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Development and validation of a highly sensitive RIA for zoledronic acid, a new potent heterocyclic bisphosphonate, in human serum, plasma and urine

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Abstract

Zoledronic acid is a new, highly potent bisphosphonate drug under clinical evaluation. A radioimmunoassay has been developed to determine zoledronic acid concentration in human serum, plasma, and urine. The assay utilizes rabbit polyclonal antisera against a zoledronic acid–BSA conjugate and a [^{125}I]zoledronic acid derivative as tracer in a competitive format adapted to microtiter plates. The assay shows a LLOQ 0.4 ng/ml in serum or plasma (interassay%CV = 17%, accuracy 97%), 5 ng/ml in urine (21%, 98%). In 23 patients receiving 4, 8 or 16 mg of zoledronic acid, drug concentrations in plasma were dose proportional and showed a multiphasic profile, followed by a prolonged gradual decline to concentrations near the LLOQ. Zoledronic acid disposition in plasma and the recovery of only 40–50% of the dose in urine are consistent with the rapid and extensive uptake by and slow release from bone in parallel with renal clearance, typically shown by bisphosphonates.

Keywords: Zoledronic acid; Biphosphonate; Radio-immunoassay; Pharmacokinetics

1. Introduction

Biphosphonates are hydrolytically stable analogs of pyrophosphate, which inhibit bone resorption as a consequence of affecting osteoclast and probably osteoblast activity [1–3]. The therapeutic efficacy of bisphosphonates in disorders of bone turnover has been shown in the treatment of Paget's disease, tumor-induced hypercalcemia (TIH), and multiple myeloma [2]. Zoledronic acid, a heterocyclic nitrogen-containing bisphosphonate (see Fig. 1 for its chemical structure) has shown significantly increased potency compared to other bisphosphonates in both in vitro and in vivo bone resorption models [2,4–6]. Zoledronic acid fully inhibits osteoclastic activity and bone resorption at low doses that do not adversely affect bone formation and mineralization, and have no appre-

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ciable impact on renal function, resulting in an improved ratio of antiresorptive versus renal effects [7]. Zoledronic acid is being developed for the treatment of tumor induced hypercalcemia, bone metastases arising from any cancer, and for the prevention of bone metastases associated with advanced breast cancer and locally advanced prostate cancer.

The expected clinical dose of zoledronic acid is 4 or 8 mg, to be administered as a 5 or 15 min intravenous infusion every 3–4 weeks. The low and infrequent dose, as well as the expected extensive uptake of zoledronic acid into human bone tissue and rapid renal clearance, based on observation of such processes in animal models [4,8,9], suggest that the circulating concentrations in human plasma will be very low, requiring a sensitive analytical method to determine the pharmacokinetics of zoledronic acid.

Bisphosphonates do not have the strong chromophores typically used for UV detection in HPLC methods. Various strategies for the determination of zoledronic acid in biological matrices were investigated, including sample preparation and derivatization procedures, separation using liquid or gas chromatography, and detection by mass spectrometry. None of these approaches gave sufficient analytical reproducibility and sensitivity.

An enzyme inhibition assay based on the inhibition of sterol biosynthesis by zoledronic acid has been reported previously [10]. However, the limit of detection of this assay (25 ng/ml) was not sufficient to allow pharmacokinetic evaluation in animals and humans.

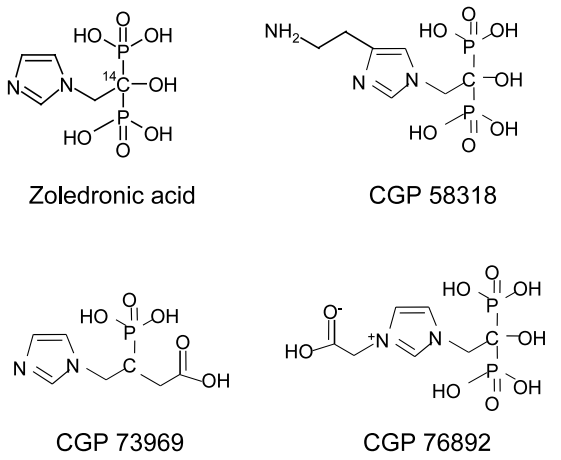

Fig. 1. Chemical structure of zoledronic acid and haptens.

