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Analytical methods for the quantification of ibandronate in body fluids and bone

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Abstract

The accurate determination of bisphosphonate levels in bone and biological fluids is important in both clinical and pharmacological/toxicological studies. Ibandronate is a potent nitrogen-containing bisphosphonate containing a tertiary amine group, which does not easily form chromophore derivatives that can be detected by UV light or fluorescence emissions. The current report describes the methodology and validation of a GC–MS assay for ibandronate in serum/plasma and urine, a similar, modified GC–MS method for measurement of bone ibandronate levels, and an ELISA for ibandronate determination in serum/plasma. The range of quantification for the GC–MS was 1–100 ng/ml and 2–7500 ng/ml in plasma or serum and urine, respectively, and 50–1600 pg/ml (potentially 10–320 pg/ml depending on sample size) for the ELISA in plasma or serum. These assays were comparable. The practical application of the assays in preclinical and clinical studies is briefly reviewed.

Keywords: Quantification; Derivatization; GC–MS; ELISA; Ibandronate

1. Introduction

Bisphosphonates are widely used in the treatment of metastatic bone disease (MBD) and postmenopausal osteoporosis (PMO). Ibandronate is a potent, nitrogen-containing bisphosphonate with proven efficacy in the treatment of MBD, including hypercalcaemia of malignancy [1–5], and in the management of PMO [6–10].

Ibandronate is administered over a wide range of therapeutic doses and as both oral and intravenous (i.v.) formulations. Therefore, investigations of the pharmacokinetic and toxicological properties of ibandronate require assays that can accurately detect and quantify a wide range of ibandronate concentrations in serum and urine. However, ibandronate and other bisphosphonates exert their pharmacological action at the bone surface. Bisphosphonates bind to bone mineral and inhibit the action of osteoclasts, the bone-resorbing cells. Thus, an assay that quantifies ibandronate in bone, the pharmacological target tissue for bisphosphonates, in addition to biological fluids is essential for comprehensive studies of the pharmacology of ibandronate and its pharmacodynamic effects.

The quantification of drug concentrations in bone tissue from animal pharmacology studies allows investigation into, and can provide data on, drug deposition throughout the entire skeleton. In contrast to the analysis of human bone biopsies, animal studies enable the analysis of the whole bone. This provides a representative drug concentration and avoids potential differences in the amount of drug accumulated in different parts of the bone, which are of trabecular and cortical nature. It therefore allows identification or investigation of issues that cannot be addressed using human bone biopsies, such as heterogeneous distribution within the different bone areas. The analysis of bone tissue, preferably, should use non-radioactive materials, thus permitting further analysis by routine laboratory methods without the precautions needed with radiochemicals.

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Such animal studies can give insight into the pharmacology and pharmacodynamics of the bisphosphonates. For example, investigations into the bone uptake of ibandronate supported the development of the total bisphosphonate dose concept for therapeutic use [11–13]. This concept postulates that, regardless of the dosing schedule, the same total cumulative dose is associated with the same total bone drug concentration and equivalent pharmacodynamic effects. This concept underlies the clinical development of intermittent oral and intravenous ibandronate dosing schedules in PMO [6–10,14–17].

Assays such as LC developed for other bisphosphonates cannot be used for ibandronate. These assays rely on the detection of UV light or fluorescence emitted by chromophore derivatives of bisphosphonates with primary or secondary amine groups, such as alendronate [18–21] and pamidronate [22–24]. Tertiary amines such as ibandronate cannot easily form chromophore derivatives with the possible exception of olpadronate [25]. However, the structure of this derivative could not be proven by mass spectrometry.

Liquid scintillation counting (LSC), using radio-labelled drug, avoids this difficulty, and is highly sensitive; however, the use of radioactive isotopes requires stringent safety precautions. Alternative methods with at least equivalent sensitivity therefore are needed. Potential alternatives are ELISA and GC–MS, which have been used to measure risedronate concentrations in serum and urine, respectively [26].

Therefore, for ibandronate, assays were required that could accurately quantify ibandronate over a wide range of concentrations in plasma or serum and urine for pharmacokinetic and toxicology studies, and in bone tissue for pharmacological studies without the use of UV or fluorescence detection. Initially, a GC–MS method was developed for ibandronate in serum and urine to accommodate the doses of ibandronate used for MBD, this was later modified for the analysis of bone tissue. Later, a more sensitive ELISA method was developed that could quantify the low concentrations of ibandronate in serum following administration of doses used for the treatment of PMO. This report describes the methodology and validation of these two methods and illustrates the practical application of the GC–MS assay for bone tissue in two pharmacological studies.

2. Experimental

2.1. Principle

The GC–MS: based on the isolation of ibandronate (and the internal standard *N*-trideuteromethyl-ibandronate) from the biological material by coprecipitation with calcium phosphate and oxidation with chlorinated water to form the amino acid *N*-pentyl, *N*-methyl-β-alanine. This amino acid is derivatized to its methyl ester and measured by GC–MS. The pseudomolecular ion MH^+, formed by chemical ionisation with ammonia, is detected at mass to charge (*m*/*z*) values of 188 and 191 for the methyl esters of the test material and the internal standard.

The ELISA: direct competition between ibandronate in the test sample and bisphosphonate conjugated to peroxidase (POD) for biotinylated anti-bisphosphonate antibody attached to streptavidine-coated tubes. When incubated with serum/plasma test samples, ibandronate becomes bound to the biotinylated antibody. The addition of bisphosphonate–POD conjugate and incubation with the POD substrate (the chromogen solution) results in a color intensity that is measured at 422 nm.

2.2. Equipment

GC–MS analyses were performed using a Hewlett-Packard mass-selective detector (model 5971A) coupled to a series II-5890 GC with a HP-7673B autosampler. The ELISA was conducted using the Enzymun-Test[1] System ES 600 with TWIN-USER software, version 1.2 (Roche Diagnostics GmbH [formerly Boehringer Mannheim GmbH], Germany).

