Development and validation of a method using ultra performance liquid chromatography coupled to tandem mass spectrometry for determination of zoledronic acid concentration in human bone

Alfredo Fabrizio Lo Faro[1], Raffaele Giorgetti[2], Francesco Paolo Busardò[3], Giovanni Lodi[1], Valeria Martini[4], Alberto Pispero[1], Marcello Iriti[5], Elena Maria Varoni[1*]

[1]Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche, Università degli Studi di Milano, Milan, Italy

[2]Section of Legal Medicine, Università Politecnica delle Marche, Ancona, Italy

[3]Unit of Forensic Toxicology (UoFT), Department of Anatomical, Histological, Forensic and Orthopedic Sciences, Sapienza University of Rome, V.Le Regina Elena 336, 00161, Rome, Italy;

[4]Ospedale Papa Giovanni XXIII di Bergamo, UO Odontostomatologia, Bergamo, Italy

[5]Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Milano, Milan, Italy

[*]Corresponding author:
Francesco Paolo. Busardò, MD, PhD
Unit of Forensic Toxicology (UoFT), Department of Anatomical, Histological, Forensic and Orthopedic Sciences, Sapienza University of Rome, Rome, Italy
Email: fra.busardo@libero.it

Highlights

- Bisphosphonates are widely used for the treatment of osteoporosis and bone malignancies
- zoledronic acid, a commonly prescribed bisphosphonate, strongly binds bone tissue and
- inhibits osteoclast function.
- A method for the extraction and quantification of zoledronic acid from human bone was set up and validated.
- This method allowed the quantification of zoledronic acid from jawbone sequestrations of patients affected by bisphosphonate-related osteonecrosis associated with ZA treatment.

Abstract

A method for the extraction and quantification of zoledronic acid (ZA) from human bone was set up and validated. This method allowed the quantification of ZA from jawbone sequestrations of patients affected by bisphosphonate-related osteonecrosis of the jaw (BRONJ) associated with ZA treatment. The analyte was extracted from the bone tissues with phosphoric acid and derivatized using trimethylsilyl diazomethane (TMS-DAM). ZA tetramethyl phosphonate was then quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), showing high accuracy, repeatability and selectivity. Lower limits of quantification and detection (LLOQ and LLQD) were 3.4 ng/mL and 1 ng/mg, respectively. This study fully described the analytical process for the determination of ZA in human bone sequestrations, representing a pivotal step for further biomedical research on ZA and BRONJ.

Keywords: bisphosphonates; osteonecrosis; liquid chromatography; mass spectrometry

1. Introduction

Bisphosphonates are widely used for the treatment of osteoporosis and bone malignancies [1, 2]. They are synthetic analogs of pyrophosphates with high affinity for hydroxyapatite, a major component of skeletal tissues [3-5]. Zoledronic acid (ZA), a commonly prescribed nitrogen containing bisphosphonate, strongly binds bone tissue, inhibits osteoclast function [6] and effectively prevents bone resorption; increasing bone mass and decreasing, the rate of skeletal fractures [7]. In cancer patients with bone metastasis, bisphosphonate treatment successfully reduces the skeletal symptoms, including severe pain [7].

A devastating adverse effect of this therapy is the development of bisphosphonate-related osteonecrosis of the jaw (BRONJ). BRONJ is a painful condition, often occurring after tooth extraction, with the presence of necrotic bone at mandibular or maxillary level, supra-infected by oral bacteria [8-10]. In ZA-treated patients, BRONJ occurrence can be as high as 10%, it is very difficult to treat and, in most severe cases, can result in marked facial disfiguration, discomfort and pain [11]. Although the pathophysiology of BRONJ remains unclear, there is a higher incidence of necrosis in the mandible compared to other bones, which is suggestive of a selective toxicity [12]. The accumulation of bisphosphonates in skeletal tissues depends on bone turnover rate and blood circulation [13]. Therefore, it is critical to determine the concentration of ZA sequestered in different types of bone to understand its long-term, local effect and toxicity.

