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Analysis of (dichloromethylene) bisphosphonate in urine by capillary gas chromatography–mass spectrometry*

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Abstract: An anion exchange extraction method of bisphosphonates from urine is described. More than 90% of the (dichloromethylene) bisphosphonate (Cl_2MBP, clodronate) was recovered from urine. The extracted bisphosphonates were trimethylsilylated and analysed with capillary gas chromatography–mass spectrometry (GC/MS). The mass spectrometric techniques used were electron ionization (EI), ammonia chemical ionization (CI), ammonia CI tandem mass spectrometry and methane negative chemical ionization (NCI). The limit of detection of Cl_2MBP was 25 pg/injection in the NCI/selective ion recording (SIR)-mode. At 100 ng ml^{-1} of Cl_2MBP the precision of the whole assay method was 17.9% ($N = 6$). The NCI/SIR technique offers a sensitive and highly selective method for the quantitation of Cl_2MBP in urine.

Keywords: *(Dichloromethylene) bisphosphonate; urine; anion exchange extraction; gas chromatography–mass spectrometry; negative chemical ionization; ammonia chemical ionization.*

Introduction

Bisphosphonates are a class of synthetic compounds (Fig. 1) used in the treatment of hypercalcaemia caused by various reasons. The gastrointestinal absorption of bisphos-

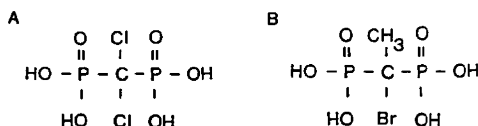

Figure 1
Chemical structures of (dichloromethylene) bisphosphonic acid, Cl_2MBP (**A**) and (C-methyl monobromomethylene) bisphosphonic acid, BrMeMBP (**B**).

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phonates is poor and has great intra- and inter-individual variations [1]. Sensitive and selective analysis methods are needed when the pharmacokinetics and clinical use of these drugs are studied.

Bisphosphonates have been extracted from plasma and urine by precipitation with calcium in basic medium [2–4]. A related compound, aminomethylphosphonic acid has been isolated from crops via chelation ion exchange and anion exchange [5].

Bisphosphonates have been analysed by decomposition of the P–C–P bond with UV-light and the inorganic phosphate released has been determined [2], or by HPLC using phosphorus selective detection after separation in an anion exchange column [3]. The phosphonic acid, (4-chlorophenyl) thiomethylene bisphosphonic acid, which contains an UV-absorbing functional group, has been analysed with reversed-phase HPLC and UV-detection [4]. Additionally, 1-hydroxyethylidene-1,1-disodium phosphonate, has been analysed in tablet formulations by gas chromatography (GC) with a flame ionization detector (FID) [6]. However, the sensitivity of the method is not sufficient for the analysis of the compound in biological fluids.

Phosphonic acids cannot be analysed readily with GC because of their low volatility and polar nature. The derivatization procedures employed include diazoalkylation [7], trimethylsilylation [6, 8, 9], and dimethyl-tert-butylsilylation [10, 11]. The limit of detection of silylated phosphonic acids is around 500 pg per injection using FID and 30–60 pg using EI/MS and SIR [11]. Gas chromatography and EI/MS of silylated phosphonates, including methylene bisphosphonic acid, has been extensively studied by Harvey and Horning [9].

Chemical ionization is a soft ionization method used both for qualitative and quantitative analyses. When ammonia, that has high proton affinity (858 kJ mol^{-1}) [12], is used as the reagent gas the resulting spectra are dominated by protonated molecular and ammonia adduct ions. The use of tandem mass spectrometry (MS/MS) offers a highly specific technique to analyse complex samples of trace specimens. In these experiments, a high molecular weight ion is separated by the first mass analyser, collided with argon in the collison chamber and the resulting ionic products are analysed with the second mass analyser [13]. The electron capture negative chemical ionization (ECNCI) is a sensitive and selective method for the analysis of compounds with high electron affinity, for example halogenated organic compounds [14].