2.3. Materials

For GC–MS analysis: strong cation exchange resin (200–400 mesh) was obtained from Bio-Rad (Munich, Germany); sodium hydroxide (titrisol ampoules), hydrochloric acid (titrisol ampoules), trichloroacetic acid, potassium dihydrogen phosphate, calcium chloride dihydrate, and sodium hydrogen carbonate (E. Merck KG, Darmstadt, Germany); 3 M methanolic hydrochloric acid from Supelco (now Sigma-Aldrich, Taufkirchen, Germany); dichloromethane and acetonitrile (HPLC-grade) from J.T. Baker (Philippsburg, NJ, USA).

For the ELISA: the following reagents were all obtained from Roche Diagnostics GmbH (formerly Boehringer Mannheim GmbH), Germany: ibandronate (1-hydroxy-3-[methylpentylamino] propylidene) bis-phosphonic acid (monosodium salt monohydrate); human control plasma (heparinized); human control serum/plasma; buffer for incubation, biotinylated antibody, PAK ⟨Bisphos⟩ S. 4650-IgG (DE)-Bi-DADOO-DSS; substrate buffer (phosphate/citrate buffer 100 mm/l pH 9.4 sodium perborate 3.2 mmol/l); chromogen (ABTS® 1.9 mmol/l; bisphosphonate–POD (Bisphos (SATP)-POD (pi 0.82, NR, MH), charge 14, 484 U); washing solution 1059475; Hepes buffer (25 mM hepes pH 6.8, 150 mM sodium chloride, 0.025 mM calcium chloride and protein stabilising additives). Deionized water was prepared using a Millipore system (Milli-Qplus, Series: F4HM59644I, Millipore Corp., USA). Uncoated and streptavidin-coated tubes were supplied by Roche Diagnostics GmbH (formerly Boehringer Mannheim GmbH), Germany. Micro test tubes supplied by Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany.

[1] Enzymun-Test is a trademark of a member of the Roche group.

2.4. Analytical procedures

2.4.1. GC–MS

2.4.1.1. Preparation of stock solutions. A 0.4% solution of chlorine in water was prepared by bubbling chlorine through water. The chlorine content was confirmed by weighing. Solutions of 25% trichloroacetic acid, 2.5 M calcium chloride, 1 M potassium hydrogen phosphate and 1% sodium hydrogen carbonate were prepared in deionized water. Stock solutions of 1 M sodium hydroxide and 2 M hydrochloric acid were prepared by dilution of sodium hydroxide titrisol (1 ampoule) and hydrochloric acid titrisol (2 ampoules), respectively, up to 1 l with deionized water.

2.4.1.2. Preparation of ibandronate and internal standard solutions for calibration and quality control standards. For serum, plasma and urine analysis, solutions of ibandronate in the range of 0.1–100 μg/ml, and the internal standard D_3-ibandronate in the range of 0.4–100 μg/ml were made in deionized water by serial dilution from 1000 μg/ml stock solutions (10.68 mg ibandronate or D_3-ibandronate monosodium salt monohydrate in 10 ml deionized water, respectively). Calibration standards of 500, 100, 50, 25, 10, 5, 2.5 and 1 ng/ml for serum and plasma and 7500, 5000, 2500, 1000, 500, 250, 100, 50, 25, 10 and 5 ng/ml for urine were prepared by appropriate dilution of the standard solutions with drug-free control serum, plasma or urine.

Quality control standards were prepared by spiking human pool serum/plasma with ibandronate to give concentrations of 5, 25 and 50 ng/ml. Urine control samples were spiked to concentrations of 10, 100, 500, 1000 and 5000 ng/ml. Samples were stored in aliquots below −18 °C.

For bone analysis, ibandronate and internal standard solutions of 100, 10 and 1 μg/ml were prepared by serial dilution in 2 M hydrochloric acid from 1000 μg/ml stock solutions in deionized water. Calibration standards ranging from 10 ng/ml to 100 μg/ml were prepared by appropriate serial dilution from the 1000 μg/ml aqueous stock solution with 2 M hydrochloric acid.

2.4.1.3. Preparation of ion exchange cartridges. A suspension of Bio-Rad AG5W (200–400 mesh) cation exchange (40 g) was prepared in sodium hydroxide solution (1 M; 200 ml) and continuously agitated. An aliquot (2 ml) was introduced into a Bio-Rad Poly-Prep cartridge and allowed to sediment. The cartridges, containing 400 mg of the cation exchange resin, were conditioned with 5 ml water, followed by 1 ml 2 M hydrochloric acid, and washed with 3 ml of deionized water.

2.4.1.4. Sample processing. Serum/plasma: Serum/plasma samples were processed according to the flow chart presented in Fig. 1. Serum/plasma samples containing ibandronate are deproteinized with trichloroacetic acid and spiked with the internal standard, *N*-trideuteromethyl-ibandronate. The addition of calcium chloride, potassium dihydrogen phosphate and sodium hydroxide results in coprecipitation of both ibandronate and the internal standard. The precipitate is dissolved in hydrochloric acid, and the precipitation and dissolution repeated. The acidic solution is passed over a strong cation exchanger to remove the calcium ions and evaporated to dryness. The residue is treated with chlorine water to form the amino acid *N*-pentyl, *N*-methyl-β-alanine, which is derivatized to its methyl ester by methylation with methanolic hydrochloric acid. The methyl ester is dissolved in acetonitrile and the solution injected into a GC–MS system.

Urine: Urine samples were similarly prepared except initially 500 μl of deionized water and 50 μl of internal standard (0.4 μg/ml stock solution) was added to 500 μl of urine and vortexed. Addition of potassium hydrogen phosphate, calcium chloride and sodium hydroxide and further sample preparation was as for serum/plasma samples.

