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Analysis of an adenine nucleotide-containing metabolite of clodronate using ion pair high-performance liquid chromatography–electrospray ionisation mass spectrometry

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Abstract

Clodronate belongs to the family of bisphosphonates, which are synthetic analogues of pyrophosphate. Bisphosphonates are widely used in the treatment of metabolic bone diseases. Some bisphosphonates, including clodronate, can be metabolized in cells into non-hydrolysable nucleotide analogues. In this paper, we describe a new method for extraction and quantitation of the clodronate metabolite in cell lysates by using ion-pairing HPLC method that is compatible with negative ion electrospray ionization mass spectrometry (ESI-MS). The method was used for detection of the metabolite of clodronate in extracts from RAW 264 macrophage cells after treatment with clodronate.

Keywords: Clodronate

1. Introduction

Clodronate belongs to the family of bisphosphonates, which are synthetic analogues of pyrophosphate. Bisphosphonates are widely used in the treatment of metabolic bone diseases, including Paget's disease of bone, osteolytic bone metastases and postmenopausal osteoporosis [1]. Until recently it was generally believed that bisphosphonates are not metabolized. However, Rogers et al. and others have shown that clodronate and other bisphosphonates that closely resemble pyrophosphate in structure, can be metabolized by amoebae of the slime mold Dictyostelium discoideum into non-hydrolysable, adenine-containing analogues of adenosine triphosphate (ATP). The metabolite of clodronate is adenosine 5′(β,γ-dichloromethylene) triphosphate ($AppCCl_2p$) (Fig. 1) [2–4]. More recently it was demonstrated that clodronate is metabolized in vitro by intact mammalian cells, such as J774 and RAW 264 macrophages [5–7], and the metabolite may

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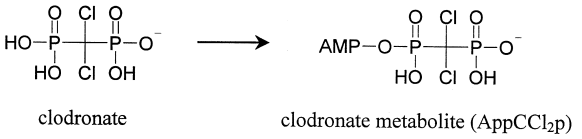

Fig. 1. An adenine nucleotide containing metabolite of clodronate is formed by a back reaction catalyzed by aminoacyl-tRNA synthetase enzymes.

actually under lie some of the cellular effects of clodronate [7]. The $AppCCl_2p$ is formed by a back reaction catalyzed by class II aminoacyl-tRNA-synthetase enzymes (Fig. 1). These enzymes can probably bind clodronate in place of pyrophosphate in the ATP binding site [2–4,8].

In initial studies the metabolite of clodronate was detected in perchloric acid cell extracts by using anion-exchange FPLC [2,3,5,7]. More recently, we developed a very sensitive analytical method to identify the metabolites of bisphosphonates in extracts of J774 macrophages [6]. This method was based on combining ion-pairing HPLC and tandem mass spectrometry. In addition to clodronate, it was found that etidronate and tiludronate are also metabolized to non-hydrolysable analogues of ATP by J774 cells [5,6], although to a lesser extent than clodronate [6]. These three bisphosphonates are non-aminobisphosphonates, which do not contain a nitrogen atom in the R_2 side chain. Nitrogen-containing bisphosphonates, such as alendronate and ibandronate are not metabolized [6], and have different mechanism of action [9].

In this paper, we describe a new method for extraction and quantitation of the clodronate metabolite in cell culture samples by using ion-pairing HPLC method that is compatible with negative ion electrospray ionization mass spectrometry (ESI-MS). The method is suitable for analysis of samples containing 0.5–40 μM of the clodronate metabolite. Nucleotides are usually separated by ion-exchange chromatography using mobile phases that are incompatible with ESI [10,11]. The use of an ion-pair is necessary for retention of $AppCCl_2p$ on the reversed-phase column because the metabolite is very hydrophilic. We tested four different ion-pairing agents; ammonia, triethylamine (TEA), tripropylamine (TPA), and dimethylhexylamine (DMHA). We chose DMHA as the ion-pairing agent and methyleneadenosine 5′-triphosphate (AppCp) as internal standard. We obtained a good retention time and a high signal intensity by using DMHA. AppCp is a very similar molecule to $AppCCl_2p$, since chlorine is replaced by hydrogen. Deoxyguanosine triphosphate (dGTP) was used for studying the effect of the buffer concentration on the signal. We also determined a few validation parameters, including linearity and reproducibility. The calibration curve appeared linear and relative standard deviation (RSD) with six replicate samples was under 10%. RAW 264 macrophage cells were treated with liposome encapsulated and free clodronate and were extracted with acetonitrile (ACN). Previously, bisphosphonates were extracted with perchloric acid (PCA) [2,3,5,7]. Compared with PCA extraction, the ACN extraction method is simple and fast, and the ACN can be removed by evaporation and thus does not interfere with the HPLC analysis, unlike the salts of perchloric acid [6,12].