In this study, the capillary gas chromatography combined with the techniques of EI/MS, ammonia CI/MS, ammonia CI/MS/MS and ECNCI/MS were used for the quantitation of (dichloromethylene) bisphosphonate (Cl_2MBP, clodronate). Additionally, this paper describes an anion exchange extraction method for the isolation of bisphosphonates from urine. The detection limit of the method was 5 ng ml^{-1} of Cl_2MBP in urine, when the ECNCI and SIR were used.

Experimental

Chemicals

The compound (dichloromethylene) bisphosphonate (Cl_2MBP) (as a disodium salt) and the ^{14}C-labelled analogue were obtained from Leiras, Pharmaceutical Company (Tampere, Finland). The internal standard (C-methyl monobromomethylene) bisphosphonic acid (BrMeMBP), was generously provided by Professor D. W. Hutchinson (University of Warwick, Coventry, UK). The reagent, N,O-bis(trimethylsilyl) trifluoro-

acetamide (BSTFA), and Dowex 1 × 2 anion exchange resin were from Fluka AG (Buchs SG, Switzerland) and acetonitrile was from E. Merck (Darmstadt, FRG).

Sample preparation

Samples for the investigation of the mass spectra of the bisphosphonates were prepared by adding 0.1 mg of Cl_2MBP and BrMeMBP in 1 ml of 0.1 M phosphate buffer (pH 7.2). The phosphonates were extracted with 150 mg of Dowex 1 × 2 anion exchange resin packed in an empty reservoir with a frit on the bottom (Analytichem International, Harbor City, CA, USA). The resin was first washed with 2 ml of water, the sample was added, the resin was washed with 10 ml of water and the bisphosphonates were eluted with 0.5 ml of 2 N HCl. An aliquot of the eluent (0.2 ml) was added to the reaction vial and the solvent was evaporated to dryness with a stream of air at 80°C. Into this vial, 0.1 ml of acetonitrile and 0.1 ml of BSTFA were added. The mixture was shaken by Vortex® and heated at 80°C for 2 h. An aliquot (1 μl) was injected into the GC.

The quantitative studies were carried out by adding known quantities of Cl_2MBP in blank urine samples (1 ml). The internal standard (BrMeMBP, 500 ng) was added to each sample. Evaluation of the assay was carried out by constructing a five-point calibration graph covering the concentration range from 50 ng ml^{-1} to 5 μg ml^{-1}. The urine samples were extracted and derivatized by the same method as the standards from phosphate buffer. The precision of the GC/MS detection was determined by six replicate injections of the same sample and the precision of the total method including extraction was tested by injecting an aliquot of six replicate samples into the chromatograph.

The recovery of the extraction method was tested by adding 5 or 10 μg of $[^{14}C]Cl_2MBP$ to 1 ml aliquots of blank urine. The samples were extracted as described above and the radioactivity of the resulting acidic extract was measured by a routine liquid scintillation procedure.

Gas chromatography–mass spectrometry

The mass spectra of BrMeMBP were obtained with a VG 250 70–SE double focusing mass spectrometer interfaced with a HP 5890 gas chromatograph. For the EI studies the ion source temperature was 220°C, electron energy 70 eV and the trap current 0.2 mA. In the NCI studies, the ion source temperature was 120°C, electron energy 150 V, emission current 0.3 mA, and the methane pressure was 5×10^{-4} mbar.