Bone: Bone (ground or as small pieces) was weighed (50–1000 mg, according to the expected ibandronate concentration) into a polystyrene tube. One millilitre of 2 M hydrochloric acid per 100 mg bone was added and the tube incubated at 50 °C for 18 h. The solution was transferred to a volumetric flask and diluted to volume with 2 M hydrochloric acid. A 100 μl aliquot was transferred to polystyrene centrifuge tube, 1 ml deionized water added and the sample vortexed. Fifty microlitres of the internal standard solution (1 μg/ml) was added and the sample vortexed. Fifty microlitres each of 1 M potassium hydrogen phosphate and 2.5 M calcium chloride, and 1 ml of 1 M sodium hydroxide were added. The sample was vortexed and centrifuged at 4000 rpm for 5 min. The aqueous phase was discarded and the precipitate dissolved in 500 μl 1 M hydrochloric acid. The solution was transferred into a cation exchange cartridge and processed as described for serum/plasma and urine.

2.4.1.5. Chromatographic conditions. Samples were introduced with a 10 μl Hamilton syringe via a splitless injection (split closed for 0.5 min). Chromatographic separations were carried out on a DB-WAX fused silica column (J&W Scientific, Folsom, USA) (10 m × 0.25 mm i.d.) with a Carbowax stationary phase (film thickness 0.25 μm), and a retention gap consisting of a fused silica deactivated capillary (0.5 m × 0.32 mm i.d.) connected to the DB-WAX main column by column connectors (ANALYT, Müllheim, Germany). The GC injection port temperature was 250 °C, and the oven temperature 80 °C (1 min) to 220 °C (3 min), at a rate of 40 °C/min. The gaseous phase was helium: flow rates were 0.5 ml/min for the carrier gas and 30 ml/min to flush the split line.

2.4.1.6. Mass spectrometry. The chemical ionization (CI) reagent gas was ammonia (99.8% pure), at a pressure of 1.2×10^4 Torr. Transfer line temperature was 240 °C. Selected ion monitoring was applied to the following ions: $m/z = 188$ for *N*-pentyl, *N*-methyl-β-alanine methyl ester, and $m/z = 191$ for *N*-pentyl, *N*-trideuteromethyl-β-alanine methyl ester.

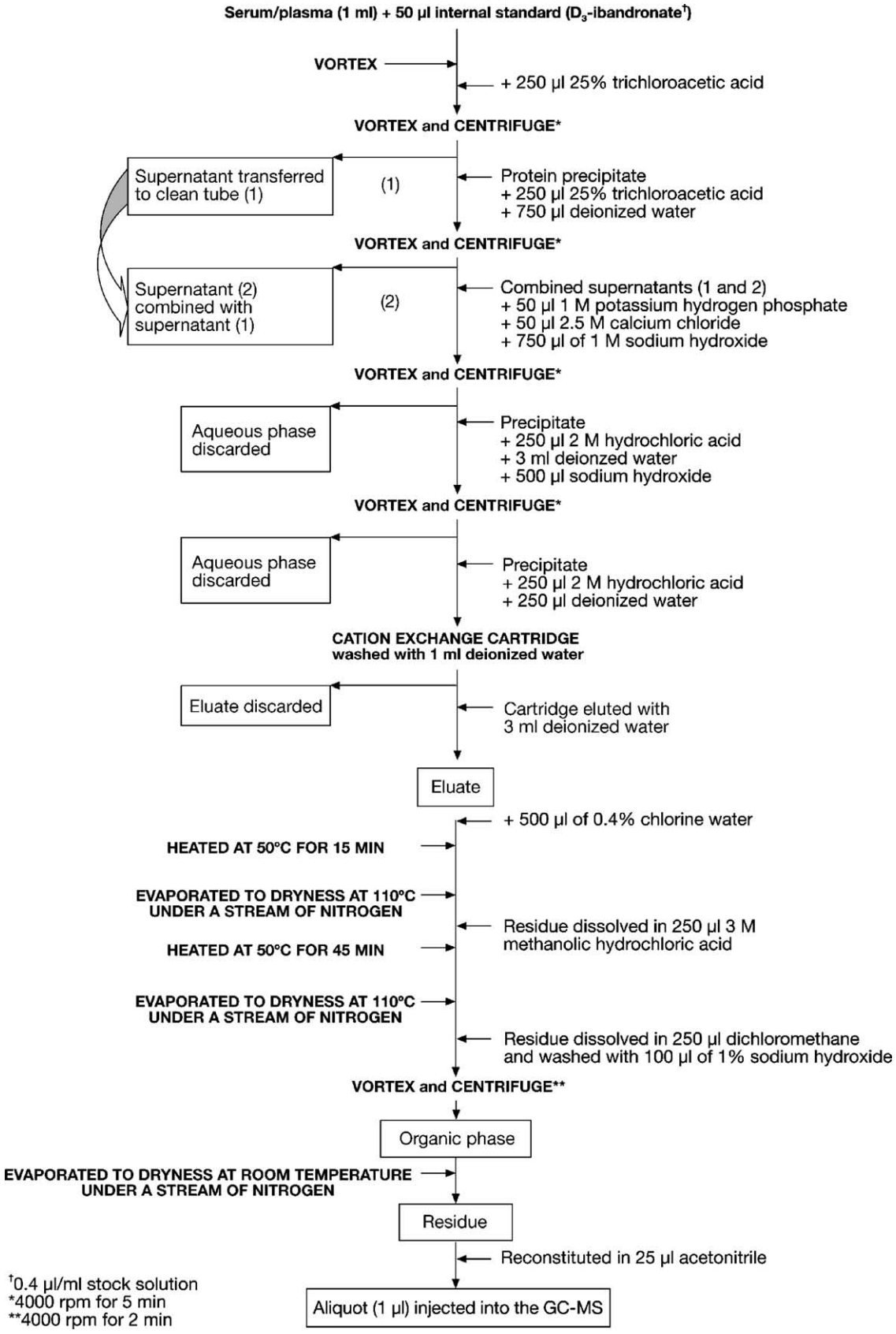

Fig. 1. Flow chart representing the sample processing for analysis by GC–MS.

