ELSEVIER

Journal of Chromatography A, 686 (1994) 205–212

JOURNAL OF CHROMATOGRAPHY A

Pharmaceutical application of liquid chromatography–mass spectrometry
II☆. Ion chromatography–ion spray mass spectrometric characterization of alendronate

Xue-Zhi Qin[a,*], Eric W. Tsai[a], Takeo Sakuma[b], Dominic P. Ip[a]

[a]*Pharmaceutical Research and Development, Merck Research Laboratories, West Point, PA 19486, USA*
[b]*PE SCIEX, Toronto, Ontario, Canada*

First received 6 May 1994; revised manuscript received 18 August 1994

Abstract

The trihydrate of alendronate sodium (MK-0217) is an important bisphosphonate drug for the treatment of a variety of bone diseases. Determination and characterization of this compound in dosage formulations is challenging since it has no chromophore, and as a highly polar and thermally labile compound, it is not amenable to electron impact mass spectrometry. Ion chromatography coupled with an ion spray mass detector (IC–ISP-MS) in the negative ionization mode was developed and applied to the characterization of this compound. Under these conditions alendronate (m/z 248, $[M - H]^-$, M = parent alendronic acid) was readily observed. The anion can form cluster anions with acid molecules including that of the alendronic acid in the gas phase, which is a distinguishing feature of the IC–ISP-MS spectrum. IC–ISP-MS–MS study of the anion shows that cleavage of the C–P bond(s) is the dominant fragmentation pathway(s) of the anion, characteristic of its structure.

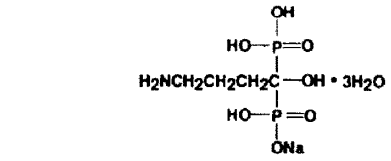
1. Introduction

The monosodium salt trihydrate of 4-amino-1-hydroxybutane-1,1-bisphosphonic acid, or trihydrate of alendronate sodium (MK-0217, **1**, Fig. 1) is an important bisphosphonate drug, which possesses therapeutic indications in the treatment of many bone diseases such as hypercalcemia of malignancy, osteoporosis and Paget's disease [2,3]. The compound has been characterized by various techniques such as the infrared transmittance spectrum (IR), magnetic resonance spectrum (NMR) and X-ray powder diffraction (XRPD) methods [4]. But, as a highly polar and thermally labile compound, it is not amenable to electron impact (EI) mass spectrometry [4]. In this paper, we report characterization of **1** by ion chromatography–ion spray (pneumatically assisted electrospray [5]) mass spectrometry (IC–ISP-MS) in the negative ionization mode.

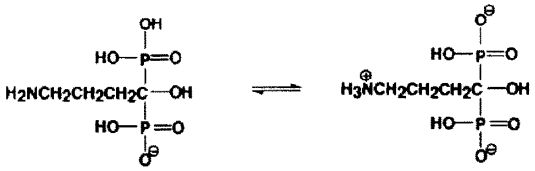
Determination and characterization of **1** in dosage formulations is challenging since it has no chromophore for UV or fluorescence detection. The determination of **1** and other bisphosphonate drugs has mainly been accomplished by reversed-phase HPLC–UV methods by intro-

* Corresponding author.
☆ For Part I, see Ref. [1].

0021-9673/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(94)00798-5



1 MK-0217



2 Alendronate 3

Fig. 1. Structures of MK-0217 and alendronate.

ducing a chromophore into their molecules using either pre- or post-column derivatization. Recently, analytical methods for determining bisphosphonate drugs without derivatization have attracted considerable attention. Examples include the development of an ion-exchange method with on-line flame photometric detection for dichloromethylene diphosphonate [6], an inductively coupled plasma (ICP) detector for specific phosphorus detection for etidronate disodium [7], an IC method with conductivity detection for alendronate [8] and an IC–indirect UV method for the direct quantitation of some bisphosphonate drugs in dosage formulations [9,10]. Mass detectors can detect compounds without a chromophore, and have been coupled with gas chromatography (GC) for the analysis of dichloromethylene bisphosphonate in urine [11], which required time-consuming extraction and derivatization. IC coupled with an ion spray mass detector, reported here, provides a convenient and specific method for the direct detection of **1** in the dosage formulations. The IC–ISP-MS method may also be useful for the study of other bisphosphonate compounds as the IC–indirect UV method [9,10].