The mass spectra of Cl_2MBP were obtained with a Finnigan MAT 45A Triple Stage Quadrupole mass spectrometer interfaced with a Finnigan MAT 9611 gas chromatograph. In the ammonia CI studies, the ionization gas pressure was 0.6 torr, ionization source temperature 100°C, emission current 0.3 mA, and electron energy 150 V. In the collision induced dissociation (CID) studies, the first quadrupole was used for selection of the protonated molecular ion produced by ammonia CI. The RF-only quadrupole was used for CID of the selected ion with argon. The collision gas pressure was 2 mtorr and the collision energy was 25 eV. The ionic collision products were analysed by scanning the third quadrupole from m/z 10 to m/z 600. In the EI studies, the ion source temperature was 120°C, emission current 0.3 mA, and electron energy 70 eV. In the NCI studies, the ion source temperature was 70°C, electron energy was 150 eV, emission current 0.3 mA, and methane pressure was 0.8 torr.

The quantitative studies using the NCI were carried out with a VG Trio 2 quadrupole

mass spectrometer interfaced with a HP 4890 gas chromatograph. The ion source temperature was 180°C, methane pressure in the source was 5×10^{-5} mbar, and electron energy was 70 eV. The quantitation was based on the SIR of the fragment ions *m/z* 424 for Cl_2MBP and *m/z* 404 for BrMeMBP.

The capillary column used in the GC was an OV-1, 25 m, i.d. 0.31 mm and film thickness 0.1 μm (Nordion, Helsinki, Finland). The injection port temperature was 250°C and the oven was programmed from 120°C min^{-1} to 230°C at 10°C min^{-1}.