Currently, the estimation of bisphosphonate concentrations in skeleton relies on the measurement of drug in plasma and urine after derivatization [14]. To the best of our knowledge, a method to detect and quantify ZA from murine bone has been recently proposed [15], but still there is no LC-MS method to quantify ZA from human skeleton, in particular from BRONJ sequestrations. These data could be correlated to the risk of BRONJ development

as well as to its severity. Nonetheless, the development of new suitable techniques to quantify bisphosphonates from blood and urine by mass spectrometry-based methods has been challenging due to the high polarity of these compounds. ZA has to be derivatized to a less polar compound before it can be retained on a reverse phase LC column and ionized effectively [16]. Recent studies have demonstrated the success of derivatizing ZA in urine and plasma with trimethylsilyl diazomethane (TMS-DAM) to produce ZA tetramethyl phosphonate [17]. The ZA tetramethyl phosphonate is less polar than its parent compound and can be used for LC-MS/MS based methods. Moreover, this approach does not allow a highly reliable determination nor the exact bisphosphonate localization within the skeleton.

In order to determine the skeletal uptake, studies have focused on the administration of fluorescent or radiolabeled derivatives of bisphosphonates to animal [18-21], showing that bisphosphonate uptake and release per unit calcium were similar in oral and appendicular bones, but lower than those in axial bones. Therefore, hydroxyapatite-bound bisphosphonate released by acid decalcification was the highest in oral, relative to axial and appendicular bones. However, some limitations arouse since it is unknown if the fluorescent or radiolabeled derivatives have the same behavior within the skeleton of the unlabeled bisphosphonates. In addition, the development of a mass spectrometry-based analytical assay would be preferable over a radioassay, due to safety conditions required for handling radiolabeled compounds.

Herein, we proposed a method to directly identify and quantify the presence of ZA in human bone sequestrations, collected from patients who received ZA therapy ho developed BRONJ and whom jaw sequestra were removed as part of BRONJ treatment.

2. Material and methods

2.1 Chemicals

Zoledronic acid (ZA) hydrate (Fig. 1), ibandronate sodium salt used as internal standard (IS) (Fig. 1), LC-MS grade acetonitrile, water, trimethylsilyl diazomethane (TMS-DAM, 2% in ether) and phosphoric acid were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

HyperSep™ SAX, strong anion exchange cartridges for solid phase extraction (SPE) were purchased from Thermo Scientific™ (Waltham, Massachussett, USA).

2.2 Chromatography

A gradient was developed to separate ZA tetramethyl phosphonate and IS from the background interference on a QTRAP 5500 System (AB SCIEX., Bresso - Milano, Italy). The LC-MS/MS was equipped with a temperature-controlled autosampler, a direct infusion syringe pump, an inline vacuum degasser and binary pump. One µL of each sample was injected into a Kinetex™ 2.6 µm C8 100 Å, LC column 30 x 2.1 mm, Ea column (Phenomenex Inc., Castel Maggiore - Bologna, Italy). The mobile phase consisted of 10 mM formic acid in acetonitrile (B) and 10 mM formic acid in water (A) (Table 1).

2.3 Mass Spectrometry

All data were acquired using electrospray ionization (ESI) on AB Sciex 5500 Q-Trap mass spectrometer (AB SCIEX., Bresso - Milano, Italy) operating in the positive mode. The instrument was controlled by the Analyst 2.1 software (AB SCIEX., Bresso - Milano, Italy). Multiple reaction monitoring (MRM) mode was used to monitor both ZA and ibandronate (IS) (Table 2).

2.4 Patient samples

In 2013, two patients were referred to the Dental Unit of the Ospedale Giovanni Paolo II (Bergamo, Italy) because of BRONJ localized at mandible; in both cases, bone removal occurred during the surgical treatment of such condition. Bone samples were washed in sterile physiologic solution, and directly stored at -80°C until analysis. In particular, patient A (female, 62 years old) received 33 infusions of zoledronate (October 2008 - May 2013), and stopped the therapy at 5 months before the bone collection. The patient B (male, 49 years old) received 9 infusions of zoledronate (May 2012 - July 2013), suspended 4 months before bone removal. They were treated with intravenous zoledronic acid for oncological disorders: patient A for metastasis related to breast cancer, while patient B for multiple myeloma. Blank human bone samples, not containing ZA, were used as controls. These

samples were prepared from bone sequestration removed in a female patient (79 years old) due to bone fracture, in therapy with risendronate for osteoporosis.

2.5 Ethical approval

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and it received the ethics approval by the Institutional Ethical Committee (Ospedale Giovanni Paolo II, Bergamo, Italy).