2.4.2. ELISA

2.4.2.1. Preparation of stock solutions. One hundred and fifty microlitres of biotinylated antibody were dissolved in 1 l incubation buffer for 1 h. Bisphosphonate–POD conjugate (484 U) was dissolved in 400 μl deionized water for 10 min, diluted in Hepes buffer to a concentration of 242 U/ml and gently mixed after 30 min. This solution was stored at −80 °C as 100 μl aliquots in micro test tubes. The working solution of conjugate was 75 μl of this solution reconstituted in 50 ml incubation buffer by gently shaking for 15 min. Chromogen solution was prepared by dissolving chromogen in substrate buffer to a concentration of 1.9 mmol/l at least 1 h before use.

2.4.2.2. Preparation of standard solutions. A 1000 μg/ml solution of ibandronate (5 mg free acid in 5 ml) was prepared in incubation buffer. Further dilutions in incubation buffer yielded a solution of 100 μg/ml from which standards ranging from 500 to 16,000 pg/ml was prepared in incubation buffer. Fifty microlitres of each standard were diluted in 450 μl serum (or plasma) to yield final concentrations of 1600, 800, 400, 200, 100, 75 and 50 pg/ml. Standards for each subject were prepared in their own drug-free serum or plasma or, if unavailable, in pooled serum or plasma diluted at least 1:10.

Quality control samples were prepared by spiking 500 μl of blank human serum/plasma with ibandronate to give concentrations of 150, 500 and 1000 pg/ml. Samples were homogenized, divided into 2 ml aliquots and stored below −18 °C.

2.4.2.3. Assay procedure. Five hundred microlitres of biotinylated antibody were incubated for 60 min at 25 °C in a streptavidin-coated tube. The tubes were washed with ELISA wash solution at an intensity of four on the wash apparatus. A 100 μl (or, for lower concentrations, 500 μl) sample was incubated with 400 μl incubation buffer (or without buffer for lower concentrations) for 60 min at 25 °C. Fifty microlitres of bisphosphonate–POD conjugate were added and incubated for 60 min at 25 °C. The washing step was repeated and 500 μl of substrate chromogen solution added and incubated for 60 min at 25 °C. Absorbance was measured at 422 nm.

2.5. Calculations

2.5.1. GC–MS

For the calculation of ibandronate concentrations in biological samples, peak area ratios were calculated for the calibration standards run in series with the test samples. Counts for peak areas were generated by the data system of the Hewlett-Packard mass-selective detector HP 5971 A and further processed by a VAX 6310 data system, using an in-house Fortran program to fit an appropriate linear or quadratic regression function to the data.

The best-fit calibration curve, judged by the correlation coefficient (r^2) and by the lowest deviation of the back calculated concentration values from the target values, was used to calculate the concentration of unknown samples.

Ibandronate concentrations in bone are usually reported as ng/mg. Thus, the original concentration data for bone samples, which are expressed in ng/ml were converted according to the following equation:

$$b = \frac{CV}{m}$$

where b is the bone concentration in ng/mg, C the concentration of the solution in ng/ml in 2 M hydrochloric acid, V the volume of the solution in ml and m the weight of the bone sample in mg.

2.5.2. ELISA

Standard curves were prepared by plotting the median absorption values of the standards versus their concentrations. The TWIN-USER software determines the calibration curve with a four-parameter function (Rodbard function). This curve was used to calculate the concentrations of ibandronate in samples and quality controls. All concentrations were calculated as the pure free compound.

2.6. Validation

2.6.1. Range of quantification

For the GC–MS and ELISA method, the range of quantification was defined as the range between the lowest and highest concentrations on the calibration curve. All data outside this range were regarded as invalid. Samples with concentrations higher than the upper limit of quantification were diluted and re-assayed.

2.6.2. Specificity

Specificity of the GC–MS method was determined by the absence of significant peaks in the mass chromatograms at the retention times of the methyl esters of the *N,N*-dialkyl substituted β-alanine derivatives. The specificity of the ELISA was demonstrated by analyzing blank human serum/plasma samples that produced background signals between 1 and 10 pg/ml when 100 μl aliquots were analyzed. However, when 500 μl aliquots were used, the background signals were reduced to 1.5–3.8 pg/ml.

2.6.3. Accuracy and precision

The overall accuracy and precision of the assays were determined from five (for the GC/MS; 5–50 ng/ml in plasma and 10–5000 ng/ml in urine) and six (for the ELISA: 100, 150 and 1000 pg/ml) successive runs (six samples per run and concentration level), corresponding to a total of 30 and 36 samples, respectively.

Intra- and inter-assay precisions were calculated as relative S.D. (%) by one-way ANOVA. Accuracy was calculated as the deviation (%) of the overall mean value from the nominal value.

2.6.4. Recovery

Recovery studies for the GC–MS method were performed in plasma and urine, using ^{14}C-labelled ibandronate with a specific radioactivity of 3091 KBq/mg. The recovery rates from spiked plasma samples were assessed at concentrations of 50, 25 and 5 ng/ml using 1 ml plasma samples processed as described above. The radioactivity was measured in the dichloromethane phase following centrifugation and transfer to the conical sample vial. Samples were analyzed in sextuplet. Aqueous solutions of ^{14}C-ibandronate corresponding to 50, 25 and 5 ng were regarded as 100% recovery reference samples and analyzed in triplicate. Recovery studies in bone were performed in a pool of ground bone from rats dosed with ^{14}C-ibandronate. GC–MS quantification of the *N*,*N*-dialkyl substituted β-alanine derivatives obtained from bone samples processed as described in Section 2.4.1.4, were compared with reference samples obtained by combustion of the bone and measurement of the radioactivity by LSC. The latter was considered to result in 100% recovery. For the ELISA, pooled serum/plasma samples were spiked with 200 or 800 pg/ml ibandronate, then diluted 1:10 with human drug-free serum/plasma and analyzed in sextuplet.