The development of the IC–ISP-MS method should be of interest in the field of IC–MS interfaces. Especially, the IC–ISP-MS method avoided the use of post-micromembrane suppressors commonly used in the IC–thermal spray (TSP) MS [12] and in an IC–ISP-MS study of organic ammonium and sulfate compounds [13].

2. Experimental

2.1. Chemicals

Trihydrate of alendronate sodium (**1**, MK-0217, $C_4H_{12}NO_7P_2Na \cdot 3H_2O$, M_r 325.1) was manufactured by Merck Research Labs. (Rahway, NJ, USA). Acetonitrile (Optima grade) and nitric acid (Optima grade) were purchased from Fisher Scientific (Philadelphia, PA, USA). Formic acid (certified ACS grade) was purchased from Aldrich (Milwaukee, WI, USA). They were used as received, without further purification. Deionized water with at least 18 MΩ purified by a Milli-Q (Bedford, MA, USA) system was used for mobile phase and sample preparations.

2.2. Sample preparation

Standard was prepared by dissolving 32.6 mg (equivalent to 25 mg free acid) of MK-0217 reference standard in 500 ml of water to yield a concentration of 0.05 mg/ml. Samples of both initial and stressed tablets [stressed at 40°C and 75% relative humidity (RH) in an open container for 2 months], at a concentration of 0.05 mg/ml, were prepared by dissolving one 5 mg MK-0217 tablet in 100 ml of deionized water.

2.3. IC–indirect UV conditions

IC–indirect UV was performed using a Dionex 4500i Bio-LC. The conditions were: a Waters IC-Pak HR anion-exchange column (75 × 4.6 mm, 6 μm particle size), ambient (about 26°C) column temperature, 1.6 m*M* HNO_3 aqueous mobile phase, flow-rate of 0.5 ml/min, injection volume of 25 μl and UV detection at 235 nm with "inverse polarity" and 0.1 AUFS (0.1 V output).

2.4. IC–ISP-MS and IC–ISP-MS–MS analysis

IC–ISP-MS and IC–ISP-MS–MS were performed on a PE Sciex (Thornhill, Canada) Model API III triple-quadruple mass spectrometer, fitted with an articulated, pneumatically assisted nebulization probe and an atmospheric pressure ionization source, which was connected to an LC system consisting of a Perkin-Elmer ISS-100 autoinjector equipped with a 200-μl loop.

The IC conditions used in IC–ISP-MS were modified from the IC–indirect UV method, which were: an Applied Biosystems AX-300 anion-exchange column (30 × 2 mm I.D., 5 μm particle size), ambient (about 26°C) column temperature, 95% water (containing 0.1% HCOOH) and 5% acetonitrile mobile phase, flow-rate of 200 μl/min, and injection volume of 10 μl.

The settings of the interface and the MS conditions were: the ion spray voltage was operated at −3.35 kV with 35–40 p.s.i. (1 p.s.i. = 6894.76 Pa) "zero" grade compressed air nebulization gas flow at 0.8 l/min. A curtain of nitrogen gas (99.999%), at a flow-rate of 1.2 l/min, was used to keep atmospheric gases out of the analyzer. The orifice potential was −60 V. Normal mass spectra were acquired by scanning Q1 after negative (deprotonated) ions (alendronate, **2**) were emitted into the quadrupole mass analyzer through a 0.0045-in. (1 in. = 2.54 cm) pinhole aperture. Product-ion spectra were taken by colliding the Q1-selected alendronate at "$m/z = 248$" with collision gas (argon, 50 eV, $400 \cdot 10^{12}$ atoms/cm) in Q2 and scan Q3 to analyze the fragment ions. The dwell time was 1.00 ms. Q1 was scanned from m/z 200 to 1000 at a step size of 0.2 u. Q3 was scanned from m/z 14 to 249. The mass spectrometer was tuned and calibrated across the m/z range 10 to 2400.