2.6 Sample pre-treatment and extraction of zoledronic acid from human bone

Prior to extraction, bone samples (20 mg) were placed into 1 mL of 0.2 M H_3PO_4 (lysis buffer). The particulate was centrifuged at 16,500 x *g* for 10 min at room temperature (RT). The supernatant was carefully removed with a pipette and placed into a 10 mL tube. 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 previously collected. Before loading the samples into a solid phase extraction (SPE) column, 9 mL buffer phosphate, pH 9.0, were added to each sample. The SAX SPE cartridges were preconditioned with 1 mL methanol and 1 mL buffer phosphate pH 9.0/0.2 M H_3PO_4 (2:1). The columns were then washed with 1 mL buffer phosphate pH 9.0, 1 mL of water and 2 mL of methanol. Immediately, 2.0 M TMS-DAM in ether (0.2 mL) and, then, methanol (0.75 mL) were directly added to the column and the reaction was allowed to proceed for 60 min at RT. The resulting ZA tetramethyl phosphonate was eluted with methanol (1mL), concentrated under nitrogen and reconstituted in 1:1 CH_3OH:H_2O (0.1 mL).

2.7 Method validation

Prior to application to real samples, the method was tested in a validation protocol following the accepted criteria for bioanalytical method validation [22-23]. Selectivity, carryover, matrix effect, linearity, limits of detection and quantification, accuracy, repeatability (precision), recovery, and stability were determined as previously reported. Validation parameters were calculated using different daily replicates of QC samples (low, medium, and high QCs) along three subsequent working days.

In brief, stock solution of ZA at 1 mg/mL was prepared in water:methanol (50:50) and stored at 4 °C. The 1 mg/mL stock solution of ZA was diluted to a 100 ng/mL calibration sample (stored at -20°C). Additional calibration samples were prepared at 1-2.5-5-10-20-50-100 ng/mL by dilution with blank matrix. All calibration samples were processed in duplicate for each daily calibration. Last-squares linear regression with the reversed square of the concentration of the analyte ($1/x^2$) was employed to define the calibration curve using the ratios of the peak area of ZA and IS. The lower limits of quantification (LLOQ) and detection (LLOD) were calculated using scalar ZA concentrations added to the blank sample. The measurements were repeated for each of the 10 calibration curves. Thus, human bone at the LLOQ and LLOD levels were analyzed in 10 times.

Ten calibration curves were assessed to check the linearity of the relationship between the ratio of the areas ZA and IS and the corresponding concentration of analyte, ranging between 1-150 ng/mg.

For accuracy, the reference concentrations were obtained throughout 10 series of bone samples, defined as "blank", then added with ZA to the following concentrations: 1-2.5-5-10-20-50-100 ng/mg. The calculation was performed by the data processing program Analyst 5.1 (AB SCIEX., Bresso - Milano, Italy) and reported as percentage between the defined value and the actual value measured multiplied by one hundred, then expressed with values higher or lower than 100%, with an acceptable range between 85% and 115%. Similarly, ten samples of blank bone spiked with ZA were evaluated for method repeatability. Six individual samples were processed to test the selectivity of the assay. These samples were processed as double blanks (without ZA and IS) and after spiking with 1 ng/mL ZA (QC-low), 5 ng/mL (QC-medium) and 10 ng/mL (QC-high), and after adding the IS (100 ng/mg). The spiked samples were also used to assess the inter-batch variation of the matrix effect.

3. Results and discussion

3.1 Chromatography and validation

This is the first method able to quantify successfully ZA from the human bone BRONJ sequestrations, using a protocol modified from a **previous one applied for studies** on mice [17]. Trimethylsilyl diazomethane (TMS-DAM) was used to derivatize ZA prior to analysis by LC-MS/MS.

Zoledronic acid and its internal standard, ibandronic acid (Figure 1) could be detected and quantified in a chromatographic run of 3.5 minutes and the investigated compound could be clearly identified and quantified in both examined patients (Figures 2 and 3).

Results obtained from the 10 calibration curves showed linear trends, with average precision for each concentration in the range of 93-103% (Table 3).

Variations of accuracy ≤ 10% were observed apart from the highest limit of the calibration range, which was associated to deviations of the accuracy of ± 15%, in agreement with international criteria [22] (Table 4).