2. Experimental

2.1. Chemicals

Clodronate (dichloromethylene-1,1-bisphosphonate) was kindly provided by Leiras Pharmaceutical Co. (Turku, Finland). Distearoylphosphatidylglycerol (DSPG) was obtained from Orion Farmos Co. (Turku, Finland), and cholesterol purchased from Sigma Chemical Co. (St. Louis, MO). Water was deionized using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC grade acetonitrile was from Rathburn (Walkerburn, UK), ammonia from Riedel-de Haen AG (Seelze, Germany), TEA from Fluka Chemie (Buchs, Switzerland), TPA and DMHA from Aldrich (Milwaukee, WI, USA), dGTP from Perkin-Elmer (Foster City, CA, USA), methanol from Labscan Ltd. (Dublin, Ireland), and formic acid from Merck (Darmstadt, Germany). The AppCp internal standard was obtained from Sigma Chemical Co. (St. Louis, MO). The $AppCCl_2p$ standard was a generous gift from Prof. G.M. Blackburn, Department of Chemistry, University of Sheffield, UK.

2.2. Liposome preparation

Clodronate was encapsulated in DSPG liposomes using 110 m*M* solution of bisphosphonate in deionized water as described previously [13]. The concentration of liposome-encapsulated suspension of clodronate was 7.1±0.07 m*M* (mean±SD, $n=3$). The molar drug:phospholipid ratio of liposomes was 0.71.

2.3. Liquid chromatography

2.3.1. The HPLC conditions for $AppCCl_2p$

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). The reversed-phase column was a Genesis C_{18} (50×2 mm) (Jones Chromatography, Lakewood, CO, USA), which was eluted with a mobile phase at a flow-rate of 100 μl/min. The signal intensities were doubled by using a flow-rate of 100 μl/min than using 200 μl/min (data not shown). The eluents were 20 m*M* DMHA formate with the pH adjusted to 7.0 with formic acid and 50% methanol containing 2 m*M* DMHA formate, pH 7.0. The HPLC gradient was from 10% to 50% methanol in 4 min, where it remained for an additional 4 min. The buffer concentration was decreased from 20 m*M* to 2 m*M* in 4 min.

2.3.2. Effect of ion-pairing compound on retention time

We tested four ion-pairing agents; ammonia, TEA, TPA and DMHA. The HPLC gradient was from 7% to 70% methanol in 12 min. The buffer concentration (pH 6.7) was decreased from 19 m*M* to 12 m*M* in 12 min.

2.3.3. Effect of buffer concentration on signal

The effect of buffer concentration was tested by infusion of 200 μ*M* dGTP (10 μl/min) via the T-split, at a flow-rate of 200 μl/min. The DMHA buffer concentrations were 0, 1, 2, 5, 10, 20, 50 and 100 m*M*. The response was measured by comparing the heights of peaks.

2.4. Tandem 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 100 μl/min was directed to the ESI source, which is designed to cope with flow-rates of up to 1000 μl/min. 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 225°C. The capillary voltage was −21 V and the tube lens offset was −5 V. The full scan MS/MS spectra were measured using 500 ms for collection of the ions in the trap. Following scan parameters were used; parent ion m/z 573: isolation width 6 mu, collision energy 24%, scan range was 160–700 and parent ion m/z 504: isolation width 2 mu, collision energy 20%, scan range was 140–600. The wide (6 mu) isolation width was used to record simultaneously transition m/z 572→225 (^{35}Cl ^{35}Cl) and transition m/z 574→227 (^{35}Cl ^{37}Cl).