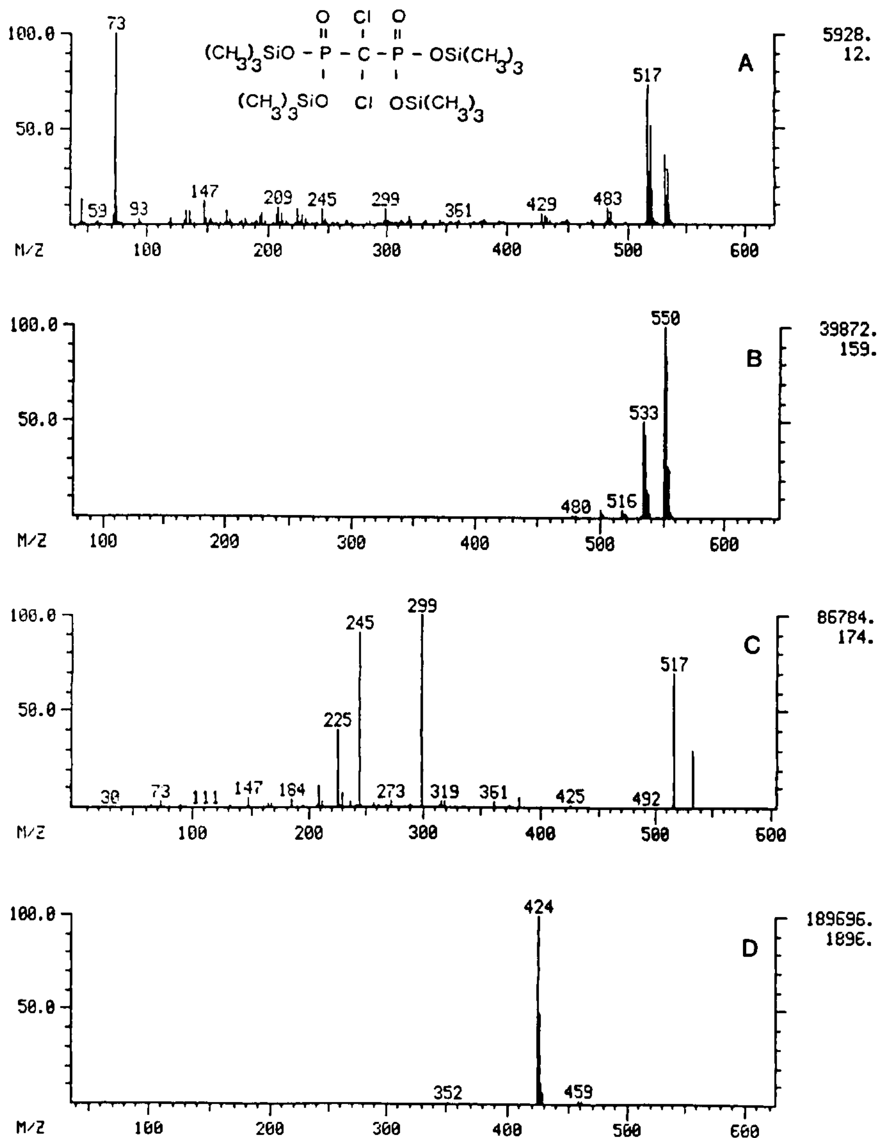

Figure 2
Mass spectra of the trimethylsilylated Cl_2MBP. (A) Electron ionization mass spectrum; (B) positive ammonia chemical ionization spectrum; (C) collision induced daughters of the protonated molecular ion (*m/z* 533); (D) negative chemical ionization spectrum.

Results and Discussion

The structures of the trimethylsilylated Cl_2MBP and BrMeMBP were verified by EI/MS. The mass spectra [Figs 2(A) and 3(A)] show molecular ions at *m/z* 532 for Cl_2MBP and at *m/z* 556 for BrMeMBP. Both the spectra show abundant $[M-15]^+$ ions formed by the loss of a methyl group from the trimethylsilyl group. Generally the fragmentation of silylated Cl_2MBP and BrMeMBP is similar to that discussed for silylated methylene bisphosphonic acid [9]. In addition, a loss of a H_2CCl-radical from the silylated Cl_2MBP gives rise to the ion at *m/z* 483. The mass spectrum of the silylated BrMeMBP shows an ion at *m/z* 477 generated by the loss of bromine from the molecular ion.

The positive ammonia chemical ionization spectrum of the silylated Cl_2MBP shows an abundant protonated molecular ion at *m/z* 533 and an ammonium adduct ion at *m/z* 550 as the base peak [Fig. 2(B)]. The CID spectrum of the protonated molecular ion [Fig. 2(C)] exhibited abundant daughter ions at *m/z* 517, 299, 245 and 225. The detection limit of the CID method, based on multiple ion recording of ions at *m/z* 517, 299 and 225, was 500 pg per injection (extracted from 0.1 M phosphate buffer). The possible structures of the monitored ions are $[M + H - CH_4]^+$, $[HO{=}P(OSiMe_3)_2SiMe_3]^+$ and $[O{=}P(OSiMe_3)_2]^+$, respectively.

The NCI spectrum of the trimethylsilylated Cl_2MBP exhibits an intensive ion at *m/z* 424 generated by the loss of a neutral fragment, Me_3SiCl, from the molecular ion [Fig. 2(D)]. The NCI mass spectrum of silylated BrMeMBP shows a corresponding ion at *m/z* 404 generated by the loss of Me_3SiBr [Fig. 3(B)]. The molecular ions are very weak in both the spectra.

The NCI/SIR of the ions at *m/z* 424 for the analyte and at *m/z* 404 for the internal standard was used for the determination of Cl_2MBP in urine. Good chromatographic

