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Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography–electrospray mass spectrometry

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Abstract

Bisphosphonates are synthetic pyrophosphate analogues, which are used as therapeutic drugs for the treatment of metabolic bone disorders. Some of these bisphosphonates can be metabolised in cells into non-hydrolysable nucleotide analogues. In this paper, we describe an ion-pairing high-performance liquid chromatography method that is compatible with negative ion electrospray mass spectrometry for the separation of these metabolites. Tandem mass spectrometry and collision-induced dissociation (CID) were used for identification of the metabolites. The CID mass spectra of bisphosphonate–adenine nucleotide adducts are very informative, because major fragment ions are formed by cleavage of the bisphosphonate moiety from the conjugate. The method was used for detection of the nucleotide metabolites of clodronate, tiludronate and etidronate in extracts from mammalian cells after treatment with bisphosphonates. © 1997 Elsevier Science B.V.

Keywords: Bisphosphonates; Adenine nucleotides; Clodronate; Tiludronate; Etidronate

1. Introduction

Bisphosphonates are analogues of pyrophosphate, in which the labile phosphoanhydride bond, H_2O_3P-O-PO_3H_2, of pyrophosphate is replaced by a stable methylene group, H_2O_3P-CR^1R^2-PO_3H_2, to which two groups (R^1 and R^2) are attached (Fig. 1). Bisphosphonates are an important class of therapeutic drugs for the treatment of metabolic bone disorders, including Paget's disease of bone, bone metastases and osteoporosis [1]. The exact mechanism by which bisphosphonates inhibit bone resorption is only just becoming clear. Until recently, it was considered that the drugs are metabolically inert. However, Rogers et al. [2,3] and others [4] have found that bisphosphonates of low potency or those closely resembling pyrophosphate (such as clodronate and tiludronate) can be metabolised by amoebae of the eukaryotic microorganism *Dictyostelium discoideum* into non-hydrolysable, adenine-containing analogues of adenosine triphosphate (ATP). This was followed by the recent discovery that mammalian cells, such as J774 macrophages, can also metabolise

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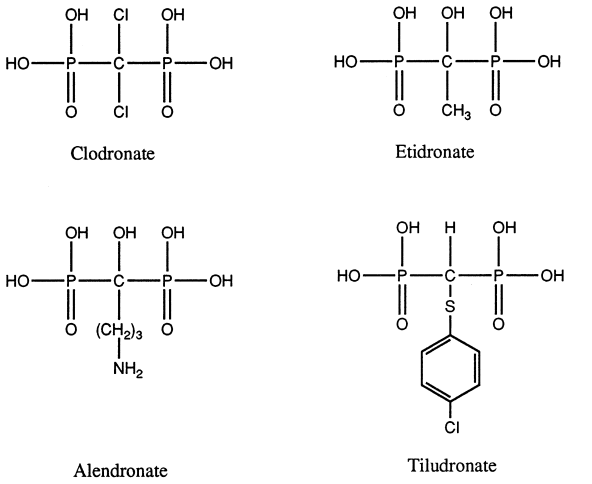

Fig. 1. Structures of the bisphosphonate drugs.

clodronate, but not the more potent bisphosphonate, alendronate [5]. The metabolite of clodronate, adenosine 5′-(β,γ-dichloromethylene)triphosphate ($AppCCl_2p$), can be detected in extracts from clodronate-treated cells on the basis of UV absorbance, after separation using anion-exchange fast protein liquid chromatography (FPLC). However, this technique is relatively insensitive and it remains unclear whether other bisphosphonates, such as etidronate or tiludronate, can be metabolised or not [3,6]. We have therefore examined the possibility of using mass spectrometry to confirm the identity of the metabolite of clodronate and to develop a more sensitive method for identifying nucleotide-containing metabolites of other bisphosphonates.