2.5. RAW 264 cell culture

Murine macrophage-like RAW 264 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 10% of fetal bovine serum (Gibco, Grand Island, NY), and 100 IU/ml of penicillin and streptomycin (Gibco, Grand Island, NY) in a 7% CO_2 atmosphere. RAW 264 cells were seeded into 6-well plates at a density of 5×10^6 cells/well. The cells were left to adhere for 2 h before treatment.

2.6. Sample preparation

The RAW 264 cells were treated for 12 h with 30 μ*M* liposome-encapsulated and 1 m*M* free clodronate. After treatment, the cells were scraped from the well, centrifuged (220 *g*, 5 min) and washed in PBS. For the acetonitrile extraction, ACN was added to cell pellets to precipitate the macromolecules; water was added within 2 min to extract the cellular content (300 μl of ACN and 200 μl of water). The soluble and precipitated fractions were separated by centrifugation (13000 g, 1 min). The soluble supernatant extract was transferred within 10 min to fresh

eppendorf tube. The organic component of the ACN/water extract was removed in 5 min by evaporation in a stream of nitrogen [12]. The aqueous samples that remained were dried down in a vacuum centrifuge and then stored at −20°C.

2.7. Measurement of clodronate metabolite

The samples were redissolved in 100 μl of 40 m*M* DMHA formate containing 25 μ*M* AppCp as internal standard. Negative ion ESI and selective reaction monitoring (SRM) was used for analysis of the compounds in the sample. The quantitation was based on fragment ions at *m*/*z* 225 and 227 obtained by MS–MS of the molecular ion of clodronate metabolite at *m*/*z* 572 and 574. Both parent ions (*m*/*z* 572 and 574) were trapped and fragmented simultaneously by using isolation width of 6 mu. The SRM chromatogram was created for mass range of *m*/*z* 224–229. The fragment ion of internal standard at *m*/*z* 406 was obtained by MS–MS of the deprotonated molecule at *m*/*z* 504. The standard curve was created by spiking extracts from untreated cells with $AppCCl_2p$ over a concentration range of 0.5 μ*M* to 40 μ*M*. The concentrations of the samples were determined using the peak areas of the SRM chromatograms and the standard curve.

3. Results and discussion

3.1. Sensitivity of ion-pairing HPLC

The retention of $AppCCl_2p$ in a C_{18} reversed-phase column was too low using ammonia or TEA as a ion-pairing compound (Fig. 2A, B). DMHA was

