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Quantitation of Zoledronic Acid in Murine Bone by Liquid Chromatography Coupled with Tandem Mass Spectrometry

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Abstract

An *in vitro* method for extraction and quantification of zoledronic acid (ZA) from murine bone was developed. Whole mouse bones were incubated in ZA solutions with predetermined concentrations and bound ZA was subsequently extracted from bone with phosphoric acid and derivatized using trimethylsilyl diazomethane (TMS-DAM). ZA tetra-methyl phosphonate was quantified by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). This resulted in a sensitive, accurate, and precise method that was linear over three orders of magnitude (0.0250–50.0 µg/mL ZA). For quality control (QC) samples, intra-and inter-day coefficients of variance were calculated and were less than 10%. This method was then applied to an *in vivo* model to quantitate ZA from the femur and mandible of three mice treated with ZA for two weeks. The mean ZA extracted from the mandible was four fold higher than that extracted from the femur (3.06 ± 0.52 vs. 0.76 ± 0.09 ng/mg respectively) indicating that ZA did not distribute equally in the skeleton and had a preference to the mandible. In conclusion, a highly sensitive method to measure ZA from mouse skeleton was developed, which can be easily adapted to multiple mammalian models including humans receiving ZA treatment.

Keywords

zoledronic acid; liquid chromatography; tandem mass spectrometry; bone

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Author Contributions

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1. Introduction

Bisphosphonates are widely used for the treatment of osteoporosis and bone malignancies [1, 2]. They are synthetic pyrophosphate analogues that retain a high affinity for hydroxyapatite, a major component of skeletal tissues [3–5]. Zoledronic acid (ZA) [1-hydroxy-2-(1*H*-imidazol-1-yl)ethane-1,1-diyl]bis(phosphonic acid) (Figure 1), a commonly prescribed nitrogen containing bisphosphonate, binds to bone and inhibits osteoclast function [6]. This effectively prevents bone resorption and results in an increase in bone mass and a decrease in skeletal fractures [7]. Bisphosphonate therapy successfully reduces skeletal symptoms, including severe pain, in cancer patients with bone metastasis. However, an unfortunate and devastating severe side effect of this treatment is the development of bisphosphonate associated osteonecrosis of the jaw (BONJ) [8–10]. BONJ occurrence can be as high as 10% in ZA treated patients [11]. It is very difficult to treat and can result in marked facial disfigurement and associated pain. Although the pathophysiology of BONJ remains unclear, there is a higher incidence of necrosis in the mandible compared to other bones, which is suggestive of a selective toxicity [12].

After intravenous administration of ZA, the compound partitions between the kidney and the skeleton [13]. It is reported that approximately 40% of the administered dose is excreted in the urine unchanged after 24 h [14]. The other 60% remains in the skeleton and binds reversibly and with high affinity to hydroxyapatite. This occurs primarily at sites of high bone turnover. As it is removed from the bone surface, ZA is then continuously excreted from the urine over a long elimination phase [15]. The half-life of this second phase could be months or even years depending on the duration of ZA treatment [16].

The concentration of bisphosphonates in a specific bone location depends on bone turnover rate and blood circulation [17]. It is critical to determine the concentration of ZA sequestered in different types of bone to understand its long-term pharmaceutical effect and toxicity. Currently, researchers rely on estimating bisphosphonate skeleton concentrations by measuring the amount of drug in plasma and urine after derivatization.[18] However, this approach does not allow for determination of concentration differences in bisphosphonate localization within the skeleton due to areas of high turnover and trabecular space. In order to determine the variability in skeletal uptake, studies have focused on the administration of fluorescent or radiolabeled derivatives of the bisphosphonates to animals [19–22]. However, these studies could also be limited because it is unknown if these modified compounds bind to and are retained within the skeleton to the same extent as the unlabeled compound. Also, the development of a mass spectrometry based analytical assay would be preferable over a radioassay due to storage purposes and the safety precautions required for handling radiolabeled compounds.

Currently, there are no LC/MS/MS methods that can extract and quantitate ZA from the skeleton. The development of acceptable methods to quantitate bisphosphonates from blood and urine using mass spectrometry based methods has been challenging due to the highly polarity of these compounds. ZA must be derivatized to a less polar compound before it can be retained on reverse phase LC columns and ionized effectively [23]. Recent studies have demonstrated the success of derivatizing ZA in urine and plasma with TMS-DAM to produce ZA tetra-methyl phosphonate [24]. The ZA tetra-methyl phosphonate is less polar than its parent and can be used for LC/MS/MS based methods.

