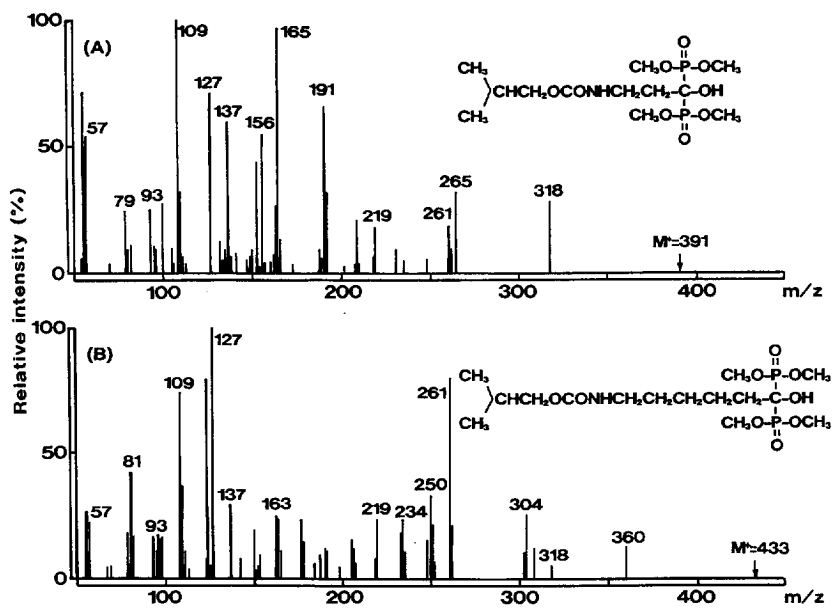

Fig. 4. Mass spectra of N-isobutoxycarbonyl methyl ester derivatives of (A) AHPrBP and (B) AHHxBP.

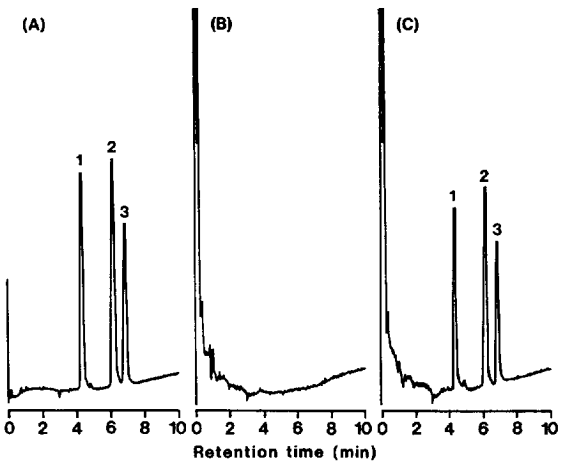

Fig. 5. Typical gas chromatograms obtained from (A) standard solution containing 100 ng of each compound, (B) non-spiked plasma (0.5 ml) and (C) plasma (0.5 ml) spiked at 200 ng/ml of each compound. Non-spiked or spiked plasma sample was deproteinized with 20% trichloroacetic acid (final concentration at 4%) and the protein-free supernatant was derivatized. GC conditions are given in Experimental . Peaks: 1=AHPrBP, 2= AHPeBP (I.S.), 3=AHHxBP.

amounts of AHPrBP and AHHxBP to give a signal three times the noise under our instrumental conditions were ca. 100 pg injected. AHPeBP, was chosen as an I.S. because it showed a similar behaviour to these bisphosphonates during the chemical reactions and was well separated from other bisphosphonates on a chromatogram. In order to test the linearity of the calibration curves, various amounts of these compounds ranging from 20 to 1000 ng were derivatized, and aliquots representing 0.2–10 ng of each compound were injected. In each instance, a linear relationship was obtained, and the relative standard deviation at each point ranged from 2.2–5.2% ($n=3$). The regression lines for AHPrBP and AHHxBP were $y=0.0084x+0.052$ ($r=0.9987$, $n=18$) and $y=0.0085x-0.173$ ($r=0.9985$, $n=18$), respectively, where y is the peak height ratio against the I.S. and x is the amount (ng) of each compound.

In order to confirm the applicability of this method to biological samples, bisphosphonates in a plasma sample was analysed after deproteinization with trichloroacetic acid. As shown in Fig. 5B and C, each compound could be analysed without any interference from other coexisting substances such as amino acids. The recoveries of AHPrBP and AHHxBP added to plasma samples were 96.8±7.2 and 101.3±8.2% ($n=3$), respectively.

4. Conclusion

A convenient and reliable method for the determination of bisphosphonates containing an amino group has been developed. By using this method, bisphosphonates in aqueous solution can be directly derivatized without evaporation of the sample. This method is rapid, selective and sensitive, and these bisphosphonates can be analysed accurately and precisely. Furthermore, a plasma sample can be quantitatively analysed without any interference from other coexisting substances. The detailed investigations on the application of this method to biological fluids such as plasma and urine are in progress.

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