The analysis of six batches of blank samples showed no interfering peak in the MRM traces for ZA and IS (ibandronate) in human bone samples. Blank responses could not be distinguished from the detector noise (signal to noise ratio < 3) for both ZA and IS, and were all below 15% of the ZA LLOQ. A threshold of 20% is required and the regular signal of IS is below 0.2%. The absence of any interference in these experiments is a proof of the high selectivity of the assay.Repeatability of the assay was also within the international requirements (Table 5). LLOQ value was 3.4 ng/mL, while LLOD value 1 ng/mg (Table 6).

The recovery of the extraction for the IS was 89 ± 15% (n = 6). Ion suppression was below 13% for all QC concentrations of ZA, and below 15 ± 6% for the IS (n = 6).

3.2 Patient samples

The developed assay was able to determine ZA levels in bone sequestrations from two oncological patients (Figures. 2-3). Noteworthy, the method was able to discriminate the drug content in samples from patient receiving a long lasting therapy (patient A, 33 infusions of ZA) with respect to patient with a shorter treatment (patient B, 9 infusions of ZA). Indeed, in patient A, we found 9.4 ng/ng ZA, while only 1 ng/ng ZA in patient B. Interestingly, both of them suspended the treatment 4 and 5

months before the bone sample collection, respectively. Although **the therapy had been suspended,** the drug was still bound to the bone tissues of patients and, therefore, detectable, as suggested by previous literature, which used indirect methods of analyses, *i.e.* the ZA urinary metabolite [17].

4. Conclusions

This is the first study able to directly detect and quantify the ZA within the bone sequestrations of patients receiving ZA therapy. The method was based on the high resolution and sensitivity of LC-MS/MS, with a simple SPE purification procedure required for the sample preparation.

High performance was demonstrated in terms of precision, sensitivity and accuracy, making the proposed method useful to detect and quantify other classes of bisphosphonates from human bone tissues. The method showed to be a valuable tool for clinical studies on BRONJ patients, to better clarify the pathophysiological role of ZA bone concentration in the development of BRONJ and related prognosis.

References

[1] J.R. Berenson, Advances in the biology and treatment of myeloma bone disease, Am J Health Syst Pharm 58 Suppl 3 (2001) S16-20.

[2] J.R. Berenson, New advances in the biology and treatment of myeloma bone disease, Semin Hematol 38(2 Suppl 3) (2001) 15-20.

[3] H.A. Fleisch, R.G. Russell, S. Bisaz, R.C. Muhlbauer, D.A. Williams, The inhibitory effect of phosphonates on the formation of calcium phosphate crystals in vitro and on aortic and kidney calcification in vivo, Eur J Clin Invest 1 (1970) 12-18.

[4] G.H. Nancollas, R. Tang, R.J. Phipps, Z. Henneman, S. Gulde, W. Wu, A. Mangood, R.G. Russell, F.H. Ebetino, Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite, Bone 38 (2006) 617-627.

[5] R.G. Russell, R.C. Muhlbauer, S. Bisaz, D.A. Williams, H. Fleisch, The influence of pyrophosphate, condensed phosphates, phosphonates and other phosphate compounds on the dissolution of hydroxyapatite in vitro and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomised rats, Calcif Tissue Res 6 (1970) 183-196.

[6] R.G. Russell, N.B. Watts, F.H. Ebetino, M.J. Rogers, Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy, Osteoporos Int 19 (2008) 733-759.

[7] D.M. Black, P.D. Delmas, R. Eastell, I.R. Reid, S. Boonen, J.A. Cauley, F. Cosman, P. Lakatos, P.C. Leung, Z. Man, C. Mautalen, P. Mesenbrink, H. Hu, J. Caminis, K. Tong, T. Rosario-Jansen, J. Krasnow, T.F. Hue, D. Sellmeyer, E.F. Eriksen, S.R. Cummings, H.P.F. Trial, Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis, N Engl J Med 356 (2007) 1809-1822.