Herein, the development of a novel method for extraction and quantitation of ZA from murine bone is reported. Similar to previous work, TMS-DAM was used to derivatize ZA prior to analysis by LC/MS/MS. This method was successfully used to quantitate ZA extracted from the mandible or femur of mice treated with ZA.

2. Materials and Methods

2.1 Chemicals

ZA hydrate and $^{15}\text{N}_2$ $^{13}\text{C}_2$ -ZA were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Supelclean® strong anion exchange cartridges for solid phase extraction (SPE) were purchased from Supelco (Belafonte, PA). ZA (Zometa®, Novartis Pharma AG, Basel, Switzerland) used in the animal studies was purchased from University of Washington Drug Services (Seattle, WA). LC/MS grade acetonitrile and water were purchased from Fisher Scientific (Pittsburgh, PA). TMS-DAM (2.0 M in ether) and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Mass spectrometry

All data were acquired using electrospray ionization (ESI) on a Waters Micromass Quattro tandem quadrupole mass spectrometer operating in the positive mode. The instrument was controlled using Micromass MassLynx 4.1 software. Multiple reaction monitoring mode was used to monitor both ZA and internal standard. ZA was monitored using the m/z 329.1 $[\text{M} + \text{H}]^+$ to m/z 203.1 transition and $^{15}\text{N}_2$ $^{13}\text{C}_2$ ZA was monitored using the m/z 333 $[\text{M} + \text{H}]^+$ to m/z 207.1 transition. The cone voltage and collision energy were optimized to 30 and 20, respectively. The dwell time was set to 400 ms for both compounds. The source temperature and desolvation temperature were set to 120 and 350 °C, respectively.

2.3 Chromatography

A gradient was developed to separate ZA tetra methyl phosphonate and $^{15}\text{N}_2$ $^{13}\text{C}_2$ ZA tetra methyl phosphonate from the background interference on a Shimadzu high performance liquid chromatograph (HPLC). The HPLC was equipped with a temperature-controlled autosampler, a direct infusion syringe pump, an inline vacuum degasser and a binary pump. The autosampler temperature was maintained at 4 °C and 3 μL of each sample was injected onto a Phenomenex, Synergi 4 u Hydro-RP 80 A column (2.0 mm $\times$ 50 mm, 4 μm). The mobile phase consisted of 10 mM formic acid (A) and 10 mM formic acid in acetonitrile (B) with a flow rate of 0.3 mL/min. The initial percentage of 2% B was held for one min, increased to 30% B over a min, held for one min, immediately increased to 90% B for 2 min, and re-equilibrated at 2% B for two min.

2.4 Preparation of zoledronic acid stock solutions

A 10.0 mg/mL stock solution of ZA was prepared in 0.1 N NaOH and used to make twelve calibration standards in H_2O ranging from 5.00 mg/mL to 0.00250 mg/mL. The calibration standards were then diluted 100 fold in 0.2 M H_3PO_4 to produce a working standard curve ranging from 50.0 $\mu\text{g/mL}$ to 0.0250 $\mu\text{g/mL}$. In order to prepare the quality control (QC) standards, a separate stock of 10.0 mg/mL ZA was prepared in 0.1 N NaOH. QC samples were dissolved in either 1 $\times$ PBS or 0.2 M H_3PO_4 to final concentrations of 0.500, 5.00, and 25.0 $\mu\text{g/mL}$. The calibration standards and QC samples were then prepared for LC/MS/MS analysis as described in section 2.8 below. A 0.500 mg/mL stock of internal standard was dissolved in H_2O and 10.0 μL of the standard was added to each sample before processing. All solutions were stored at -20 °C until used.