3. Results and discussion

3.1. IC–ISP-MS conditions

The IC conditions used in IC–ISP-MS were modified from the IC–indirect UV method, which was developed in this laboratory for the determination of bisphosphonate drugs. The indirect UV detection monitors the decrease in UV absorbance of the nitric acid eluent (maximum absorption near 220 nm) due to the replacement of nitrate by bisphosphonate molecules. Both initial and stressed MK-0217 tablets were studied by the IC–indirect UV method. Inset a of Fig. 2 shows an IC chromatogram of a 5-mg MK-0217 initial tablet, obtained under the optimized IC–indirect UV conditions described in the Experimental section. Alendronate was eluted at a retention time of ca. 10 min. There was an additional peak observed at ca. 6.4 min, which is attributed to the solvent. The assignments were confirmed by studying the MK-0217 standard. Inset b of Fig. 2 shows an IC chromatogram of a 5-mg MK-0217 stressed tablet. In addition to the solvent and the alendronate peaks, a small peak was observed, eluting at ca. 8.8 min. This peak was identified as a degradate —an alendronate/excipient adduct from a detailed study, which will be reported in a separate paper. It should be pointed out that the stressed tablets were used to demonstrate the method specificity. The tablets were made by a non-commercialized manufacturing process and the alendronate/excipient adduct was formed under the stress conditions only from these tablets. Tablets made by the final commercial formulation and process are very stable and the adduct under the stress conditions was not observed.

Although the IC–indirect UV method could separate alendronate and its potential degradate, it did not provide structural information. In order to characterize their structures, the IC was interfaced with ISP-MS. Rapid on-line MS characterization of the degradate is preferred to isolation because isolation of the very low level degradate in dosage formulations was difficult and time-consuming.

Since the ionization efficiency of the ISP interface is promoted by weak solvation by a volatile solvent, the mobile phase of the IC–indirect UV method was modified. First, it was found that when a very strong mineral acid, nitric acid, was used in the mobile phase, the ion spray needle flew toward the sample injector under the applied high voltage. To have an ion