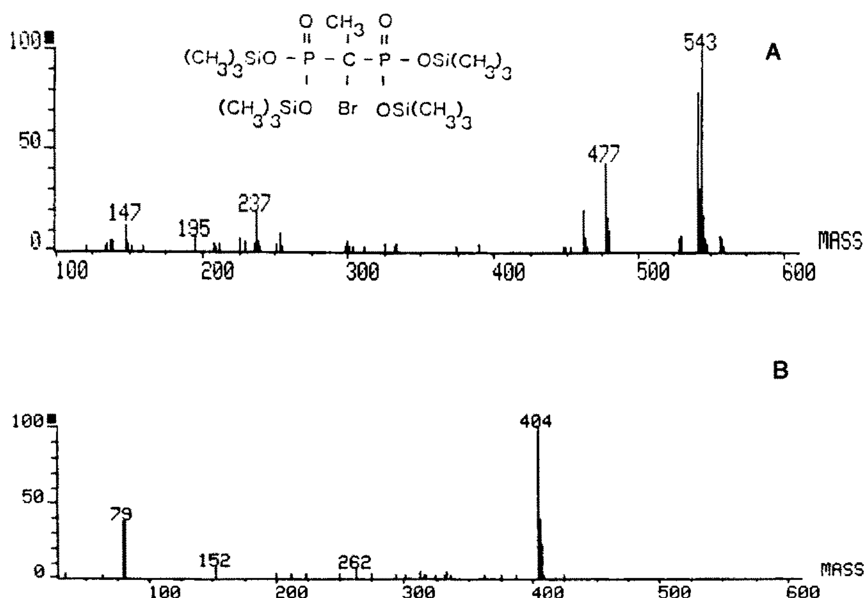

Figure 3
Mass spectra of trimethylsilylated BrMeMBP. (A) Electron ionization spectrum; (B) negative chemical ionization spectrum.

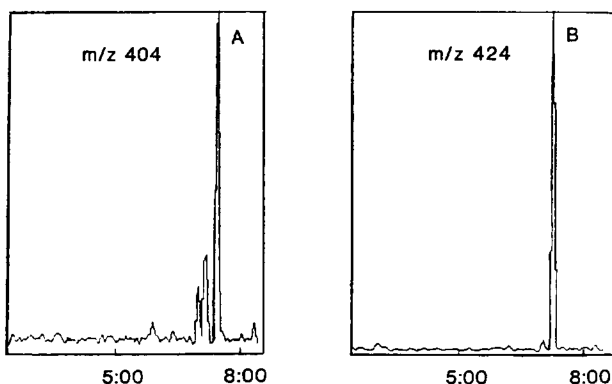

Figure 4
Selected ion chromatograms of trimethysilylated BrMeMBP at *m/z* 404 (A), retention time 7:32, and trimethylsilylated Cl_2MBP at *m/z* 424 (B), retention time 7:20. The concentrations of the compounds in a spiked human urine sample were 500 and 50 ng ml^{-1}, respectively.

separation from the background peaks was achieved for silylated Cl_2MBP and BrMeMBP; the retention times were 7:20 and 7:32, respectively (Fig. 4). A linear relationship was obtained between the concentration of the analyte (X) and the analyte/internal standard peak area ratio (Y) over a wide concentration range of Cl_2MBP in urine (50 ng ml^{-1} to 5 μg ml^{-1}). The regression equation of the linear relationship was $Y = 1.830X - 0.096$ ($r^2 = 0.9998$). The confidence limits on the slope were 1.830 ± 0.092 ($p = 0.01$). The relative standard deviation (RSD) of the GC/MS-detection of Cl_2MBP in urine (500 ng ml^{-1}, $N = 6$) was 13.2%. The precision of the whole assay method was determined for the concentration of 100 ng ml^{-1} ($N = 6$), the RSD was 17.9%. The detection limit of the method was 25 pg of Cl_2MBP injected, which corresponds to 25 ng ml^{-1} in urine (signal-to-noise ratio 5).

The solid phase extraction method based on anion exchange was simple and rapid. The recovery of the extraction method was tested for concentrations 5 and 10 μg ml^{-1} ($N = 6$), the mean recoveries were 92.6% (SD = 2.2%) and 94.0% (SD = 3.2%), respectively.

Electron ionization and ammonia CI produce intense ions at high *m/z* values. However, SIR by these ionization methods was not suitable for the analysis of Cl_2MBP in urine because of inadequate sensitivity and selectivity. Ammonia CI/MS/MS is a highly selective method, but the sensitivity is not good enough. The NCI/MS combines high sensitivity and selectivity and offers a potential method for the analysis of halogenated bisphosphonates in biological samples.

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