2.5 Zoledronic acid adsorption to bone, in vitro

An *in vitro* model of ZA absorption to bone was developed by incubating femurs, mandibles, or cranium obtained from untreated mice in a solution containing ZA concentrations of 0.500, 5.00, and 25.0 $\mu\text{g/mL}$ in 1 $\times$ PBS. Bones were dissected surgically from mice and excess soft tissue was removed and the bones were stored in 1 $\times$ PBS containing 0.02% sodium azide solution at 4°C until use. On the day of experiment, bones

were removed from storage and washed in 1× PBS, dried to remove excess moisture, weighed, and placed into 2 mL of ZA solutions. The bones were incubated at room temperature (RT) for 48 h with vigorous shaking, then removed from solution, washed with 1× PBS (2×, 0.5 mL), and processed as described below in the next section. Due to the lack of total adsorption of ZA to the bone surfaces, the wash and ZA solution were combined in a 15 mL falcon tube, internal standard was added ($^{15}\text{N}_2$ $^{13}\text{C}_2$ ZA, 10.0 μL , 0.500 mg/mL), labeled as “ZA from spiked bone”, and kept for further processing on an SPE column.

2.6 Treatment of CD-1 mice with zoledronic acid

Six-week old male CD-1 mice (N = 3) (Charles River Laboratories International, Inc., Wilmington, MA) were treated with three weekly doses of 160 μg (200 μL) ZA administered by subcutaneous injection. As a control, subcutaneous injections of saline (200 μL) were administered to age- and gender- matched CD-1 mice (N = 3) at the same time as ZA. A week after the last injection, mice were euthanized with CO_2 and sacrificed by cervical dislocation. In order to generate an *in vitro* model of ZA adsorption, additional 6 week old male CD-1 mice were sacrificed (N = 7). Mandibles, femurs, and cranium were dissected and rinsed with saline. The bones were stored in a 1× PBS solution containing 0.02% sodium azide at 4 °C. The protocol for mouse care, the method by which they were sacrificed, and experimental protocols were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC).

2.7 Extraction of zoledronic acid from mouse bone

Prior to extraction, bones from treated animals and spiked bone samples were removed from their storage solution, washed with 1× PBS (2×, 0.5 mL), blotted with a tissue, and weighed. The wash was collected and processed to ensure that ZA did not leach into the storage solution. Bones from treated or spiked ZA samples were first flash frozen in liquid nitrogen and placed into 1 mL of 0.2 M H_3PO_4 containing internal standard ($^{15}\text{N}_2$ $^{13}\text{C}_2$ ZA, 0.01 mL, 0.500 mg/mL) and Teflon beads (MO BIO Laboratories Inc.). The bones were placed into a Bertin Technologies, Precellys 24 lysis and homogenization system coupled with the Cryolys advanced temperature controller. A dry ice and acetone bath was used as the coolant. The bones were then homogenized at 6500 rpm 2×10 s until only a fine particulate remained. The particulate was centrifuged at RT for 10 min at 13,000 $\times g$. The supernatant was carefully removed with a pipette and placed into a 15 mL tube labeled “ZA pellet”. The remaining pellet was resuspended in 1 mL of 0.2 M H_3PO_4 , vortexed, and centrifuged again. The supernatant was removed and combined with the supernatant from the first pellet.

2.8 Preparation of zoledronic acid samples for LC/MS/MS analysis

Before addition of the samples onto the SPE column, 10 mM NH_4HCO_3 was added to each sample. The SAX SPE cartridges were preconditioned with methanol (1 mL) and then 2:1 10 mM NH_4HCO_3 :0.2 M H_3PO_4 (1 mL). The columns were then washed with 10 mM NH_4HCO_3 (1 mL), H_2O (1 mL), and methanol (2 mL). Immediately following the methanol washes, the stopcock was closed and 2.0 M TMS-DAM in ether (0.1 mL) was added directly to the column. This was followed immediately by the addition of methanol (0.75 mL) and the reaction was allowed to proceed for 60 min at RT. The resulting tetra methyl ZA phosphonate was eluted with methanol (2 mL), concentrated under nitrogen with a water bath heated to 40 °C, and reconstituted in 1:1 methanol: H_2O (0.2 mL).