Nucleotides are usually separated by ion-exchange chromatography or ion-pairing chromatography using mobile phases that are incompatible with electrospray ionization (ESI) [7,8]. Generally, the retention factor (k) of nucleotides is increased by the use of more hydrophobic ion-pairing compounds, by increasing the pH of the mobile phase or by increasing the concentration of the ion-pairing compound [9]. High k values of an analyte make it possible to use higher concentrations of organic modifier in the high-performance liquid chromatography (HPLC) eluent. The high organic modifier content of the eluent is critical in the ESI process as it decreases the droplet surface tension and enhances vaporization of the solvent [10].

The most frequently used ion-pairing compounds are involatile tetraalkylammonium salts, and their use is generally ruled out in HPLC–MS. However, tetrabutylammonium (TBA) has been successfully used for the analysis of five cyclic nucleotides by HPLC–ESI-MS, by using low flow-rates and low concentrations of the ion-pairing compound [11]. Ion-pairing HPLC–ESI-MS has also been used for the analysis of food dyes. The use of 5 m*M* TBA as the ion-pairing agent resulted in a ten-fold decrease in sensitivity, and the main ions detected in the mass spectra were adducts formed by the addition of one or two TBA units to the analyte [12]. One solution to the compatibility problem is to use more volatile tertiary amines for ion-pairing, in combination with ESI-MS, for example, the separation of oligonucleotide metabolites using 5 to 20 m*M* tripropylamine (TPA) [13] or the separation of oligonucleotides [14] and nucleotides using triethylamine (TEA) [14,15]. The concentration of TEA acetate buffer needed to completely separate the analytes was 100 m*M*. At this concentration, the buffer significantly suppressed the electrospray signal. By using hexafluoroisopropanol (HFIP) instead of acetate as the counter-ion, signal suppression was avoided, presumably due to the high volatility of HFIP [14,15]. However, HFIP is a highly corrosive substance and must be used very carefully. The concentration of the ion-pairing agent can be decreased by increasing the alkyl chain length of the amine. Smyrnis et al. [16] developed a HPLC–ESI-MS method for the analysis of phosphorylated penciclovir derivatives using dimethylhexylamine (DMH) as the ion-pairing agent. The analytes were successfully separated using as low as 1 m*M* DMH in the buffer.

In this paper, we report an ion-pairing HPLC method that is compatible with negative ion ESI-MS for the separation of nucleotide adduct metabolites of bisphosphonates. Tandem mass spectrometry (MS–MS) and CID were used for identification of the metabolites. This study confirms that the $AppCCl_2p$ metabolite of clodronate is formed in cultured murine J774 macrophage-like cells. Similar types of ATP analogues were also detected in cell cultures treated with tiludronate and etidronate, but not in cells treated with alendronate.

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile was from Rathburn (Walkerburn, UK). Distilled and deionized water was obtained with a Milli-Q system (Millipore, Molsheim, France). DMH was from Aldrich (Milwaukee, WI, USA), ammonium hydroxide, 25% solution, was from Riedel deHaën (Seelze, Germany), TEA was from Fluka Chemie (Buchs, Switzerland) and formic acid was from Merck (Darmstadt, Germany). The ATP standard was from Perkin-Elmer (Foster City, CA, USA). The $AppCCl_2p$ standard was a generous gift from Prof. G.M. Blackburn, Department of Chemistry, University of Sheffield, UK.

2.2. HPLC

On-line HPLC–ESI-MS measurements were carried out on a Rheos 4000 pump (Flux Instruments, Danderyd, Sweden) and a Rheodyne 7725 injector with a 20-μl loop (Cotati, CA, USA). Off-line HPLC–UV measurements were performed using a Merck Hitachi combination (Tokyo, Japan) consisting of a L6200 pump, L4500 diode-array detector and an AS2000 autosampler.