In addition to the difficulty of developing a sufficiently sensitive assay, the physicochemical properties of zoledronic acid generate analytical problems. The bisphosphonate moiety is highly negatively charged and binds strongly to the surface of calcium crystals to form insoluble calcium salts. A consequence of binding to calcium, and other divalent cations, is a modification of the chemical structure of zoledronic acid. This may affect binding to proteins, and particularly antibodies. In addition, precipitation of calcium adducts of zoledronic acid has been observed in the calcium-containing phosphate buffers normally used to perform immunoassays. EDTA, which is frequently used to prepare plasma, may affect the binding of zoledronic acid to calcium, in turn affecting the molecular conformation [11–15].

Zoledronic acid has a low molecular weight (272.1 Daltons) and is not immunogenic, requiring conjugation of the molecule to a protein carrier to induce antibody response in animals. Depending on the site of conjugation and type of carrier, antibodies of differing avidity, specificity, and suitability for immunoassay may be generated. A suitable tracer capable of interacting with the antibody, and of adequate specific activity to measure the expected low in vivo concentrations of drug was obtained by incorporating ^{125}I into a chemical derivative of zoledronic acid containing an amino group (see Fig. 1). With the appropriate combination of antibody and tracer, a competitive radioimmunoassay (RIA) has been developed. The assay conditions have been optimized to reduce variation. The specificity of the method has been studied in rats dosed with ^{14}C zoledronic acid, by comparing the ^{14}C concentrations in plasma and urine with those obtained by the radio-immunoassay. The absence of metabolism of zoledronic acid in the rat was established by liquid chromatographic analysis of all radioactive components in the urine. The assay has been applied for the measurement of zoledronic con-

centrations in plasma and urine of cancer patients with bone metastases who had received a single 5 or 15 min infusion of 4, 8, or 16 mg zoledronic acid monohydrate for injection (ZOMETA®).

2. Materials and methods

2.1. Antibody production

2.1.1. Immunogen preparations

Different immunogens were prepared using several analogs of zoledronic acid containing either a primary amino group (CGP 58318) or carboxyl group (CGP 73969, CGP 76892), for conjugation with a protein carrier. The functional groups were introduced in different positions of the zoledronic acid molecule (see Fig. 1), allowing different presentation of the antigen.

CGP 58318 was conjugated to bovine serum albumin (BSA) (immunogen 1) or to Diphteria toxoid (immunogen 2) according to the method described by Reichling [16]. Briefly, 12 mg CGP 58318 and 76 mg of carrier protein were dissolved in 4 ml of borate buffer. One milliliter the addition of a 12.5% glutaraldehyde solution was added dropwise and the mixture was shaken for 2 h at room temperature. The reaction was stopped with the addition of 20 mg glycine. The conjugates were dialyzed 24 h against water and then lyophilized.

CGP 73969 was conjugated to BSA (immunogen 3), and to Diphteria toxoid (immunogen 4) according to a modification of the procedure described by Erlanger [17]: 17 mg CGP 73969 were dissolved in 1 ml of borate buffer; 14 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 10 mg of *N*-hydroxysulfosuccinimide were added, and the mixture was shaken for 1 h at room temperature. 125 mg protein were dissolved in 3 ml of borate buffer and added to the first solution. After overnight agitation at +4 °C, the reaction was stopped with 20 mg of glycine. The conjugates were dialyzed for 24 h against water and then lyophilized.

CGP 76892 was conjugated to Keyhole Limpet hemocyanin (immunogen 5) using a modification of the procedure described above [17].