2.9 Method validation

Plotting peak height normalized to internal standard versus ZA concentration and calculating the equation of the line determined a linear relationship between twelve calibration standards of ZA and peak height. The linearity was deemed acceptable if the calculated

coefficient of determination (R^2) was 0.997. Analyzing three separate determinations of the calibration curve on the same day and then repeating the procedure twice more on subsequent days determined the intra- and inter-day variation in slope and y-intercept. The range of the calibration curve was determined by the upper and lower limits of the calibration standards and a data point outside of this range could not be quantitated. Quality control (QC) standards at three different concentrations of ZA (low, medium, and high) that contained bone or no bone were used to determine the accuracy and precision of the method. For the no bone QCs, intra- and inter-day determinations were performed by conducting five replicates of the QCs and analyzing the samples on the same day and then preparing one replicate and analyzing them on three subsequent days. The stability of ZA in 0.2 M H_3PO_4 was determined by preparing QC samples $1\times$ PBS and derivatized on column using the method described above. There was no difference between QC samples derivatized from $1\times$ PBS or 0.2 M H_3PO_4 . The QC samples prepared with bone were compared to no bone QCs to determine if there were any matrix effects due to extraction from bone tissue or stability issues with ZA over the incubation period of 48 h used to prepare the bone QCs. The percent coefficient of variance (%CV) was considered acceptable only if the calculated value was within $\pm 15\%$. The stability of ZA was determined by analyzing three subsequent freeze ($-20^\circ C$) thaw cycles of the calibration curve and left out at RT for 6 h. The specificity of the method was assessed by determining the absence of peaks corresponding to the ZA tetra methyl phosphonate in methanol compared to a spiked ZA sample in methanol that had been derivatized with TMS-DAM).

3. Results and Discussion

3.1 Mass spectrometry

Due to bisphosphonates being polar, hydrophilic molecules with a strong affinity for metal ions, they must first be derivatized to a more hydrophobic molecule in order to be retained on a reversed phase HPLC column and analyzed by ESI mass spectrometry [24]. Several publications have used diazomethane to improve the volatility of carboxylic acid containing compounds for use in liquid and gas chromatography mass spectrometry [25]. However, the synthesis and storage of diazomethane is difficult due to its chemical instability and explosive nature. As a safer alternative to diazomethane, a more stable form, TMS-DAM, was utilized to derivatize ZA [26, 27]. Derivatization of 25.0 $\mu g/mL$ ZA in methanol using 0.1 mL TMS-DAM yielded the tetra methyl phosphonate that could easily be ionized by ESI to yield the $[M + H]^+$ m/z 329 as the predominant ion (Figure 2A). Other peaks were also observed and determined to be the penta methylated ZA phosphonate, $[M + H]^+$ m/z 351, $[M + Na - \text{imidazole}]^+$ m/z 283, $[M + H - \text{imidazole}]^+$ m/z 261. Tandem mass spectrometry parameters were then optimized to fragment the $[M + H]^+$ of the tetramethyl derivative and production of the daughter ion at m/z 203.1 (Figure 2B).

3.2 Chromatography

Standards of ZA and the internal standard, $^{15}N_2$ $^{13}C_2$ ZA, were derivatized in methanol with TMS-DAM (2.0 M in ether) at RT for 1 h to verify peak identity and to determine optimal chromatographic conditions. A Synergi Hydro-RP 80 A column (2.0 mm $\times$ 50 mm, 4 μm) was used because this column is suitable for chromatographic analysis using high aqueous mobile phase conditions. The mobile phase consisted of water and acetonitrile with formic acid to help with the ionization of the ZA tetra-methyl phosphonate and maximize signal intensity. A representative chromatogram of ZA tetra-methyl phosphonate extracted from the mandible of a treated mouse is shown in Figure 3D. A sample containing only 0.2 M H_3PO_4 and bone was used as a negative control to determine any interfering peaks from the biological matrix (Figure 3B). There was a minor peak at a retention time similar to the ZA methyl phosphonate in a sample containing 0.2 M H_3PO_4 derivatized with TMS-DAM on

column (Figure 3C). However, this peak could not be quantitated using the calibration curve and yielded a concentration of the ZA methyl phosphonate that was $<0.0003 \mu\text{g/mL}$. Furthermore, this peak was not present in chromatograms that contained only methanol and therefore is not system contamination or carryover, but is a result of the derivatization method used. This peak was less than 20% of the lower limit of quantitation (LLOQ), which was $0.0250 \mu\text{g/mL}$ of the ZA tetramethyl phosphonate (Figure 3B and C). Therefore the method was still considered to be selective for the detection of the ZA tetra-methyl phosphonate at this retention time. [28] The internal standard, $^{14}\text{N}_2$ $^{13}\text{C}_2$ ZA tetra methyl phosphonate, was used to determine any losses due to the handling, extraction, and derivatization of ZA (Figure 3E). No interfering peaks were present in the MRM channel for the internal channel (Figure 3F). The average retention time for the ZA tetra-methyl phosphonate was 2.74 ± 0.10 min throughout an 8 h run (Figure 3C). The average retention time for the internal standard was $2.74 \text{ min} \pm 0.10 \text{ min}$ throughout a 4 h run (Figure 3F).