The reversed-phase column used in the studies (Merck Purospher C18e, 5 μm, 125×3 mm I.D.) was eluted with the mobile phase at 500 μl min^{-1} at a temperature of +23°C. The gradient eluent system used for on-line HPLC–ESI-MS comprised two eluents. Eluent A was 10 m*M* DMH formate, with the pH adjusted to 5.0 with formic acid. Eluent B was 50% acetonitrile and 50% of 20 m*M* DMH formate solution, pH 5.0. The gradient was from 4 to 20% of B in 10 min and then to 80% of B in 30 min. The concentration of the ion-pairing compound, DMH, in the eluent was first optimized using the HPLC–UV system and measuring the *k* value of $AppCCl_2p$ (20 μl, 1 m*M*). In these studies, the acetonitrile concentration was kept at 15% and the concentrations of DMH added in 10 m*M* ammonium formate buffer were zero, 2, 5 and 10 m*M* (pH 5.0). The use of TEA as the ion-pairing compound was also tested by the addition of 10 m*M* TEA to the formate buffer (pH 5.0). Reproducibility of the retention time was tested by analysing eight different $AppCCl_2p$ samples with the ion-pairing HPLC–ESI-MS system.

2.3. Electrospray ionization mass spectrometry

Negative ion mass spectra were acquired using an LCQ quadrupole ion trap mass spectrometer equipped with an ESI source (Finnigan MAT, San Jose, CA, USA). The total eluent flow of 500 μl min^{-1} was directed to the ESI source, which is designed to cope with flow-rates of up to 1 ml min^{-1}. When biological samples were analysed, the flow was diverted to waste for the first 4 min, to avoid contamination of the ion source. The system was tuned by infusion of ATP from a T-split to the eluent flow. The spray needle potential was set to −4.5 kV. The spray was stabilized using a nitrogen sheath flow, with the value set to 90. The stainless steel inlet capillary was heated to 200°C. The capillary voltage was −39 V and the tube lens offset was 55 V. The full scan mass spectra from m/z 200 to 700 were measured using 200 ms for collection of the ions in the trap; four microscans were summed.

CID parameters were optimized by infusion of the $AppCCl_2p$ standard (10 μ*M*) and using the collision energy tune option of the LCQ. Typically, the following parameters were used for identification of the compounds in the biological samples by CID: collision energy, 18%; isolation width, four mass units; scan range, from m/z 160 to 700.

2.4. Sample preparation

J774 macrophage-like cells were treated with bisphosphonates as described by Frith el al. [5]. These cells were chosen as a model, because bisphosphonate metabolism has been established previously in this particular cell line using FPLC [5]. Cultures were incubated for 48 h with 250 μ*M* clodronate, or for 24 h with 750 μ*M* etidronate, 50 μ*M* tiludronate or 50 μ*M* alendronate. Deproteinised cell extracts were then prepared from approximately 10^8 cells after clodronate treatment or from 5×10^7 cells after treatment with other bisphophonates using perchloric acid [2,3,5]. The extracts were neutralised with potassium bicarbonate and lyophilised. The

samples were then redissolved in 5 ml of 50 m*M* DMH formate, pH 5.0, for HPLC–ESI-MS analysis. In addition, lyophilised extracts from clodronate-treated cells were also redissolved in distilled water and analysed by FPLC to obtain the fraction containing $AppCCl_2p$. This fraction was collected, lyophilised and redissolved in 100 μl of 50 m*M* DMH formate, pH 5.0, for LC–ESI-MS analysis.

3. Results and discussion

3.1. Ion-pairing HPLC

The retention of $AppCCl_2p$ in a C_{18} reversed-phase column could be increased by the addition of volatile DMH to the eluent. When 15% acetonitrile and 10 m*M* ammonium formate (pH 5.0) were used as the mobile phase, the analyte was not retained in the stationary phase and eluted with the solvent front. The addition of 10 m*M* of the ion-pairing compound increased the *k* value to six. Higher concentrations were not tested since increasing the concentration of buffer ions above 10 m*M* rapidly decreases the sensitivity of ESI [17]. The addition of 10 m*M* TEA to the eluent instead of DMH did not increase the *k* value to two (data not shown). The concentration of DMH needed to increase the retention was higher compared to the concentration of tetrabutylammonium salts (50 μ*M* to 2 m*M*) that is normally used for ion-pairing [7,11]. The reproducibility of the retention time of $AppCCl_2p$ in the ion-pairing gradient system was adequate (18.4 ± 0.18 min, $n=8$), especially as highly selective MS is used for detection. However, the injection of samples containing acetonitrile resulted in a total loss of retention of $AppCCl_2p$ on the reversed-phase column (data not shown). Also, the high amounts of salts and other impurities in cell extracts result in peak shape deterioration (Fig. 3D). These problems were overcome to some extent by injecting the samples in aqueous buffer containing an excess of the ion-pairing agent (50 m*M*) and by dilution of the sample.