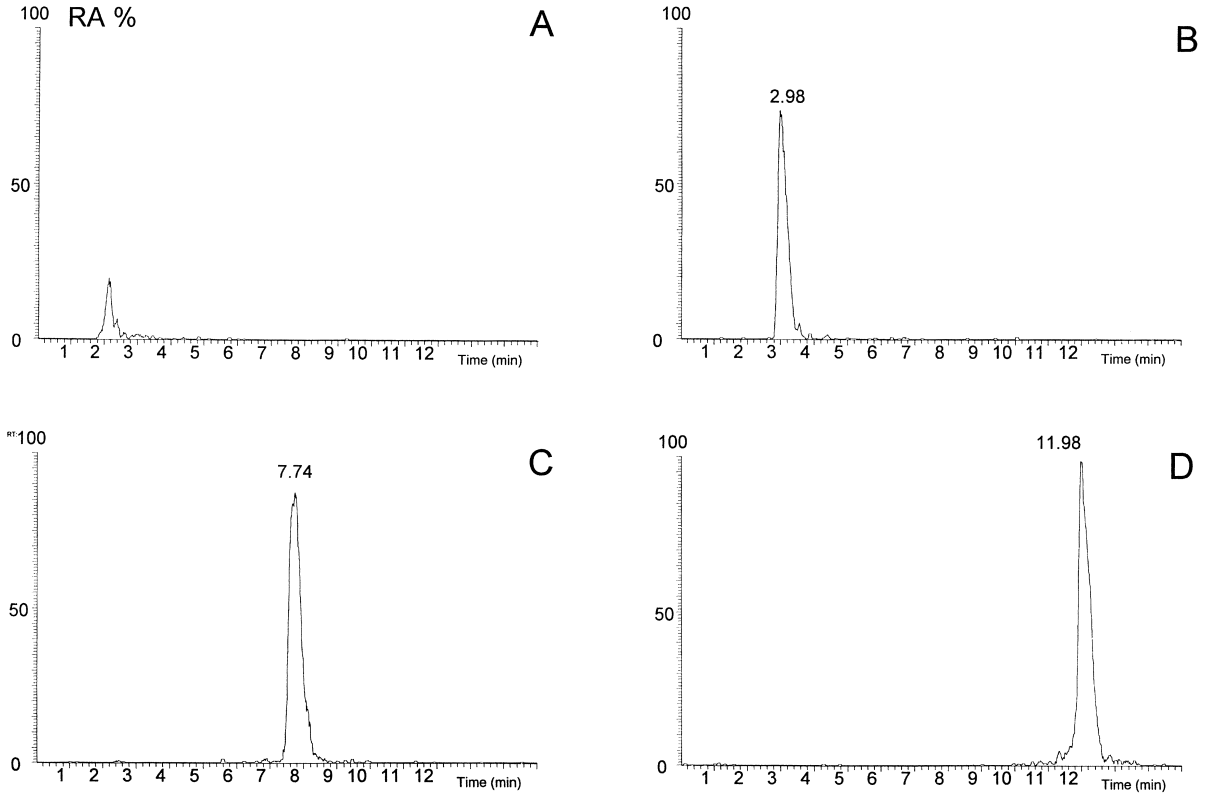

Fig. 2. The effect of ion-pairing compound on retention time. Selected reaction monitoring (SRM) chromatograms obtained with $AppCCl_2p$ standard using as ion-pairing agents (A) ammonia, (B) TEA, (C) TPA and (D) DMHA. Conditions: Genesis RP C_{18} column, 50×2 mm. The gradient was from 7 to 70% methanol and 19–12 m*M* buffer (pH 6.7) in 12 min, at a flow-rate of 100 μl/min.

the most efficient ion-pairing agent of these four compounds (Fig. 2D). The use of DMHA resulted also in the highest signal intensity (peak areas) (Fig. 3).

High DMHA concentration is needed for the ion-pairing. When the DMHA concentration was decreased from 16 m*M* to 2 m*M* in 4 min, sensitivity was 2.5 (2 μ*M*) and 3.5 (0.5 μ*M* $AppCCl_2p$) times bigger than using 20 m*M* DMHA continuously (data not shown). $AppCCl_2p$ was eluted from the column in 50% methanol and 2 m*M* buffer, which is a favorable composition for the ionization process (Fig. 4).

3.2. Mass spectrometry of the clodronate metabolite

The full scan negative ion mass spectra of $AppCCl_2p$ and the internal standard show deprotonated molecular ions $(M-H)^-$ as the base peaks (Figs. 5A and 6A). The isotopic pattern of the metabolite, showing major ions at *m*/*z* 572, 574 and 576, is typical for a compound containing two chlorine atoms (Fig. 5A). The corresponding sodium adducts ions $(M-2H+Na)^-$ can be seen at *m*/*z* 594, 596 and 526 (Figs. 5A and 6A). The quantitation of metabolite was based on fragment ions at *m*/*z* 225 and 227, which are formed by cleavage of the bisphosphonate moiety from the conjugate (Fig. 5B). The ion monitored for the internal standard with MS–MS was 406 (Fig. 6B) which was originated by loss of phosphoric acid from the deprotonated molecule at *m*/*z* 504 (Fig. 6A). The highest signal intensities were obtained by using these fragment ions.

The SRM chromatograms of untreated cell extract, $AppCCl_2p$ standard, cells treated with liposome encapsulated clodronate, and the internal standards are shown in Fig. 7. The major components did not show up in the chromatogram of untreated cell extract (Fig. 7A). The substantial peaks showed up in the chromatogram of $AppCCl_2p$ standard and the treated cells (Fig. 7C and E, respectively), and also the chromatograms of AppCp internal standards of all samples (Fig. 7B, D, F). The HPLC peaks of compounds eluted with their own channels and the retention times were 8.3 min ($AppCCl_2p$) and 8.1 min (AppCp). The treated cell extract contained 24.4 μ*M* of the metabolite, redissolved in 100 μl volume (Fig. 7E).