3.3 Development of an *in vitro* model of zoledronic adsorption to mouse bone

In order to generate an LC-MS-MS based method to extract ZA from bone, an *in vitro* model of ZA adsorption to bone was required to develop a suitable extraction procedure. All bones were removed from storage in $1 \times \text{PBS}$ containing 0.02% sodium azide. Bones samples must be processed wet because ZA could not be detected from dry bones using this extraction method. Therefore, whole mouse bone, dried with a tissue paper, was weighed before placing into $1 \times \text{PBS}$ solution containing either 0.500, 5.00, or $25.0 \mu\text{g/mL}$ ZA. The samples were incubated for 48 hr at RT with shaking. To begin the extraction procedure, bones were flash frozen in liquid N_2 , immersed in 1 mL of 0.2 M H_3PO_4 containing internal standard, and homogenized to a fine powder. A previous study reported the removal of hydroxyapatite bound pamidronate by co-precipitation of calcium phosphate using a strong acid and subsequent solubilization of the pellet in H_3PO_4 [29]. For this study, H_3PO_4 , at a concentration of 0.2 M, was chosen to remove ZA bound to bone by simultaneously decalcifying and solubilizing calcium salts.

The samples were centrifuged, the supernatant was removed, and washed once with 1 mL of 0.2 M H_3PO_4 . Bones must be homogenized in tubes containing 2.8 mm ceramic (zinc oxide) beads. The use of metal beads during the homogenization process resulted in a significant loss of ZA. The supernatant could be stored for up to two weeks at 4°C without significant degradation of ZA which confirms the stability of ZA. After homogenization, SPE facilitated the removal of impurities and the derivatization of ZA for analysis by LC/MS/MS. In order to determine the suitability of the *in vitro* adsorption model, the efficiency of ZA and internal standard extraction was calculated by comparing the amount of compound extracted from bone compared to ZA spiked into 0.2 M H_3PO_4 and extracted. However, even after incubation of the bones in spiked ZA solutions for 48 h, complete adsorption of ZA to the bone surface could not be achieved. This was determined by detection of residual ZA in the spiked $1 \times \text{PBS}$ solution, which suggested that saturation of ZA adsorbing to the bone surface occurred and does not reflect an accurate measurement of ZA uptake *in vivo* [30]. The average ($\pm$ S. D.) amount of ZA extracted from bone pellets from quality control (QC) samples spiked and incubated with either $0.500 \mu\text{g/mL}$ ($N = 7$), $5.00 \mu\text{g/mL}$ ($N = 8$), or $25.0 \mu\text{g/mL}$ ($N = 7$) was $0.449 \pm 0.030 \mu\text{g/mL}$, $3.90 \pm 0.33 \mu\text{g/mL}$, and $14.0 \pm 1.5 \mu\text{g/mL}$, respectively. This was in comparison to QC samples spiked with same amount of ZA but containing no bone. The average ($\pm$ S. D.) amount of ZA extracted from QC samples spiked with either $0.500 \mu\text{g/mL}$ ($N = 8$), $5.00 \mu\text{g/mL}$ ($N = 8$), $25.0 \mu\text{g/mL}$ ($N = 8$) in 0.2 M H_3PO_4 was $0.492 \pm 0.031 \mu\text{g/mL}$, $4.94 \pm 0.07 \mu\text{g/mL}$, and $25.1 \pm 0.8 \mu\text{g/mL}$, respectively. The extraction efficiencies (average $\pm$ SD) of ZA from the pellet of three concentrations of samples were: $0.500 \mu\text{g/mL}$, $67.3\% \pm 11.3$ ($N=7$), $5.00 \mu\text{g/mL}$, $66.5\% \pm 12.9$ ($N = 8$), and $25.0 \mu\text{g/mL}$, $69.2\% \pm 19.6$ ($N = 7$) and the extraction efficiencies for the internal standard the

concentrations were: $72.2\% \pm 13.8$, $74.9\% \pm 22.0$, $70.1\% \pm 15.8$, respectively. There was no significant difference between the extraction efficiency of ZA and that of the internal standard. This validated the use of the internal standard in this assay to effectively decrease any discrepancies in extraction of ZA from the bone due to differences in bone type and size.