3.2. Mass spectrometry of bisphosphonate metabolites

The full scan negative ion mass ES spectrum of $AppCCl_2p$ standard (calculated monoisotopic molecular mass, 573.1 Da) showed a signal of the deprotonated molecule $(M-H)^-$ as the base peak, and no significant fragment ions. The isotopic pattern showing major ions at *m*/*z* 572, 574 and 576 is typical for a compound containing two chlorine atoms (Fig. 2). The corresponding sodium adduct ions $(M-H+Na)^-$ can be seen at *m*/*z* 594 and 596. Tandem mass spectrometry in the ion trap was used to induce the formation of fragment ions, which could also be used for identification of the metabolites in the samples. The major fragment ions detected in the CID mass spectrum of $AppCCl_2p$ standard could be found at *m*/*z* 225 and 227. These ions are very informative, because they are formed by cleavage of the bisphosphonate moiety from the conjugate (Fig. 4A). This type of fragment ion is similar to the $HP_2O_6^-$ ion formed by the CID of ATP conjugates, described by Gibson et al. [18]. The ion trap technique limits the lower scan range to approximately *m*/*z* 160 and, thus, it was not possible to detect if ions typical of the adenine base [11] were formed during CID.

Ion-pairing HPLC separation and negative ion

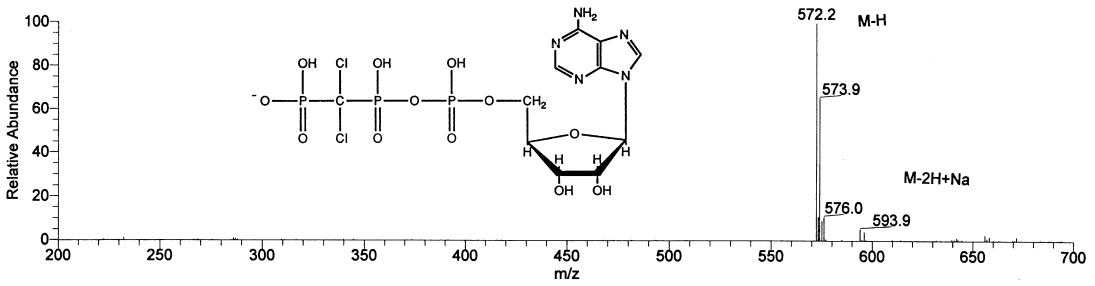

Fig. 2. Molecular structure and full scan negative ion mass spectrum of $AppCCl_2p$ standard (2 nmol/injection).