We applied this analysis technique to study the kinetics of clodronate metabolism in RAW 264 cells. Five million cells were exposed to clodronate for 1–24 h. The metabolite was found already 1 h after

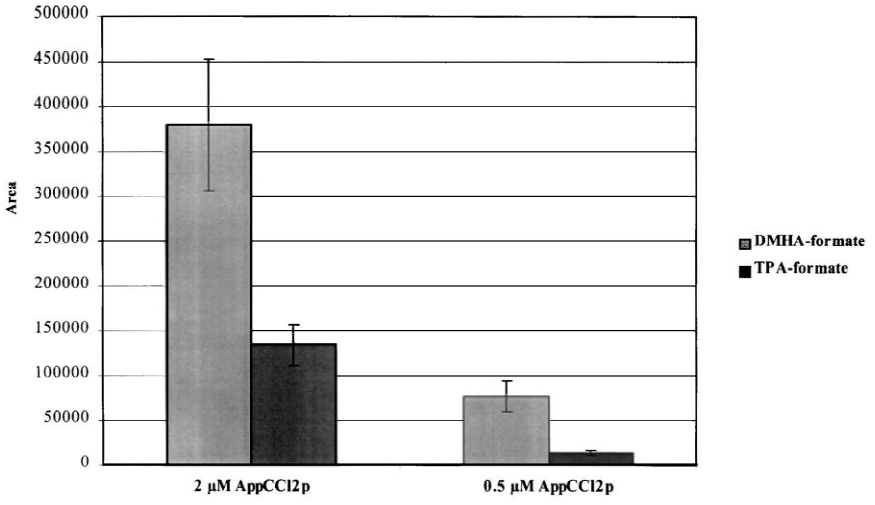

Fig. 3. The effect of DMHA and TPA ion-pairing agent on metabolite peak area. Conditions: Genesis RP C_{18} column, 50×2 mm. Eluent A was 10% methanol, 16 m*M* buffer (pH 7). Eluent B was 50% methanol, 2 m*M* buffer (pH 7). The gradient was 0–100% B in 4 min, where it remained for an additional 7 min, at a flow-rate of 100 μl/min.

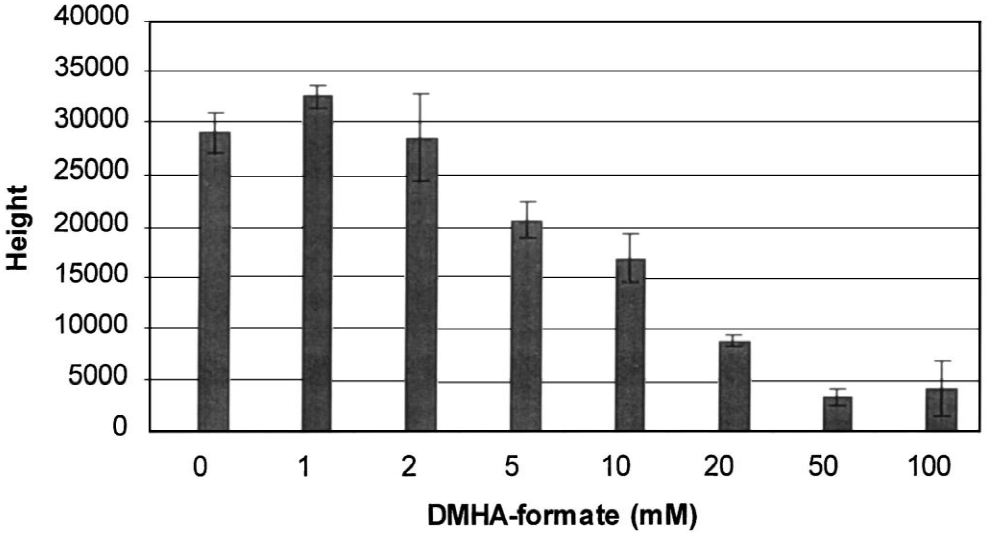

Fig. 4. The effect of buffer concentration on the ionization process using 200 μ*M* dGTP. dGTP was infused via T-split (10 μl/min) to the buffer eluent (200 μl/min in 30% methanol).