2.6.5. Limit of quantification

For both the GC–MS and ELISA methods, the limit of quantification is defined as the lowest concentration in the calibration curve and must not exceed 20% deviation in accuracy and precision (coefficient of variation; CV).

2.6.6. Sample stability

To determine long-term stability, plasma and urine samples were spiked to relevant concentrations, deep frozen at −25 °C and analyzed by GC–MS 3 months and 1 year later. Freeze–thaw stability was determined in serum/plasma and urine samples immediately after spiking and after 1, 2 and 3 freeze–thaw cycles. Bench-top stability of serum/plasma and urine samples was determined 0 and 4 h after spiking. Processed sample stability was determined by analysis of processed spiked urine samples either immediately after processing or 18 h later to simulate the length of time between processing and analysis of the last sample in the sample tray. All measurements were conducted in sextuplet.

2.6.7. Cross-validation of GC–MS and ELISA in human serum/plasma

Each method was used to analyze, in at least sextuplet, pooled serum/plasma samples spiked with ibandronate. For the ELISA, the serum/plasma samples were diluted 1:10 with the same drug-free serum/plasma.

2.7. Application of GC–MS in in vivo preclinical studies

2.7.1. Concentration of ibandronate in rat femurs after 104 weeks oral administration in rats

The GC–MS method was used to quantitatively determine ibandronate in bone samples from rats as part of a long-term safety study. Details of the study protocol are described in Lalla et al. [27]. The right femurs from 10 animals per group were removed and frozen at −20 °C in 0.9% sodium chloride solution. For previous analyses other than ibandronate bone concentrations, this bone tissue underwent various freeze–thaw cycles and partial embedding in plexiglas. To determine ibandronate concentrations, non-embedded bone samples (proximal metaphyses plus epiphyses, representing about one third of the entire femur length) were separated from the plexiglass socket, dried to constant weight at 80 °C and their dry weight recorded after adaptation to room temperature. The bone samples were then processed and their ibandronate content determined by GC–MS as described above. Statistical evaluation was performed using the stratified Wilcoxon test (strata = dose) and the two-way ANOVA model.

2.7.2. Gender-related concentration of ibandronate in vertebrae and long bones of aged rats after repeated subcutaneous (s.c.) administration

Aged (9-month-old) Sprague-Dawley rats of both sexes were given 0.1 mg/kg ibandronate (free acid equivalents) in isotonic saline and adjusted to pH 7.4, by s.c. injection for 7 consecutive days. Twenty-four hours after the last ibandronate administration, the animals were sacrificed by exsanguination.

Both femurs and L1–L5 lumbar vertebrae were removed, cleaned of soft tissue and dried to constant weight at 80 °C. After adaptation to room temperature, the proximal and distal parts of the right femur were separated from the midshaft to produce three parts of equal length consisting of different trabecular and cortical compositions, which were stored separately. Of the lumbar vertebra, only L5 was used for analysis. The dry weight of all bones and bone parts was recorded and the samples processed and analyzed by GC–MS as described above.

Statistical evaluation was conducted using non-parametric equivalence tests, with equivalence criteria as defined by Golditz et al. [28], and the Wilcoxon Mann-Whitney estimate with a 95% two-sided confidence interval.

3. Results

3.1. Analysis of serum, plasma and urine by GC–MS

Data for precision and accuracy, recovery and limit of quantification of ibandronate in serum/plasma and urine for the GC–MS method are presented in Table 1. The specificity of the assay was confirmed by the absence of significant peaks from the mass chromatograms at the retention times of the methyl esters of the *N*,*N*-dialkyl-β-alanine derivatives in multiple ion detection modes at the appropriate *m*/*z* ratios (Fig. 2). Fig. 3 shows the corresponding peaks for ibandronate and the internal standard in a plasma sample taken

Table 1
Summary of validation data for GC–MS and ELISA

	GC–MS		ELISA
	Plasma	Urine	Serum
Range of quantification	1 to 500 ng/ml	2 to 7500 ng/ml	50 to 1600 pg/ml (100 μl sample)
Precision			
Overall[a]	4.0 to 7.4%	3.9 to 8.4%	5.9 to 8.7%
Inter-assay	6.5 to 9.7%	5.1 to 21.4%	12.2 to 20.0%
Intra-assay	3.2 to 7.6%	1.3 to 5.6%	3.2 to 4.6%
Overall accuracy[a]	+0.1 to +7.4%	−7.7 to +3.7%	−1.1 to −6.7%
Recovery in plasma (%)	14.7 to 17.2%	20.1 to 24.4%	100.0 to 106.0%
Limit of quantification	1 ng/ml	2 ng/ml	50 pg/ml (100 μl sample) 10 pg/ml (500 μl sample)

[a] See Tables 2–4 for further details.