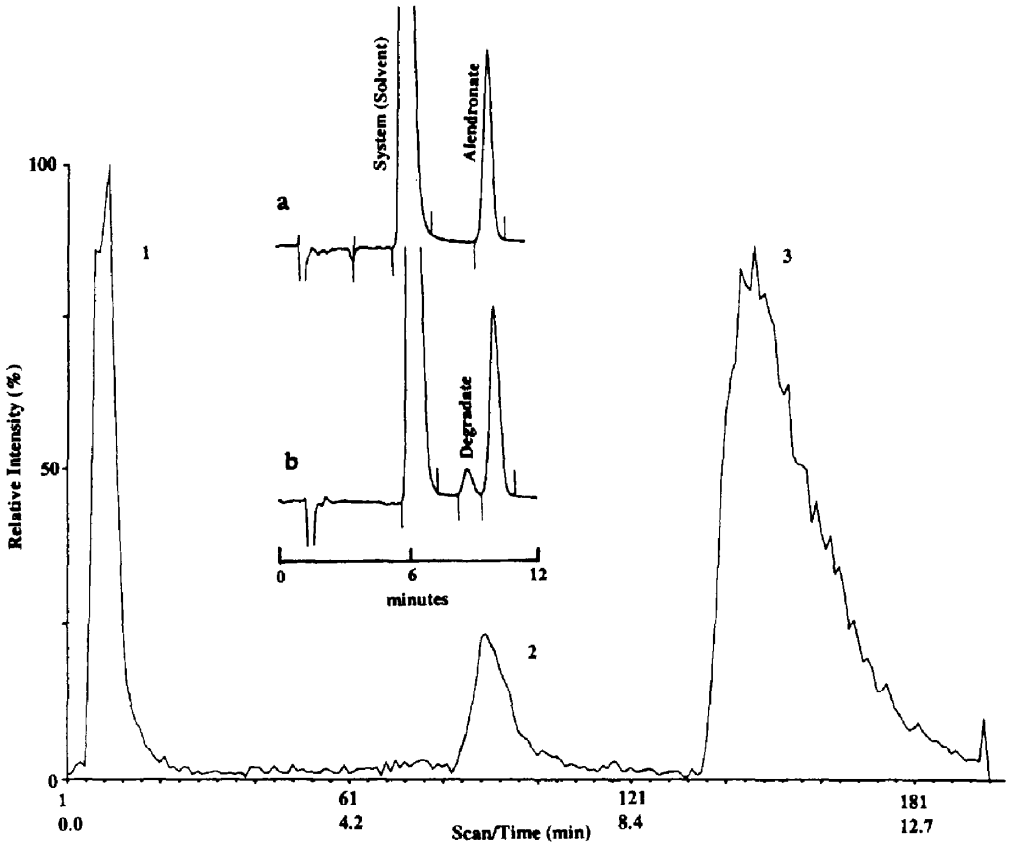


Fig. 2. Total ion current chromatogram (TIC) of a 5-mg MK-0217 stressed tablet; and IC–UV chromatogram of a 5-mg MK-0217 initial tablet (inset a) and stressed tablet (inset b).

current going towards the MS interface, the 1.6 m*M* nitric acid was replaced by 0.1% formic acid that is more volatile. Secondly, the 0.1% formic acid aqueous solvent was mixed with 5% acetonitrile, which is preferred over 100% aqueous mobile phase since a finer mist could be formed in the interface as the mixture had a lower surface tension than pure water. These modifications did not affect the IC separation of alendronate and the alendronate/excipient adduct. The desired pH of the mobile phase (pH 3) was readily achieved by using formic acid. The IC was coupled with ISP-MS in the negative ionization mode, which was chosen simply because alendronate and its excipient adduct were negatively charged in the mobile phase. Because no non-volatile salts were used in the mobile phase, a micromembrane suppressor [12,13] was deemed unnecessary between the IC column outlet and the ISP-MS inlet, and not used.

Fig. 2 shows a total ion current (TIC) chromatogram of a 5-mg MK-0217 stressed tablet, obtained under the IC–ISP-MS conditions. The first peak (ca. 1 min) is an excipient peak, which is not observed as a positive peak in the IC–indirect UV chromatogram (inset b). It is probable that the excipient peak was included in the negative peak of the indirect UV chromatogram (ca. 1.5 min). The second peak (ca. 6 min) is the alendronate/excipient adduct peak, which was observed at ca. 8.8 min with indirect UV detection. The third peak observed at ca. 10 min is clearly assigned to the alendronate, which was also observed at ca. 10 min with indirect UV detection. The peak was broad, but it is typical

in IC. In a separate experiment, alendronate was isolated and examined by fast atom bombardment (FAB) MS, which showed that no other species co-eluted with alendronate. The IC–ISP-MS method is stability-indicating since it well separated alendronate from excipients and one potential degradate. Only three peaks were observed in TIC. The solvent (water) peak observed with indirect UV detection was not seen in TIC, indicating that water anions were successfully declustered by nitrogen curtain gas and free jet expansion at the orifice in the ISP interface.

3.2. IC–ISP-MS of alendronate

According to the observed molecular mass by IC–ISP-MS and fragmentation pattern by IC–ISP-MS–MS, alendronate and the alendronate/excipient adduct shown in TIC were both characterized. As mentioned above, this paper focuses on alendronate.

Fig. 3 shows the IC–ISP-MS spectrum of the alendronate peak in TIC. Alendronic free acid has bifunctional groups, namely, the acidic OH and the basic NH_2 groups. The pK_a values for the parent bisphosphonic acid, obtained by potentiometric titration in water with 0.1 *M* sodium hydroxide, are $pK_{a1} < 2$; $pK_{a2} < 2$, $pK_{a3} = 6.2$, $pK_{a4} = 9.9$ and $pK_{a5} = 10.2$ [4]. The basic primary-amine group has a pK_b value less than 4 (for example, CH_3NH_2 has a pK_b value of 3.38 at 25°C). Thus, in the mobile phase used in IC–ISP-MS, which had a pH about 3, the zwitterionic alendronate mono-anion **3** was formed. In the ISP interface in the negative ionization mode, the mono-anions **3** were transported from the IC effluent (liquid phase) into the mass analyzer (gas phase). The most intense peak in the IC–ISP-MS spectrum shown in Fig. 3 has *m/z* 248, which is clearly attributed to the alendronate mono-anion. Whether the mono-anion in the gas phase is still a zwitterionic anion is an interesting question, which cannot be