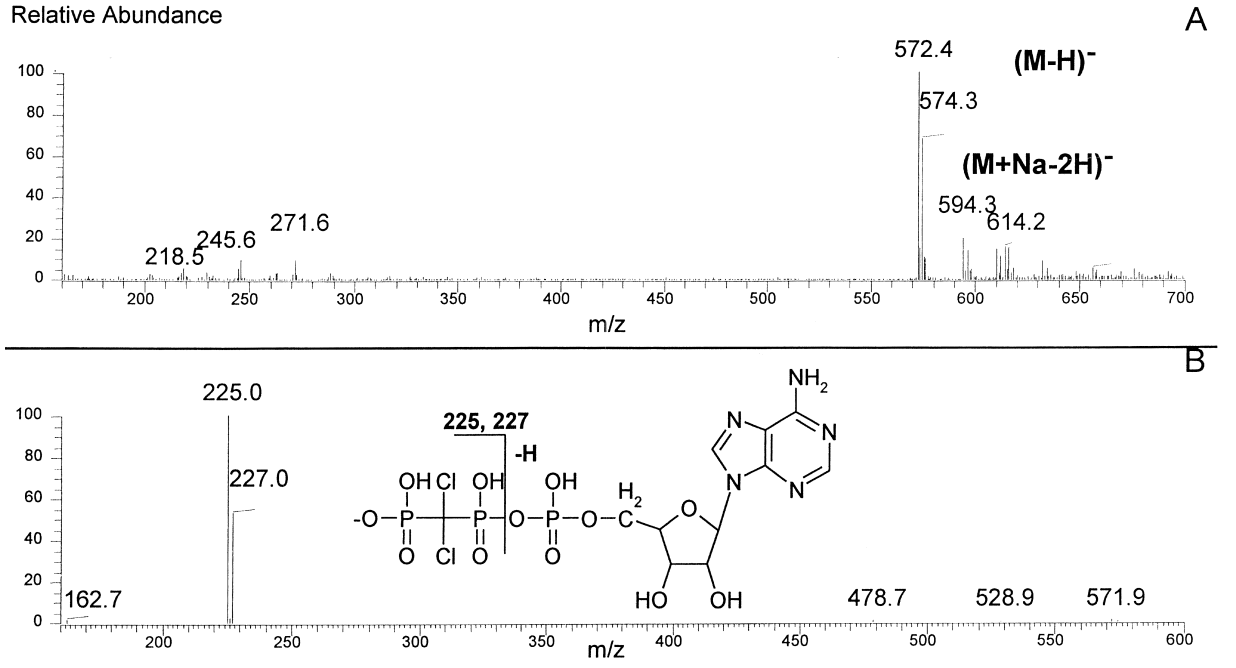

Fig. 5. (A) Full scan negative ion ESI mass spectrum of the $AppCCl_2p$ standard and (B) MS–MS spectrum of $AppCCl_2p$. Conditions: Genesis RP C_{18} column, 50×2 mm. Eluent A was 20 m*M* DMHA buffer (pH 7). Eluent B was 50% methanol, 2 m*M* buffer (pH 7). The gradient was 20–100% B in 4 min, where it remained for an additional 4 min, at a flow-rate of 100 μl/min.