[8] G.L. Borromeo, C.E. Tsao, I.B. Darby, P.R. Ebeling, A review of the clinical implications of bisphosphonates in dentistry, Aust Dent J 56 (2011) 2-9.
[9] A.O. Hoff, B. Toth, M. Hu, G.N. Hortobagyi, R.F. Gagel, Epidemiology and risk factors for osteonecrosis of the jaw in cancer patients, Ann N Y Acad Sci 1218 (2011) 47-54.
[10] M. Pavkovic, G. Petrushevska, R. Jovanovic, O. Karanfilski, L. Cevreska, S. Stankovic, A. Stojanovic, Osteonecrosis of the jaw in patients with multiple myeloma treated with bisphosphanates, Prilozi 31 (2010) 39-49.
[11] S.B. Woo, J.W. Hellstein, J.R. Kalmar, Narrative [corrected] review: bisphosphonates and osteonecrosis of the jaws, Ann Intern Med 144 (2006) 753-761.
[12] V.S. Grewal, E.P. Fayans, Bisphosphonate-associated osteonecrosis: a clinician's reference to patient management, Todays FDA 20 (2008) 38-41, 43-46.
[13] P. Masarachia, M. Weinreb, R. Balena, G.A. Rodan, Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones, Bone 19 (1996) 281-290.
[14] M.R. Allen, Skeletal accumulation of bisphosphonates: implications for osteoporosis treatment, Expert Opinion on Drug Metabolism & Toxicology 4 (2008) 1371-1378.
[15] B.S. Raccor, J. Sun, R.F. Lawrence, L. Li, H. Zhang, M.J. Somerman, R.A. Totah, Quantitation of zoledronic acid in murine bone by liquid chromatography coupled with tandem mass spectrometry, J Chromatogr B Analyt Technol Biomed Life Sci 935 (2013) 54-60.
[16] L.S. Zhu, V.N. Lapko, J.W. Lee, Y.J. Basir, C. Kafonek, R. Olsen, C. Briscoe, A general approach for the quantitative analysis of bisphosphonates in human serum and urine by high-performance liquid chromatography/tandem mass spectrometry, Rapid Commun Mass Spectrom 20 (2006) 3421-3426.
[17] K. Veldboer, T. Vielhaber, H. Ahrens, J. Hardes, A. Streitburger, U. Karst, Determination of zoledronic acid in human urine and blood plasma using liquid chromatography/electrospray mass spectrometry, J Chromatogr B Analyt Technol Biomed Life Sci 879 (2011) 2073-2080.
[18] F. Legay, S. Gauron, F. Deckert, G. Gosset, U. Pfaar, C. Ravera, H. Wiegand, H. Schran, Development and validation of a highly sensitive RIA for zoledronic acid, a new potent heterocyclic bisphosphonate, in human serum, plasma and urine, J Pharm Biomed Anal 30 (2002) 897-911.
[19] J. Lin, L. Qiu, W. Cheng, S. Luo, L. Xue, S. Zhang, Development of superior bone scintigraphic agent from a series of (99m)Tc-labeled zoledronic acid derivatives, Appl Radiat Isot 70 (2012) 848-855.
[20] H.M. Weiss, U. Pfaar, A. Schweitzer, H. Wiegand, A. Skerjanec, H. Schran, Biodistribution and plasma protein binding of zoledronic acid, Drug Metab Dispos 36 (2008) 2043-2049.
[21] D. Wen, L. Qing, G. Harrison, E. Golub, S.O. Akintoye, Anatomic site variability in rat skeletal uptake and desorption of fluorescently labeled bisphosphonate, Oral Dis 17 (2011) 427-432.
[22] S.M.R .Wille, W. Coucke, T. De Baere, F.T. Peters. Update of Standard Practices for New Method Validation in Forensic Toxicology. Curr Pharm Des 23 (2017) 5442-5454.
[23] F.T.. Peters, D.K. Wissenbach, F.P. Busardo, E. Marchei, S. Pichini. Method Development in Forensic Toxicology. Curr Pharm Des 2017;23:5455-5467.

Figure legends

Figure 1. Chemical structures of (1) zoledronic acid ($C_5H_{10}N_2O_7P_2$ - MW 272.090) and (2) ibandronic acid ($C_9H_{23}NO_7P_2$ - MW 319.229).

Figure 2. LC-MS/MS chromatograms of bone samples from patient A (female, 62 years old) showing peak for zoledronic acid and ibandronate (internal standard)

Figure 3. LC-MS/MS mass chromatograms of bone samples from patient B (male, 49 years old) showing peak for zoledronic acid and ibandronate (internal standard)

Table 1. Mobile phase

	Total time (min)	Flow rate (µL min^{-1})	A%	B%
0	0	500	100	0
1	1.5	500	100	0
2	2.0	500	85	15
3	3.5	500	70	30
4	5.5	500	0	100
5	7.5	500	0	100
6	7.6	500	100	0
7	10	500	100	0

Table 2. Multiple reaction monitoring (MRM) mode used to monitor both zoledronic acid and ibandronate.