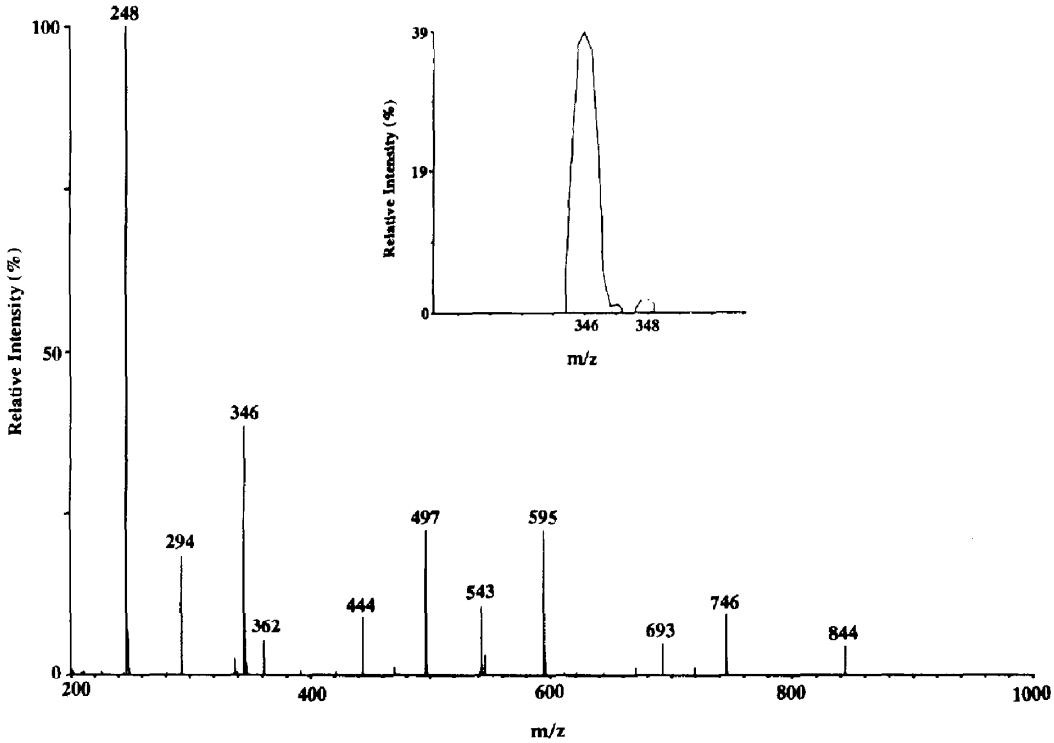


Fig. 3. IC–ISP-MS spectrum of alendronate.

answered on the basis of the m/z value since both the zwitterionic anion **3** and the non-zwitterionic anion **2** have the same m/z value. In the IC–ISP-MS–MS study discussed below, the fragmentation pattern(s) of the anion was interpreted in terms of the non-zwitterionic anion **2** although this did not prove it since rapid proton transfer could occur during the fragmentation process.

A distinguishing feature of the IC–ISP-MS spectrum shown in Fig. 3 is that in addition to the strongest peak of the alendronate mono-anion, there are additional weaker peaks, which all have m/z values greater than 248 of alendronate. These peaks except for the peak of the m/z ratio of 362 are attributable to the non-covalent cluster ions formed by hydrogen bonding of alendronate with acid molecules in the gas phase [14–16]. The peak of m/z 362 is not identified, but it is a very weak, insignificant peak.

The identifications of the cluster ions are summarized in Table 1. All the cluster ions can be accounted for by a series m/z $[248 + (46n) + (249m) + (98p)]$. The acid molecules having mass 46 and 249 are attributed to formic acid used in the mobile phase, and the parent alendronic acid, respectively. The acid molecule having mass 98 is attributed to sulfuric acid, which was a contaminant from the column according to the manufacturer's information. The observation of M + 2 isotope ions, as exemplified by the ion of m/z 346 (see the inset in Fig. 3), confirms this assignment. Phosphoric acid, which also has the mass 98, was excluded.