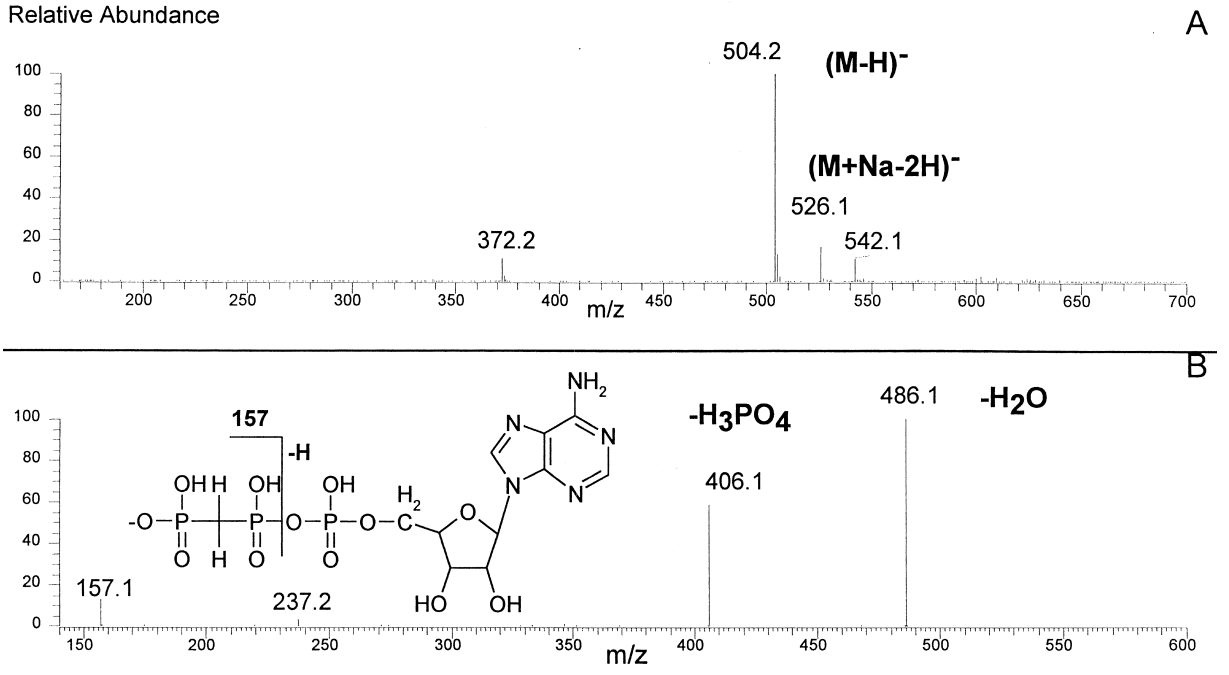

Fig. 6. (A) Full scan negative ion ESI mass spectrum of the AppCp internal standard and (B) MS–MS spectrum of AppCp. Conditions as in Fig. 5.

treatment, and the concentration gradually increased during the first 12 h of clodronate treatment, then reaching a plateau (Fig. 8).

3.3. Linearity and reproducibility

The weighting factor of the calibration curve was chosen as 1/concentration. The calibration curve was created by spiking extracts from untreated cells over a concentration range of 0.5 μ*M* to 40 μ*M* $AppCCl_2p$ (six point curve, three replicate standards). The calibration curve appeared linear and the equation of regression line was $y=-0.0384537+0.0911912x$ ($R^2=0.9807$).

The reproducibility of the analysis method was tested by multiple injections of 2 μ*M* and 20 μ*M* quality control sample ($n=6$), the RSDs were 4.9% and 6.7%, respectively. The reproducibility of the extraction method was tested by analyzing six replicate samples treated with 1 m*M* free clodronate for 12 h, the RSD was found to be 8.3%.

This analysis technique is sensitive and reproductive enough to study the kinetics of clodronate metabolism over a concentration range of 0.5 μ*M* to 40 μ*M* ($AppCCl_2p$) in RAW 264 macrophages.

4. Conclusions

Ion-pairing HPLC–ESI-MS is a sensitive method for quantitation of the adenine nucleotide-containing metabolite of clodronate ($AppCCl_2p$) in cell extracts. Acetonitrile extraction is simple and very suitable for HPLC analysis. This technique can be applied to study the metabolism of bisphosphonates in vitro and in vivo.