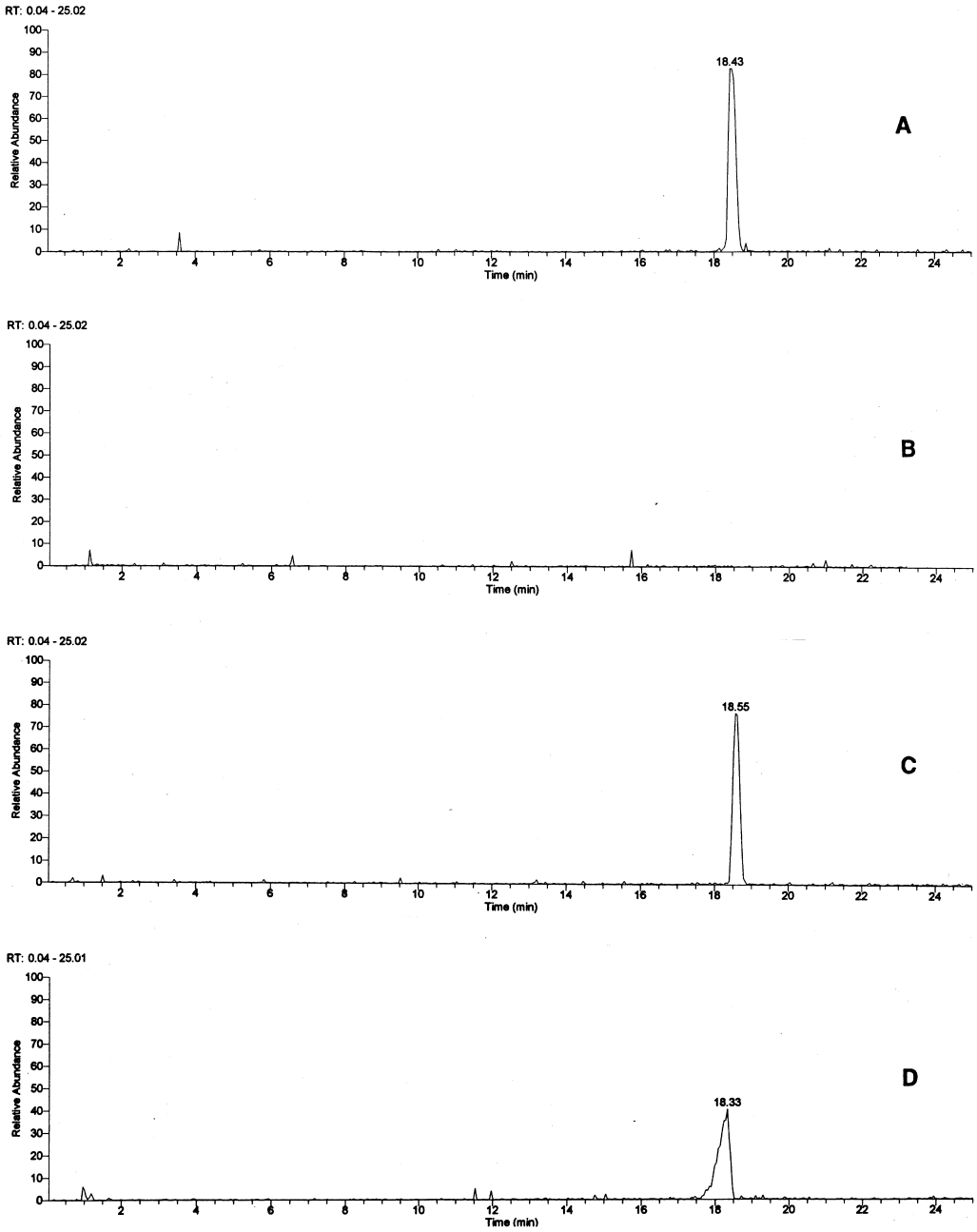

Fig. 3. Full scan negative ion HPLC–ESI-MS–MS chromatograms of (A) 200 μ*M* $AppCCl_2p$ standard, (B) untreated cell extract, (C) cells treated with clodronate, sample extracted with perchlorate and the metabolite was purified by FPLC prior to HPLC–ESI analysis and D) cells treated with clodronate, sample extracted with perchlorate. Conditions: Purospher RP18e column, 125×3 mm I.D., 5 μm. Eluent: 10 m*M* dimethylhexylamine formate–acetonitrile, pH 5.0. The gradient was from 2 to 10% acetonitrile in 10 min, then to 40% acetonitrile in 30 min, at a flow-rate of 500 μl min^{-1}.