It is well documented that there are two competing processes in the negative ionization mode: deprotonation to form molecular anion and anion attachment to form cluster ions via the formation of hydrogen bonding. The latter process can be thermodynamically favorable [17]. Nevertheless, under the IC–ISP-MS conditions alendronate was the predominant anion, which facilitates the characterization and quantitation of the anion. The cluster ions formation might depend on factors such as the concentration of alendronate and injection volume. However, in this study, the relatively higher concentration of alendronate and injection volume were necessary to the MS and MS–MS detection of the low-level alendronate/excipient adduct.

Table 1
Cluster ions of alendronate with acid molecules

m/z	Cluster ions
294	Alendronate + formic acid
346	Alendronate + sulfuric acid
444	Alendronate + two sulfuric acid
497	Alendronate + alendronic acid
543	Alendronate + alendronic acid + formic acid
595	Alendronate + alendronic acid + sulfuric acid
693	Alendronate + alendronic acid + two sulfuric acid
746	Alendronate + two alendronic acid
844	Alendronate + two alendronic acid + sulfuric acid

3.3. IC–ISP-MS–MS of alendronate

The product ion spectrum of the alendronate mono-anion of m/z 248, obtained by the use of the IC–ISP-MS–MS conditions described in the Experimental section, is shown in Fig. 4. The spectrum shows five main fragment ions with m/z 166, 148, 81, 79 and 63, respectively. The ion with m/z 166 was the most intense fragment ion. As shown in Fig. 5, the C–P bond(s) breaking in alendronate (**2**) initially leads to two fragment anions $\mathbf{a}_1$ (m/z 166) and **b** (m/z 81), depending where the negative charge was contained during the fragmentation:

$$\mathbf{2} \rightarrow (\mathbf{b} + \mathbf{H}^+) + \mathbf{a}_1 \ (m/z166); \text{ or}$$

$$\mathbf{2} \rightarrow (\mathbf{a}_1 + \mathbf{H}^+) + \mathbf{b} \ (m/z\ 81)$$

The fragmentation was suggested to occur in a concerted process involving either of the five-membered ring transition states illustrated in Fig. 5. In the process, the pentavalent phosphorus was changed to trivalent phosphorus.

The anions $\mathbf{a}_1$ and **b** underwent further fragmentation as indicated by the more fragment ions observed in the product ion spectrum. The ion with m/z 148 is suggested to be the six-membered ring $\mathbf{c}_1$ formed by the loss of a water from $\mathbf{a}_1$. The anion $\mathbf{c}_1$ possessed a P–N bond. P–N bonds are stable and occur widely in phosphorus-containing compounds [18]. The ion with