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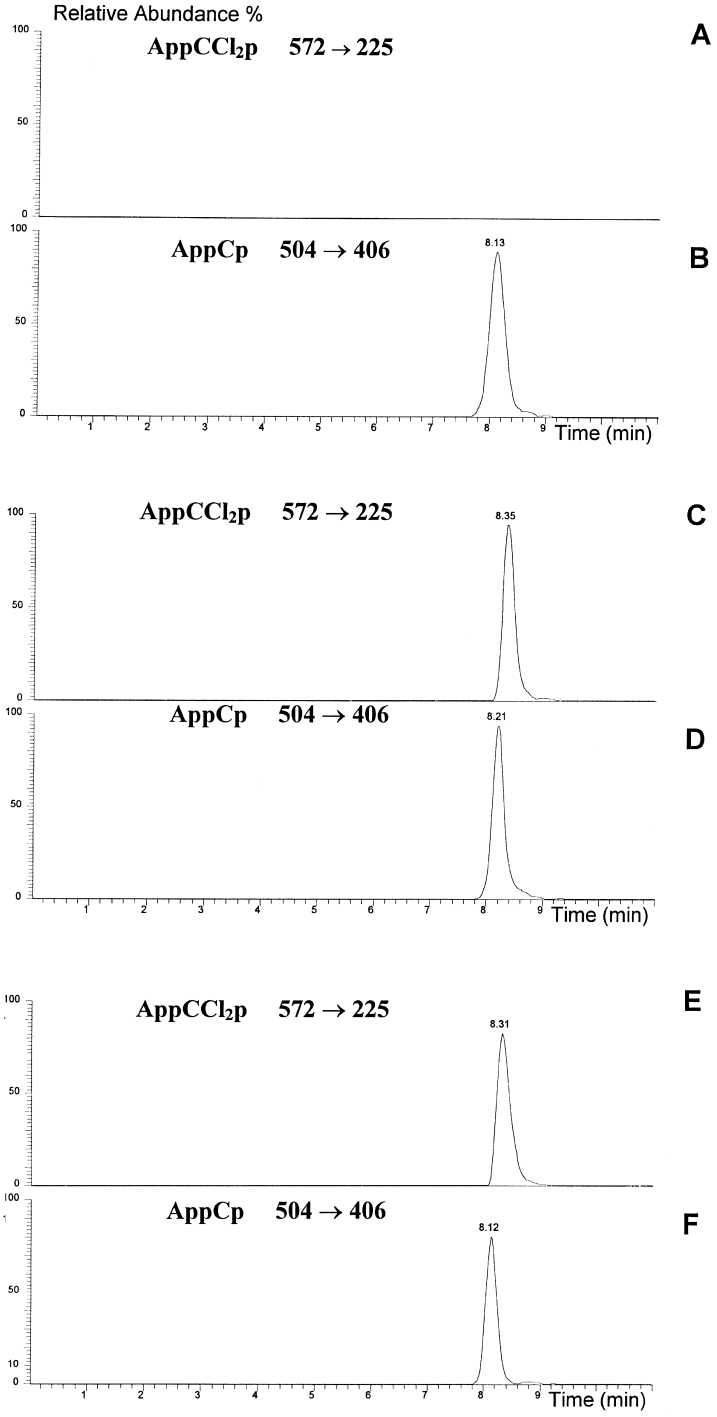

Fig. 7. Selective reaction monitoring chromatograms of $AppCCl_2p$ and AppCp. (A) untreated cell extract, (B) internal standard in the untreated cell extract, (C) cell extract spiked with 20 μM $AppCCl_2p$, (D) internal standard in the spiked cell extract, (E) cells treated with 30 μM liposome encapsulated clodronate for 12 h and (F) internal standard in the treated cells. Conditions as in Fig. 5.

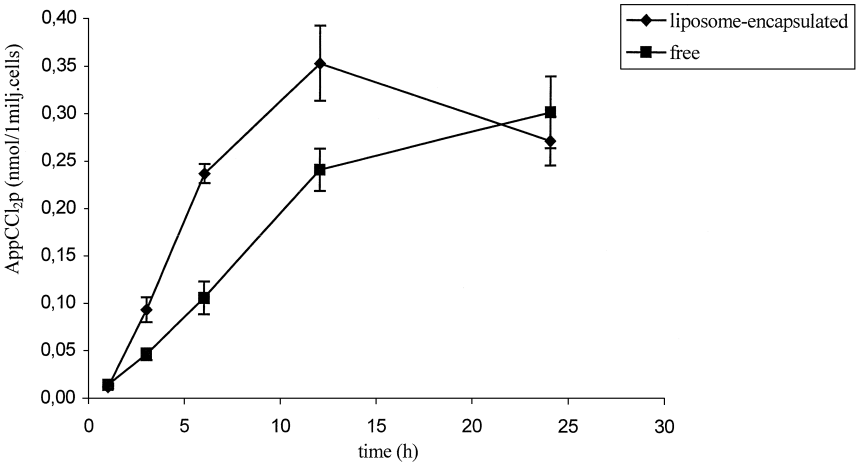

Fig. 8. The accumulation of the metabolite of clodronate in RAW 264 macrophage cells after liposome-encapsulated (30 μ*M*) and free clodronate (1000 μ*M*) treatment ($n=3$). The metabolite ($AppCCl_2p$) has been calculated as molar amount per million cells.

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