3.4 Performance

In order to validate an analytical method it must be reproducible, precise, and accurate [31]. A standard curve containing twelve different concentrations of ZA was desired in order to effectively determine the linearity and robustness of this method. The limit of detection was 2.50 pg of the ZA tetra-methyl phosphonate on column. The LLOQ was determined to be 0.0250 $\mu\text{g/mL}$ of ZA due to the back calculated concentration being within $\pm 20\%$. In order to quantitate ZA from murine bone, standard curves were prepared by spiking known concentrations of ZA into 0.2 M H_3PO_4 . The standard curves obtained were linear over three orders of magnitude from 0.0250 $\mu\text{g/mL}$ – 50.0 $\mu\text{g/mL}$. For analysis, curves were split into a low curve from 0.0250 $\mu\text{g/mL}$ – 0.500 $\mu\text{g/mL}$ and the total curve from 0.0250 $\mu\text{g/mL}$ – 50.0 $\mu\text{g/mL}$. The intraday and interday variability of the slope and intercept $\pm$ S.D. for the low curve was 0.290 ± 0.007 and 0.00260 ± 0.0004 ($N = 3$) and 0.286 ± 0.008 and 0.00220 ± 0.0007 ($N = 5$), respectively. The intraday and interday variability of the slope and intercept for the total curve was 0.291 ± 0.007 and 0.0354 ± 0.0059 ($N = 3$) and 0.290 ± 0.008 and 0.0150 ± 0.0295 ($N = 5$) respectively. All regression coefficients were $R^2 > 0.999$. The ZA tetra-methyl phosphonate standard curve was stable after three freeze (-20°C) thaw cycles.

Intraday and interday accuracy and precision was assessed using QC samples at three concentrations: 0.500 $\mu\text{g/mL}$ (low QC), 5.00 $\mu\text{g/mL}$ (middle QC), and 25.0 $\mu\text{g/mL}$ (high QC). The QC samples were prepared either by spiking ZA directly into 0.2 M H_3PO_4 or incubating ZA with cranium, mandible, or femur in $1\times$ PBS for 48 h at RT. There was no change in QC samples prepared in $1\times$ PBS and immediately extracted in terms of accuracy. ZA QC samples prepared from whole mouse bone were weighed before placing into spiked ZA solution to normalize any difference in the amount of bone added to each sample. As shown in Table 1 and 2, for both intra- and inter-day the %CV for all samples was less than 10% and the percent accuracy ranged from 89–101%. There were no differences between QC samples extracted with or without bone in terms of intra- and inter- day precision and accuracy (Table 1 and 2). Therefore, the preparation of a standard curve from spiked ZA in 0.2 M H_3PO_4 is acceptable for quantitation of ZA from treated mice.

3.5 Biological samples

ZA was extracted from the mandible and femur of three treated mice and the results are presented in Figure 6. The amount of ZA extracted was normalized to the weight of the bone and reported as ng/mg. The concentration of ZA in the mandible of treated mice was four fold higher than in the femur (Figure 4). The mean ZA extracted from the mandible was 3.06 ± 0.52 ng/mg and the mean from the femur was 0.76 ± 0.09 ng/mg. A Student's unpaired t test was used to determine if there was a significant difference between the amount of ZA in the mandible versus the femur. A significant difference was defined by a P value < 0.05 . There was a significant difference between the amount of ZA extracted from the mandible compared to that of the femur ($P = 0.0017$).