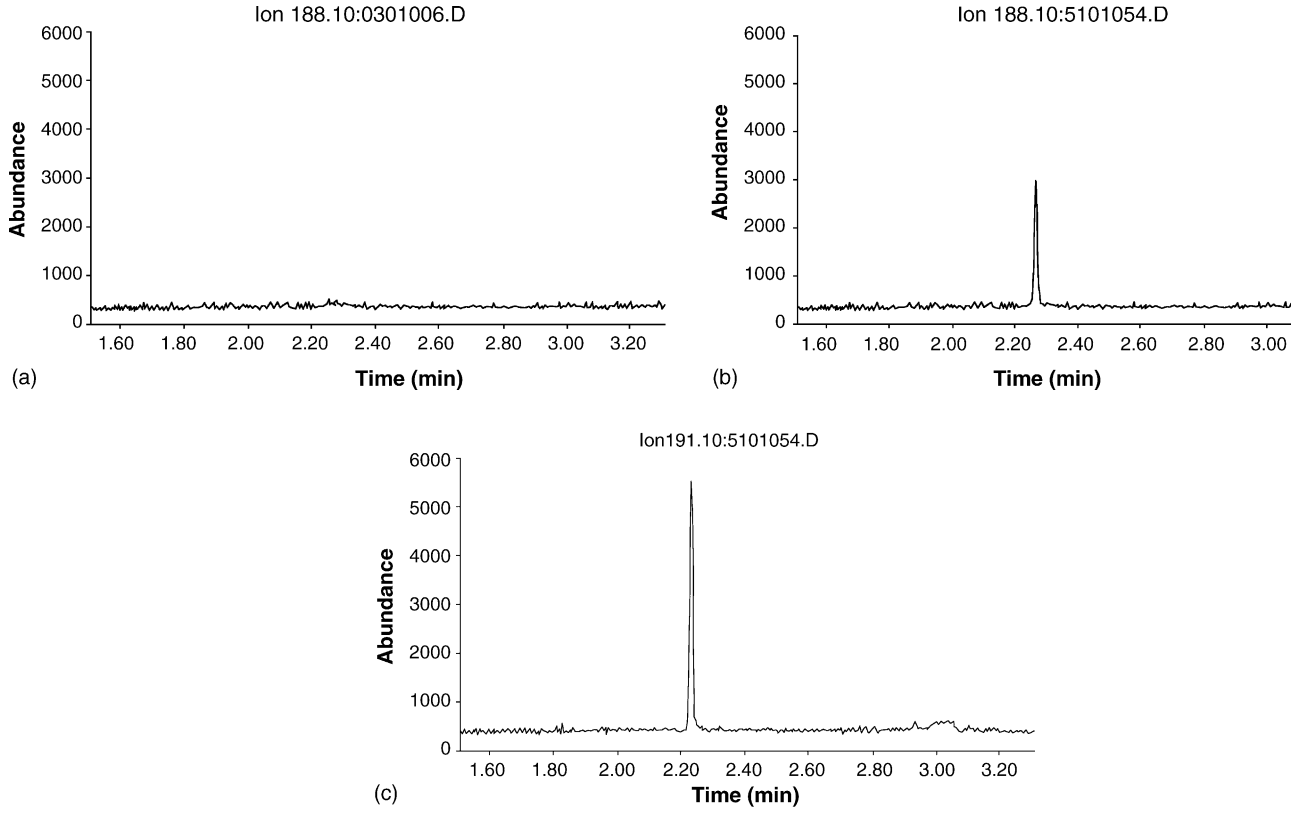

Fig. 2. GC–MS mass chromatographs showing (a) blank serum, and spiked with (b) ibandronate, and (c) internal standard (D_3-ibandronate) peaks.

from a healthy volunteer that had received oral ibandronate (50 mg). Accuracy and precision data for serum/plasma and urine samples are presented in Tables 2 and 3 , respectively.

Overall precision was 4.0–7.4% for serum/plasma and 3.9–8.4% for urine. The overall accuracy varies between +0.1 and +7.4% for serum/plasma, and −7.7 and +3.7% for urine.

Table 2
Accuracy and precision data for ibandronate in human plasma by GC–MS

Ibandronate concentration level (ng/ml)	Precision (%)			Overall accuracy (%)
	Intra-assay	Inter-assay	Overall	
5	5.8	9.7	6.5	7.4
25	7.6	6.5	7.4	2.0[a]
50	3.2	7.2	4.0	0.1

Thirty serum samples per concentration (five groups of six replicates).
[a] $n = 28$.

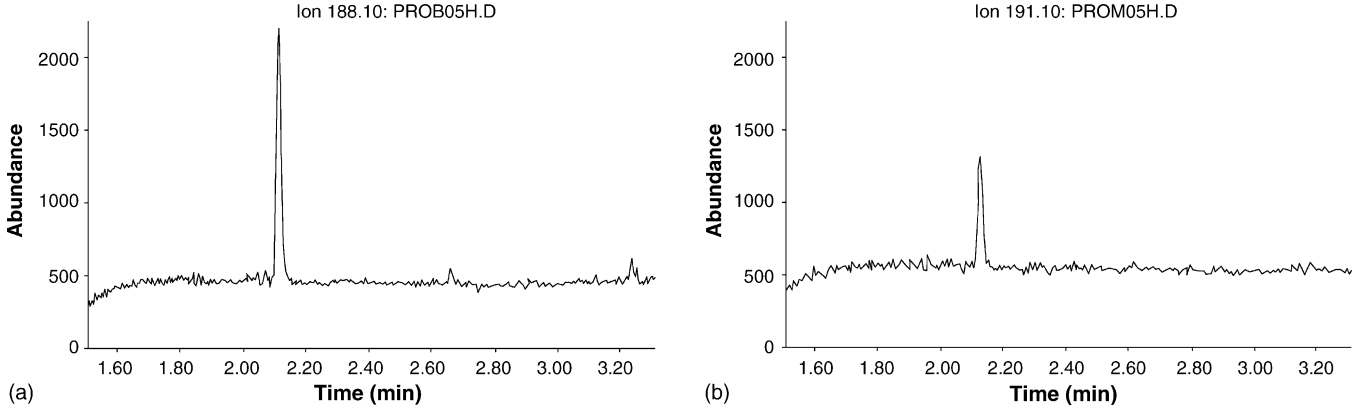

Fig. 3. GC–MS mass chromatographs of plasma sampled from a volunteer taken 30 min after ingestion of 50 mg ibandronate showing peaks for (a) ibandronate and (b) the internal standard, D_3-ibandronate.

Comparison of mean values for samples spiked to the same concentrations demonstrated that the method performs equally well in either plasma or serum.