Compound	Q1 mass	Q3 mass	DP	CE	CXP
ZA[1]-1	329.1	203.3	85	29	15
ZA[2]-2	329.1	135.1	85	40	21
IS-1	376.5	250.7	85	25	5
IS-2	376.5	114.2	85	31	7

[1]Zoledronic acid

[2]Internal standard, ibandronate

Table 3. Calibration curves of ZA obtained in ten different replicates

Concentration range 1-150 ng/mg		
	Equations	*r*
1	y=0.0085x+0.0071	0.9998
2	y=0.0085x+0.0156	0.9996
3	y=0.0088x+0.0075	0.9996
4	y=0.0081x+0.0177	0.9996
5	y=0.008x+0.0307	0.9987
6	y=0.0079x+0.0237	0.9999
7	y=0.0088x+0.0037	0.9999
8	y=0.0081x+0.0193	0.9998
9	y=0.0092x+0.005	0.9999
10	y=0.0083x+0.028	0.9999

Table 4 . Accuracy of the assay at all ZA concentrations.

Theoretical Concentration ng/mg	1	2.5	5	10	20	50	100
Measured Concentration ng/mg*	0.83-1.11	2.3-2.8	4.7-5.3	8.5-11	19.7-21.6	48.6-51.3	96.6-101.9
Accuracy % min-max	99.2-100.6	87.1–103.3	96.9 – 101.5	85.0-109.7	98.3-107.8	97.2-102.6	97-101.9

*Measured Concentrations were obtained from 10 different replicates

Table 5. Repeatability (Precision) of the assay at all ZA concentrations

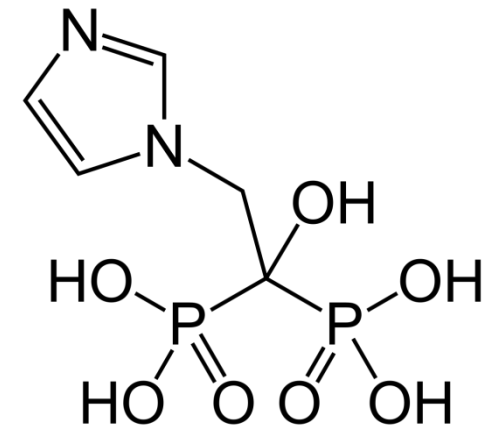
N=10	Mean measured concentrations ng/mg*	Standard deviation	CV%
1 ng/mg	1.0	1.1334	113.3
2.5 ng/mg	2.57	0.0884	3.44
5 ng/mg	4.6	0.2179	4.73
10 ng/mg	10	0.8770	8.77
20 ng/mg	20.2	0.6129	3.03
50 ng/mg	50.0	1.1449	2.29
100 ng/mg	99.9	1.4058	1.41
150 ng/mg	150.1	0.8512	0.57

*Measured Concentrations were obtained from 10 different replicates

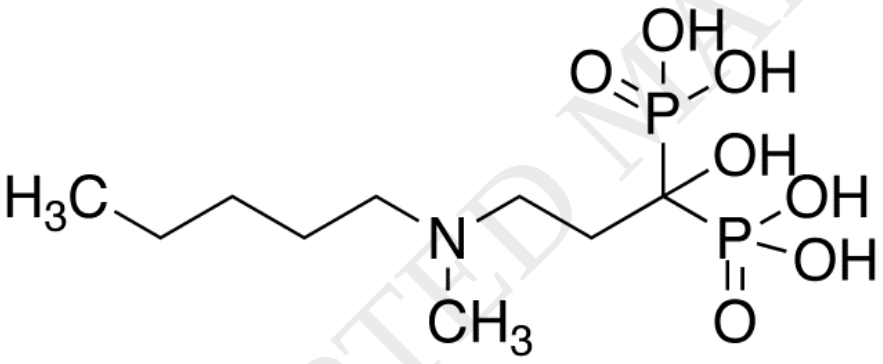
Table 6. ZA concentrations in "blank" bone