4. Conclusion

In conclusion, a sensitive and reproducible method to quantitate ZA from murine skeleton was developed. This is the first report of a sensitive LC/MS/MS based method for the quantitation of ZA from murine bone. The key features of this assay include the use TMS-

DAM to derivatize ZA into a compound suitable for separation and ionization using LC/MS/MS. In order to extract ZA, whole bones were homogenized in 0.2 M H₃PO₄ and impurities were removed using SPE. The accuracy and reproducibility of this method were determined using QC samples that were prepared either by extracting ZA from pre-incubated whole bone samples or spiked solutions of ZA in 0.2 M H₃PO₄. This method was then applied to quantitate the amount of ZA in the skeleton in mice that had been treated with ZA. The higher concentration of ZA noted in mandibular extracts maybe indicative of the sequestering of ZA at this site and maybe responsible for the selective toxicity in the mandible. This method is suitable to quantitate ZA from other mammalian skeleton including jaw bone biopsies from patients treated with ZA.

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Abbreviations

ZA	zoledronic acid
LC/MS/MS	liquid chromatography tandem mass spectrometry
TMS-DAM	trimethylsilyl diazomethane
HPLC	high performance liquid chromatograph
ESI	electrospray ionization
QC	quality control

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Zoledronic Acid

$^{15}\text{N}_2$ $^{13}\text{C}_2$ Zoledronic Acid**Figure 1.**

Chemical structure of zoledronic acid and $^{15}\text{N}_2$ $^{13}\text{C}_2$ zoledronic acid.

Figure 2.

Zoledronic acid mass spectra. (A) Scan showing $[M + H]^+$ ($m/z = 329.0$) and other ions obtained after derivatization of zoledronic acid with trimethylsilyl diazomethane. (B) Spectra showing product ion obtained after fragmentation of $[M + H]^+$ ($m/z = 329.0 > 203.0$). The spectra were acquired by infusing 25.0 $\mu\text{g/mL}$ of derivatized zoledronic acid onto the mass spectrometer.

Figure 3.

Representative liquid chromatography coupled with tandem mass spectrometry chromatograms: (A) Methanol wash showing the absence of a zoledronic acid peak at 2.74 min. (B) Extraction and derivatization of 0.2 M H_3PO_4 with trimethylsilyl diazomethane (C) Zoledronic acid at the lowest limit of quantitation (0.0250 $\mu\text{g/mL}$) obtained after extraction and derivatization with trimethylsilyl diazomethane (D) Extraction of zoledronic acid from murine mandible and the resulting specific peak at 2.7 min following derivatization. (E) Extraction and derivatization of 0.2 M H_3PO_4 showing the absence of $^{15}\text{N}_2$ $^{13}\text{C}_2$ zoledronic acid (internal standard). (F) Extraction and derivatization with trimethylsilyl diazomethane of $^{15}\text{N}_2$ $^{13}\text{C}_2$ zoledronic acid in 0.2 M H_3PO_4 at a concentration of 50 $\mu\text{g/mL}$.

Figure 4. Zoledronic acid extracted from the bones of treated mice. Data are represented as the mean $\pm$ S.D. of zoledronic acid concentration extracted from three mice. There was a significant difference between zoledronic acid concentration in the mandible compared to the femur as determined by a Student's unpaired t-test ($P = 0.0017$).

Table 1

Intra- and inter-day precision and accuracy of zoledronic acid quality control samples in 0.2 M H₃PO₄.

	Intra-day (N= 5)				Inter-day (N= 8)			
Concentration (µg/mL)	0.500	5.00	25.0	0.500	5.00	25.0	25.0	25.0
Mean	0.481	4.91	25.1	0.492	4.93	25.2	25.2	25.2
CV (%)	7.07	0.733	3.27	6.32	1.35	2.91	2.91	2.91
Accuracy (%)	96.2	98.2	100	98.4	98.6	101	101	101

Intra- and inter-day precision and accuracy of zoledronic acid quality control samples from spiked bone samples extracted in 0.2 M H₃PO₄.^a

Table 2

	Intra-day (N = 3)			Inter-day		
	0.500	5.00	25.0	0.500	5.00	25.0
Concentration (µg/mL)				(N = 7)	(N = 8)	(N = 7)
Mean	0.506	4.71	23.3	0.481	4.45	22.6
CV (%)	2.37	1.08	0.3	7.61	5.95	5.90
Accuracy (%)	101	94.1	93.2	96.1	89.0	90.4

^aTotal zoledronic acid extracted from bone was calculated by: Amount of zoledronic acid extracted + amount of zoledronic acid unextracted