3.2. Analysis of serum and plasma by ELISA

Data for precision and accuracy, recovery and limit of quantification of ibandronate for ELISA in serum/plasma are presented in Table 1. All blank serum/plasma samples yielded values below the lower quantification limit, demonstrating the specificity of the assay. Because of the non-specific matrix effect of serum/plasma, calibration was performed in the individual blank serum/plasma of the respective volunteer. The limits of quantification of ibandronate were 50 and 10 pg/ml in a 100 and 500 μl sample, respectively. Accuracy and precision data for serum/plasma are presented in Table 4.

3.3. Stability of plasma and urine samples

Drug concentrations remain stable in unprocessed plasma and urine samples stored at −25 °C for up to 1 year, or stored at −25 °C and subjected to 1–3 repeated thawing cycles, or when kept at room temperature for 4 h. Processed samples showed no decrease in the analytical signal when kept at room temperature for up to 18 h.

3.4. Comparison of GC–MS and ELISA methods

The cross-validation of the GC–MS and ELISA methods yielded comparable results, with no significant difference between the two techniques and with comparable accuracy at ibandronate concentrations of 1.5, 5 and 10 ng/ml in serum (Table 5).

Table 3
Accuracy and precision data for ibandronate in human urine by GC–MS

Ibandronate concentration level (ng/ml)	Precision (%)			Accuracy (%)
	Intra-assay	Inter-assay	Overall	
10	5.6	11.9	6.9	3.7
100	2.6	21.4	8.4	−1.2[a]
500	1.3	10.8	4.2	−2.4
1000	3.5	11.5	5.4	−2.5
5000	3.6	5.1	3.9	−7.7

Thirty urine samples per concentration (five groups of six replicates).
[a] $n = 29$.

Table 4
Accuracy and precision data for ibandronate in human serum by ELISA

Ibandronate concentration level (ng/ml)	Precision (%)			Accuracy (%)
	Intra-assay	Inter-assay	Overall	
150	4.6	20.0	8.7	−1.1
500	3.2	17.1	7.2	−6.7
1000	3.8	12.2	5.9	−3.7

Thirty-six serum samples per concentration (six groups of six replicates), sample size: 100 μl.

Table 5
Comparison of the GC–MS and ELISA methods for accuracy

Ibandronate concentration (ng/ml)	ELISA[a] ($n = 10$)	GC–MS ($n = 6$)
1.5	1.4 ± 0.1	1.4 ± 0.1
5	4.3 ± 0.2	4.7 ± 0.2
10	8.7 ± 0.4	10.1 ± 0.2

[a] Samples diluted 1:10 before assay. Data are mean values of indicated sample numbers.

3.5. Analysis of ibandronate in bone by GC–MS

A comparison of ibandronate concentrations in ground rat bone by measurement of ^{14}C-labelled ibandronate by LSC and GC–MS demonstrated a 107.5% recovery of ibandronate from bone calculated by GC–MS (by definition recovery of ibandronate by LSC was 100%). Accuracy and precision calculated for the GC–MS method were +7.5 and +12.9%, respectively, at the 0.5 ng/mg level. These data were comparable with those obtained by LSC. The limit of quantification is dependent on weight of bone and volume in which the sample is dissolved. However, as the limit of quantification of aqueous solutions of ibandronic acid was 1 ng/ml, the limit of quantification in bone is calculated using the equation described in Section 2.4.1. For example, the limit of quantification for a 100 mg bone sample dissolved in 2 ml is 20 pg/mg when a 1 ml aliquot of the bone solution is processed. These data confirm that the presence of bone matrix does not affect the accurate quantification of ibandronate by GC–MS.

3.6. Analysis of ibandronate concentrations in bone from in vivo studies in rats

In the 2-year study, in which the animals received daily oral administration of ibandronate from 6 weeks of age, the uptake of ibandronate was found to be dose-dependent and linear for both male and female rats. However, bones from male animals incorporated about twice the amount per mg dry weight than those from females ($p < 0.001$ for gender and dose differences; Fig. 4). No saturation of bone uptake

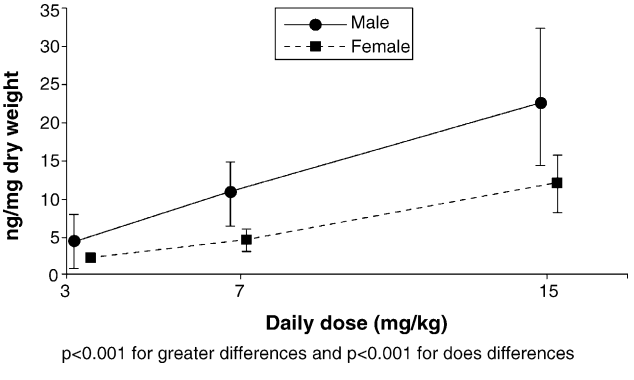

Fig. 4. Linear increase in ibandronate concentration in bone (proximal femur) of rats with increasing dose of daily oral ibandronate administered for 104 weeks to rats.

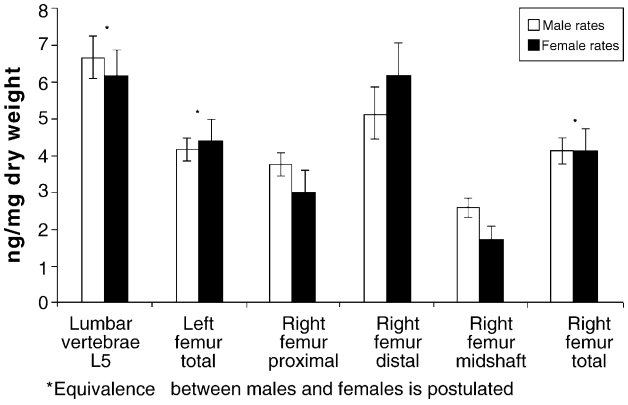

Fig. 5. Concentration of ibandronate in various bones of aged rats following daily s.c. injection of 0.1 mg/kg ibandronate for 7 days.

was observed during lifelong administration of ibandronate, even at high doses of up to 15 mg/kg/day.