ESI-MS were successfully used for detection of bisphosphonate metabolites in cell extracts. Fig. 3A shows the full scan MS–MS chromatogram of $AppCCl_2p$ after injection of 200 μ*M* standard (CID of molecular ion *m*/*z* 572 and 574). The $AppCCl_2p$ standard eluted with a retention time of 18.4 min. Fig. 4A shows the corresponding full scan MS–MS spectrum of the standard peak. The formation of $AppCCl_2p$ in J774 cells treated with clodronate was verified using two approaches. One of the samples was first analysed using anion-exchange FPLC, as described by Frith et al. [5], and the UV-absorbing metabolite peak appearing in the FPLC chromatogram was collected and subjected to HPLC–ESI-MS analysis. The chromatogram obtained from this FPLC fraction shows one major peak with a retention time of 18.5 min (Fig. 3C). The MS–MS mass spectrum (Fig. 4B) of this peak was identical to that of $AppCCl_2p$ standard. Secondly, the cell extract was also analysed by HPLC–ESI-MS without FPLC purification of the $AppCCl_2p$ peak (Fig. 3D). In this case, the chromatographic peak is wide and fronting, possible due to high concentrations of salts and impurities in the sample. However, the MS–MS mass spectrum (Fig. 4C) clearly indicates the presence of the metabolite in this sample. When the

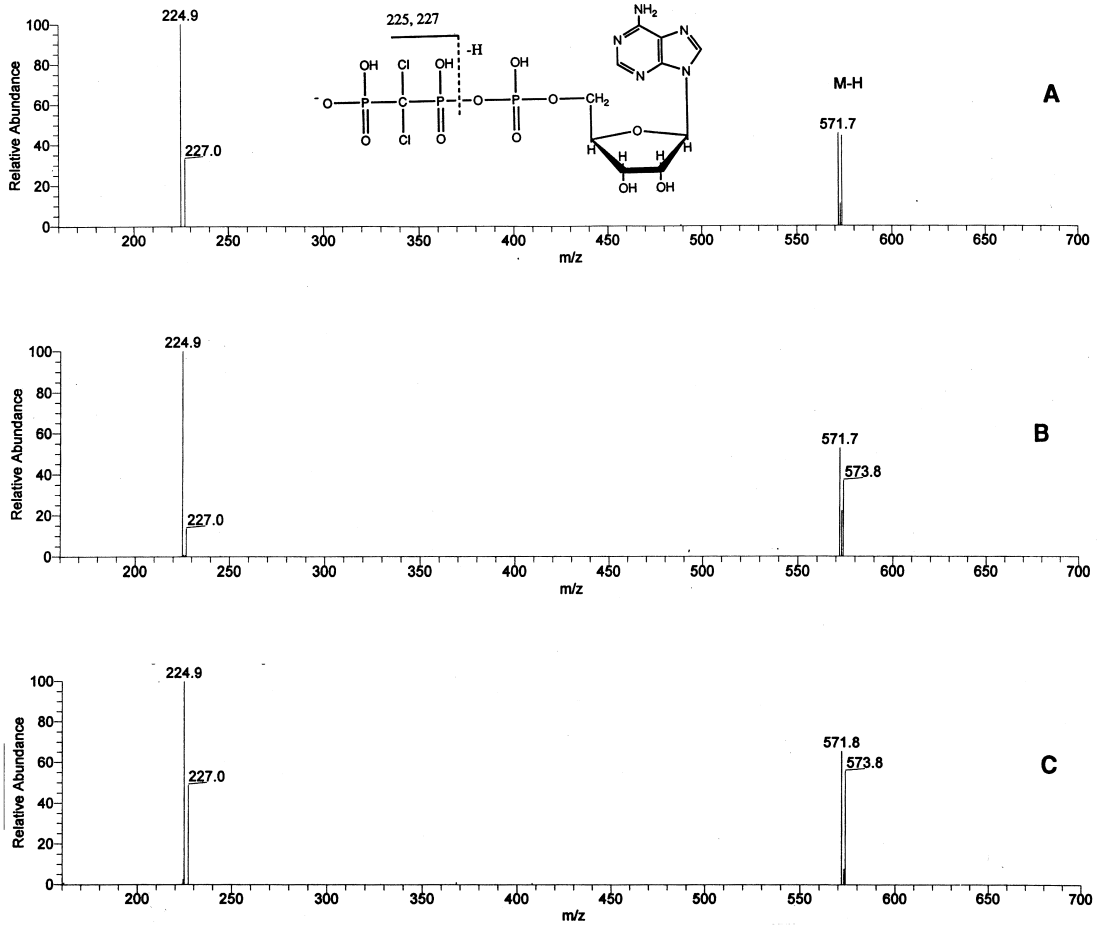

Fig. 4. Full scan negative ion MS–MS spectra of *m*/*z* 573 (width, four mass units). (A) $AppCCl_2p$ standard (200 μ*M*); (B) metabolite found in the fraction that was purified by FPLC; (C) metabolite found after the cells were treated with clodronate and the sample was extracted with perchlorate. Conditions as in Fig. 3.