N	Average F.
1	0.49
2	0.46
3	0.325
4	0.98
5	0
6	0.47
7	0
8	0.66
9	0
10	0.75
Average	**0.413**
Standard deviation	**0.3378**
LOD (3*standard deviation)	**1.0**
LOQ (10* standard deviation)	**3.4**

Figure 1



Zoledronic Acid



Ibandronic Acid

Figure 2

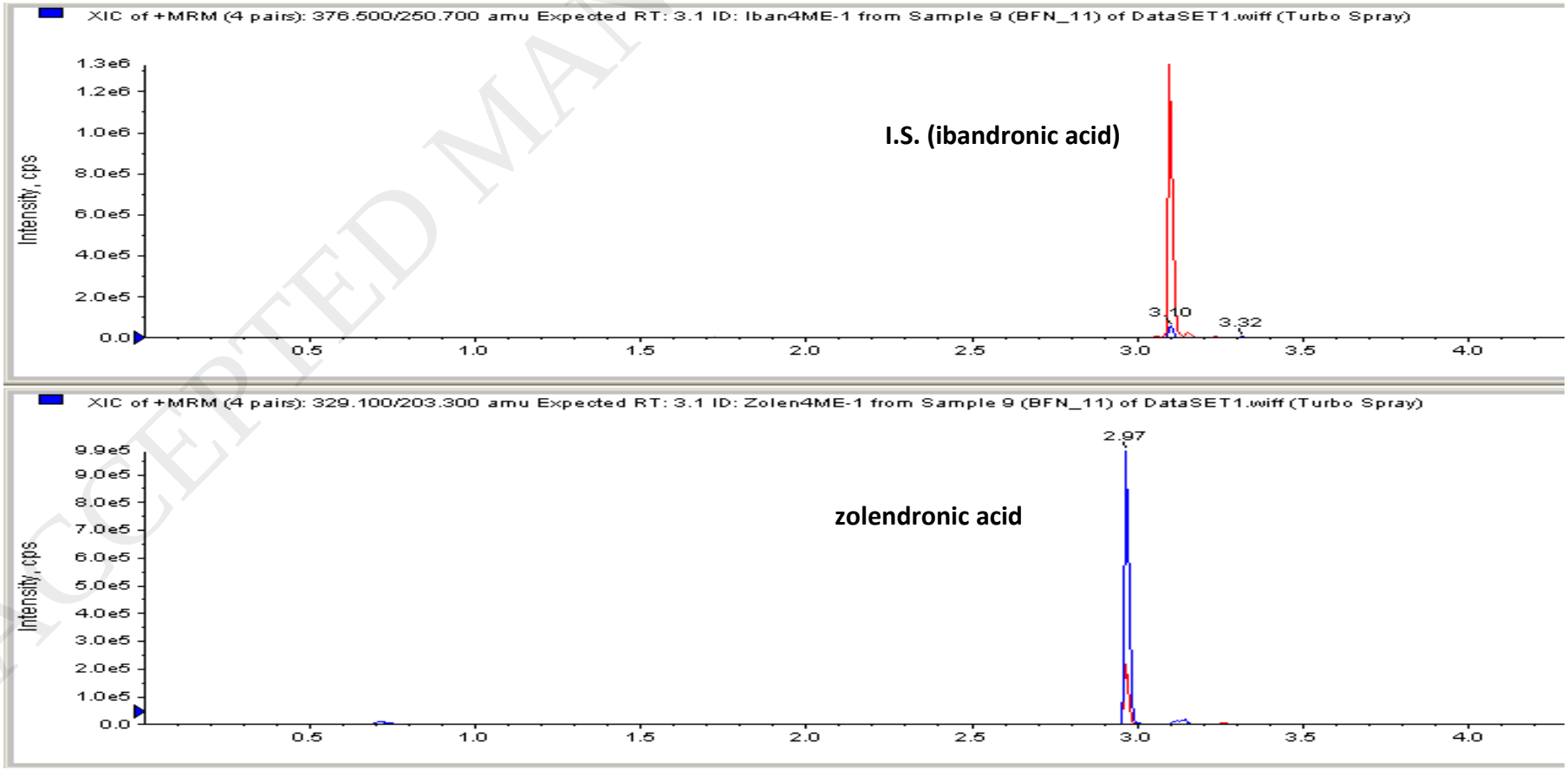


Figure 3