In the second study in aged rats, no gender difference in bone uptake was observed for ibandronate in the total bone of both lumbar vertebra and whole femurs taken from skeletally mature animals of both genders following a week's s.c. administration of ibandronate. However, ibandronate concentrations were higher in the distal and proximal femur (containing both cortical and trabecular bone) than in the midshaft (containing only cortical bone). However, no gender differences were observed for the uptake of ibandronate into vertebrae and long bones (total femur; Fig. 5).

4. Discussion

Unlike conventional bisphosphonates, ibandronate cannot easily be derivatized with a chromophore, and therefore cannot be detected by UV light or fluorescence with the sensitivity needed for a bio-analytical assay. Although a high-performance ion exchange chromatographic method has been recently described to detect ibandronate complexed with copper [29], the limit of detection is poor (6 mg/l) and insufficiently sensitive for clinical use. Additionally, although a chromophore derivative of the bisphosphonate olpadronate, which also has a tertiary amine structure, has been described [25], the derivative is not characterized in detail, needing semi-automation, and has a higher limit of quantification of 5 ng/ml for urine and 10 ng/ml for serum. There was, therefore, a need for assays to detect and quantify ibandronate in biological and bone matrices that could be applied over a wide range of concentrations (to accommodate the different therapeutic doses used for MBD and PMO) without UV or fluorescence detection.

Both GC–MS and ELISA are established, sensitive methods that were developed because UV and fluorescence detection are unsuitable for ibandronate, and to avoid the need to handle radioisotopes. GC–MS and ELISA methods have also been reported for risedronate, which contains a pyridine

moiety [26,30,31] and thus can also be analyzed by HPLC with UV detection [32].

The GC–MS method described here requires a greater number of sample preparation steps than the UV or fluorescence detection methods used for alendronate [18–21] and pamidronate [23–24], for example. Each stage of the sample preparation results in drug loss; consequently, overall recovery is low (15–25%). However, the use of a deuterated internal standard corrects for this low recovery, as demonstrated by comparable findings when bone ibandronate levels were measured using both GC–MS and LSC of ^{14}C-labelled ibandronate. ELISA has a lower limit of quantification and, therefore, is more sensitive than GC–MS. Moreover, because no sample preparation is needed the recovery of ibandronate in serum and plasma approaches 100%. The use of standards either in the patient's own drug-free serum or plasma, or diluted in pool plasma or serum, corrects for inter-individual matrix effects. Despite the differences in complexity between the two methods, when directly compared over the same range of ibandronate concentrations they produce similar results.

The GC–MS and ELISA ibandronate assays developed and described in this report are at least as sensitive and reproducible as those obtained with other bisphosphonates, including alendronate [18–21], pamidronate [22–24,33], risedronate [26,30–32], olpadronate [25], minodronate [34], tiludronate [35], and zoledronate [36–38]. The GC–MS method performs at least as well as those using HPLC and UV/fluorescence detection of chromophore bisphosphonate derivatives. Whereas the ELISA, with its lower limit of quantification of 0.01 ng/ml (with a 500 μl sample), is not only more sensitive than the assay for risedronate [26,30,31] (lower limit of quantification 0.15 ng/ml) but also to that of a RIA for zoledronate, (lower limit of quantification 0.4 ng/ml) [36,37].

The utility of both these ibandronate assays over a wide concentration range has been demonstrated in phase I pharmacokinetic studies with oral and intravenously administered ibandronate in healthy subjects, postmenopausal women and patients with MBD [39]. In addition, the ELISA has been used in clinical studies to determine levels of ibandronate in serum [40] and plasma [41]. In the recent Monthly Oral Pilot Study (MOPS) [42] with monthly oral ibandronate (up to a dose of 150 mg), ELISA and GC–MS were used to quantify serum and urine concentrations of ibandronate, respectively.

In addition to its use for biological samples, the GC–MS assay has also been used in animal studies to determine bone ibandronate concentrations in rats [12] and monkeys [43], as well as in the two rat studies described herein. The two preclinical studies described in this report illustrate the practical use and value of accurate quantification of ibandronate in bone. In the first study, a linear uptake of ibandronate by bone was clearly demonstrated at doses even far in excess of that intended for therapeutic use. In addition, although the amount of ibandronate deposited in bone was greater in male rats, this was considered to be a result of the different growth rate rather than a true difference between the genders. This was confirmed by the second study in skeletally adult animals, which demonstrated a gender-independent uptake and heterogeneous distribution of ibandronate in bone. This pattern of distribution was considered to be a result of metabolic differences in different types of bone (cortical and trabecular).

Previously reported preclinical studies with ibandronate have given rise to and supported the total dose concept [11–13], i.e., that ibandronate uptake by bone is related to the total dose regardless of whether it is administered continuously or intermittently. This concept and further information about skeletal accumulation and distribution, and about potential gender differences in uptake, would be impossible to obtain without the analysis of bone ibandronate levels. Such preclinical studies also support the intermittent dosing of ibandronate, which is now in the late stages of clinical development.

5. Conclusions

The described GC–MS and ELISA methods enable accurate and reproducible determination of ibandronate in bone and biological fluids with sensitivity at least comparable to that of assays available for other bisphosphonates, while avoiding the need to handle and use radiochemicals. In the clinical setting, these ibandronate assays enable quantification of the low ibandronate serum levels in women with PMO, as well as the higher levels in patients with MBD and hypercalcaemia of malignancy. The use of GC–MS also eliminates the need for a separate ELISA assay for urine samples. In the experimental setting, the quantification of bone ibandronate levels has led to greater understanding of the bone kinetics of ibandronate and the total dose concept, thus supporting the use of intermittent dosing schedules in clinical trials.

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