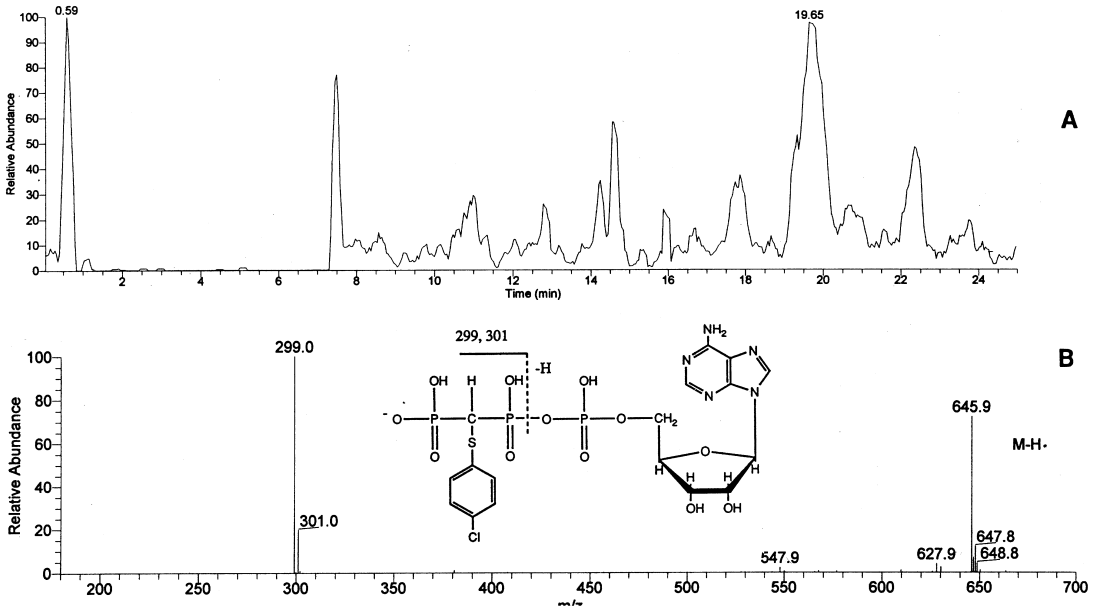

Fig. 5. (A) Full scan negative ion HPLC–ESI-MS–MS chromatogram of the sample obtained by extracting tiludronate treated cells with perchlorate (Precursor ion m/z 647). (B) Full scan negative ion MS–MS spectrum of the metabolite eluting at a retention time of 19.6 min.