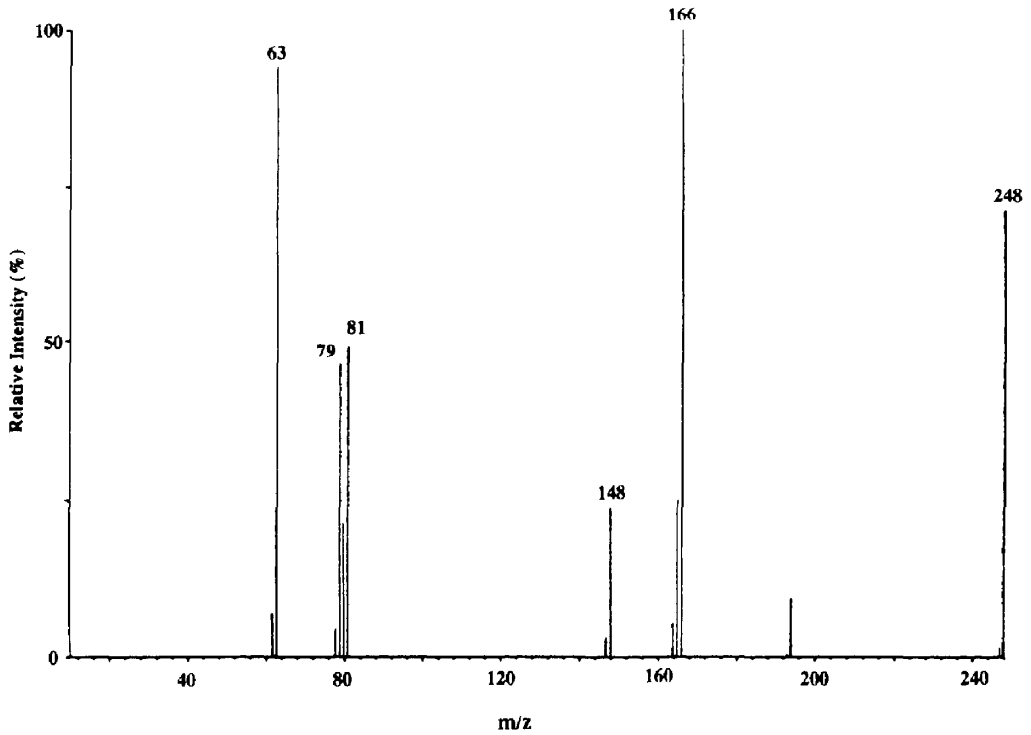


Fig. 4. Product ion spectrum of alendronate.

m/z 79 is suggested to be **d** formed by the transfer of two H from **b** to other molecules. It could also be formed by the loss of H_2 from **b**. But, the loss of H_2 from a secondary fragment ion is generally thermodynamically unfavorable. It is worth indicating that **d**, metaphosphate anion, is an important intermediate in the phosphate chemistry [19]. It is highly reactive in aqueous solution [20], but relatively stable and unreactive in gas phase [21]. The ion with m/z 63 is suggested to be **e** formed by the loss of a water from **b**. The characteristic fragmentation patterns provide structural identification of alendronate.

It should be pointed out that $\mathbf{a}_1$ could undergo cyclization to form $\mathbf{a}_2$, which also has m/z 166, and then $\mathbf{a}_2$ could lose a water to form $\mathbf{c}_2$ which has m/z 148. Although these species cannot be absolutely excluded, they are considered energetically less favorable than $\mathbf{a}_1$ and $\mathbf{c}_1$ because of the ring strain of their five-membered ring structures.

4. Conclusions

Alendronate, an important bisphosphonate drug (in the form of trihydrate of alendronate sodium **1**), has been successfully detected and characterized by IC–ISP-MS in the negative ionization mode. To the best of the authors' knowledge, there is no MS report of alendronate and other bisphosphonate drugs in the literature. The present study has demonstrated the advantage of the soft ionization of the ISP technique in this application to pharmaceutical research and development.

Acknowledgement

The authors thank Dr. M.A. Brooks for critical review and helpful discussion of the paper before its publication. The authors also thank reviewers in the Merck Research Laboratories and referees for critical comments.

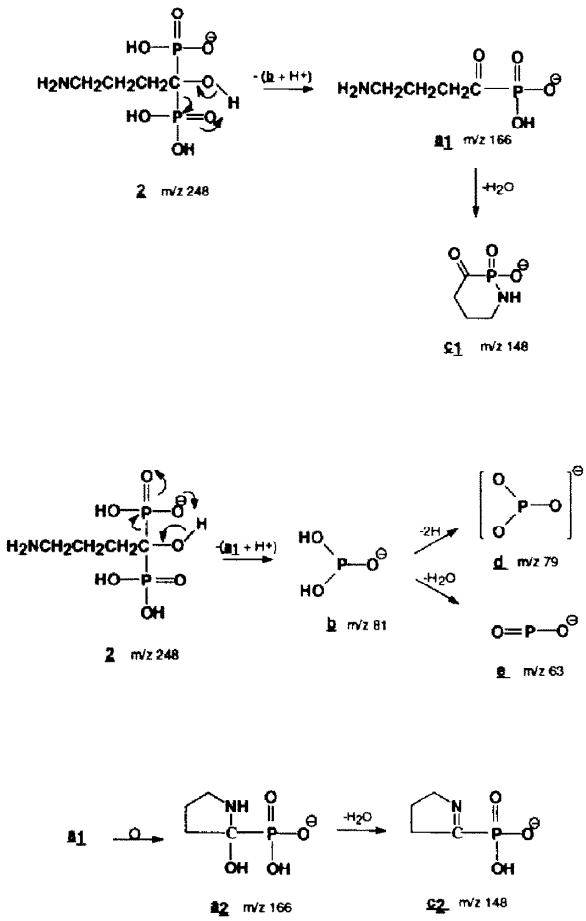


Fig. 5. Fragmentation pathways of alendronate.