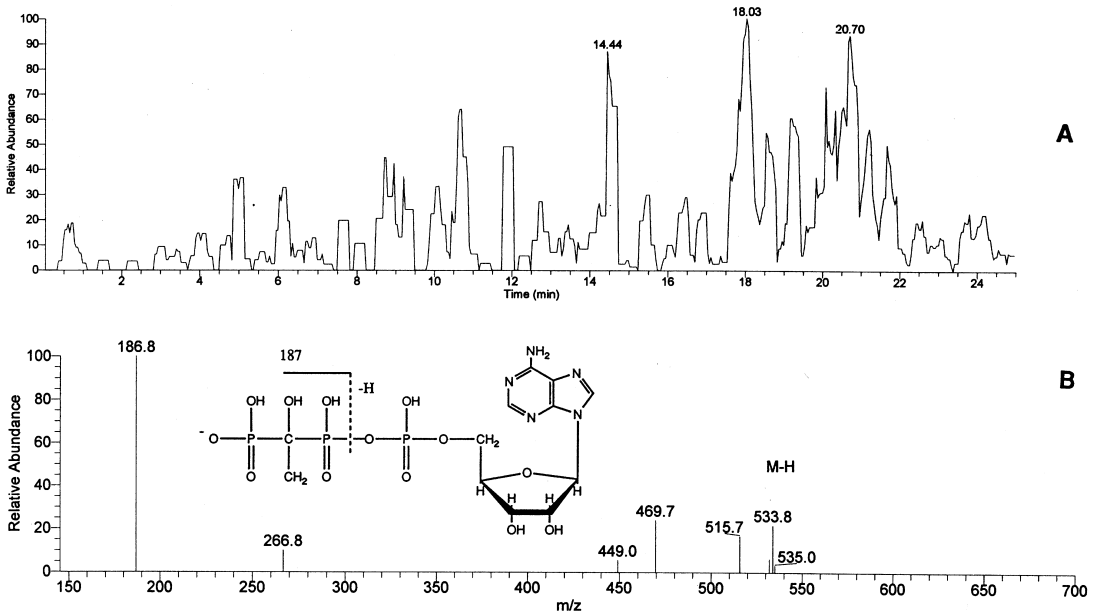

Fig. 6. (A) Full scan negative ion HPLC–ESI-MS–MS chromatogram of the sample obtained by extracting etidronate-treated cells with perchlorate (precursor ion, m/z 534). (B) Full scan negative ion MS–MS spectrum of the metabolite eluting at a retention time of 18.0 min.

extract from untreated cells was analysed using the same method, no major components showed up in the full scan MS–MS chromatogram (Fig. 3B).

Cell extracts from J774 cells treated with the other bisphosphonates were also analysed by FPLC with UV detection, but no major metabolite peaks could be detected in the chromatograms, confirming previous studies [3,6]. The FPLC chromatogram of etidronate-treated cells showed one minor new peak, but no bisphosphonate metabolite could be identified in the collected fractions (data not shown). The crude cell extracts were then also analysed by HPLC–ESI-MS to identify possible bisphosphonate metabolites. The molecular masses of these potential metabolites were calculated and were screened using full scan MS–MS mode. The values searched were m/z 646 (tiludronate), m/z 534 (etidronate) and m/z 577 (alendronate). In the sample treated with tiludronate, it was possible to find one metabolite at a retention time of 19.6 min (Fig. 5A) that had a molecular weight of 647 Da and that gave an isotopic pattern that was typical for a compound containing one chlorine atom. The full scan MS–MS spectrum shows two major fragment ions at m/z 299 and 301. This fragmentation is indicative of the presence of the tiludronate moiety in the molecule (Fig. 5B). The corresponding metabolite was found also in the etidronate-treated cells at m/z 534, with a retention time of 18.3 min. However, in this case, the signal intensity was two orders of magnitude lower than that of the tiludronate metabolite (Fig. 6A). The MS–MS spectrum of the etidronate metabolite also shows an ion formed by the loss of water at m/z 516, in addition to the bisphosphonate-derived ion at m/z 187 (Fig. 6B). By using the same approach for the analysis of extracts from alendronate-treated cells, no metabolites were found in the sample (data not shown).

In future studies, authentic standards of the metabolites of tiludronate, etidronate or alendronate would be useful for comparison of retention times, for mass spectral identification and for quantitation of the compounds. However, this study confirms that clodronate can be metabolised to AppCCl_2p by mammalian cells. Furthermore, we have found that etidronate and tiludronate are also metabolised, although to a lesser extent than clodronate. In agreement with previous studies, alendronate does not appear to be metabolised [3,5,6].

4. Conclusions

Ion-pairing HPLC–ESI-MS is an excellent new analytical method for studying the metabolism of bisphosphonate drugs. The major advantages compared to previously used techniques (FPLC and NMR) are the higher sensitivity of MS detection, and the simple sample preparation needed prior to HPLC–ESI-MS analysis.

Acknowledgements

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