References

[1] X.-Z. Qin, D.P. Ip, K. H.-C. Chang, P.M. Dradransky, M.A. Brooks and T. Sakuma, *J. Pharm. Biomed. Anal.*, 12 (1994) 221–234.

[2] G. Heynen, P. Delwaide, O.L.M. Bijvoet and P. Franchimont, *Eur. J. Clin. Invest.*, 11 (1982) 29–35.

[3] H.P. Sleeboom, O.L.M. Bijvoet, A.T. van Oosterom, J.H. Gleed and J.L.H. O'Riordan, *Lancet*, ii (1983) 239–243.

[4] Merck Research Laboratories, unpublished results.

[5] A.P. Bruins, T.R. Covey and J.D. Henion, *Anal. Chem.*, 59 (1987) 2642–2646.

[6] T.L. Chester, E.C. Lewis, J.J. Benedict, R.J. Sunberg and W.C. Tettenhorst, *J. Chromatogr.*, 225 (1981) 17–25.

[7] K.A. Forbes, J. Vecchiarelli, P.C. Uden and R.M. Barnes, in P. Jandik and R.M. Cassidy (Editors), *Advance in Ion Chromatography*, Century International, Franklin, MA, 1989, pp. 487–502.

[8] E.W. Tsai, D.P. Ip and M.A. Brooks, *J. Chromatogr.*, 596 (1992) 217–224.

[9] E.W. Tsai, S.D. Chamberlin, R.J. Forsyth, C. Bell, D.P. Ip and M.A. Brooks, *J. Pharm. Biomed. Anal.*, 11 (1993) 513–516.

[10] E.W. Tsai, D.P. Ip and M.A. Brooks, *J. Pharm. Biomed. Anal.*, 12 (1994) 983–991.

[11] S. Auriola, R. Kostiainen, M. Ylinen, J. Monkkonen and P. Ylitalo, *J. Pharm. Biomed. Anal.*, 7 (1989) 1623–1629.

[12] W.M.A. Niessen, R.A.M. van der Hoeven, J. van der Greef, H.A. Schols, G. Lucas-Lokhorst, A.G.J. Voragen and C. Bruggink, *Rapid Commun. Mass Spectrom.*, 6 (1992) 474–478.

[13] J.J. Conboy, J.D. Henion, M.W. Martin and J.A. Zweigenbaum, *Anal. Chem.*, 62 (1990) 800–807.

[14] J.B. Fenn, M.C. Mann, K. Meng, S.F. Wong and C.M. Whitehouse, *Science*, 246 (1989) 64–71.

[15] T.R. Covey, R.F. Bonner, B.I. Shushan and J.D. Henion, *Rapid Commun. Mass Spectrom.*, 2 (1988) 249–256.

[16] D. Suckau, Y. Shi, S.C. Beu, M.W. Senko, J.P. Quinn, F.M. Wampler, III and F.W. McLafferty, *Proc. Natl. Acad. Sci. U.S.A.*, 90 (1993) 790–793.

[17] C.E. Parker, R.W. Smith, S.J. Gaskell and M.M. Bursey, *Anal. Chem.*, 58 (1986) 1661.

[18] H. R. Allcock, *Chem. Rev.*, 72 (1972) 315.

[19] B. Ma, Y. Xie, M. Shen, P.v.R. Schleyer and H.F. Schaefer, III, *J. Am. Chem. Soc.* 115 (1993) 11169–11179.

[20] D. Herschlag and W.P. Jencks, *J. Am. Chem. Soc.* 111 (1989) 7579.

[21] R.G. Keesee and A.W. Castleman, Jr., *J. Am. Chem. Soc.* 111 (1993) 9015.