2.1.2. Immunization procedure

Immunizations were performed according to a standard protocol: 1 mg of conjugate was injected subcutaneously into rabbits or guinea pigs using Freund's Complete Adjuvant for the first injection and Incomplete Freund's Adjuvant for subsequent injections.

Immunogens 1 and 3 were applied to three rabbits each on day 1 and at weeks 2, 4, 8, 12, 16, 35, 50, 62, 84 and 104. Blood samples for titer determinations were collected on day 1 and at weeks 6, 10, 14, 18, 20, 36, 38, 51, 53, 63, 65, 86, 88 and 106.

Immunogens 2 and 4 were applied to 10 guinea pigs each on day 1 and at weeks 3, 5 and 9. Blood samples were collected on day 1 and at weeks 7 and 12.

Immunogen 5 was applied to three rabbits on day 1 and at weeks 3, 5, 9, 13, 17 and 24. Blood samples were collected on day 1 and at weeks 6, 10, 14, 18, and 25.

2.1.3. Antibody titration

The principle of the assay was based on a competitive ELISA. CGP 58318 was labeled with peroxidase according to Nakane and Kawaoi [18]. Microtiterplates (NUNC, Maxisorb, Ref 439454) were coated with either 100 μl of a solution of sheep anti-rabbit IgG antibody (Sigma R3631) diluted at 10 μg/ml in a 0.1 mol/l pH 9.6 carbonate buffer, or 100 μl of rabbit anti-guinea pig IgG antibody (Sigma G7892) diluted at 10 μg/ml in a 0.1 mol/l pH 9.6 carbonate buffer. The plates were incubated overnight. After washing, they were blocked for 2 h with 200 μl PBS-gelatin. After washing, 100 μl of rabbit or guinea-pig antiserum diluted 1:10 000 in phosphate buffer were added and incubated for 4.5 h. After washing 100 μl of CGP 58318-peroxidase conjugate diluted 1:1000 in phosphate buffer and 50 μl of human serum were added to each well. After overnight incubation at 4 °C, the plates were washed and a substrate solution was prepared: 10 mg of tetramethylbenzidine (Fluka 87748) was dissolved in 1 ml of dimethylsulfoxide (Merck 9678) and diluted in 100 ml of substrate buffer (4.5 g of sodium acetate was mixed with 100 ml of bidistilled water and adjusted to pH 3.5 with citric

acid). Fourteen microliters of H_2O_2 (Merck 7209) were added to the mixture immediately before use, and 100 μl of this solution was distributed in each well. After a 30 min incubation, the reaction was stopped by addition of 100 μl 1N H_2SO_4 per well. The optical density was measured at 450 nm in what instrument?

2.1.4. Tracer preparation

An enzymatic iodination on the -NH_2 functional group of CGP 58318 was performed using the lactoperoxidase method by ANAWA Laboratories (Wangen, Switzerland), according to the method described by Marchalonis [19]. The tracer shelf-life was 2 months from the date of synthesis.

2.2. RIA method

2.2.1. Preparation of calibrators and quality control (QC) samples

A stock solution of zoledronic acid at 100 μg/ml was prepared in water. This solution was kept in a polypropylene tube to avoid non-specific adsorption on glass.

2.2.2. Plasma/serum calibration solutions

The stock solution was diluted to 1000 ng/ml with drug-free human serum or heparinized plasma. Nine calibration samples were prepared by serial dilutions to final concentrations ranging from 40 to 0.04 ng/ml. Quality control samples were prepared from an independent stock solution, by serial dilutions to final concentrations of 0.1, 1 and 4 ng/ml.

2.2.3. Urine calibration samples

The stock solution was diluted to 10 μg/ml with drug-free reference human urine. Nine calibration samples were prepared by serial dilutions to final concentrations ranging from 1000 to 1 ng/ml. Each sample was pre-diluted 1:10 in blank urine before use. Quality control samples were prepared from an independent stock solution, by serial dilutions to final concentrations of 5, 10 and 100 ng/ml.

2.3. RIA procedure

The format of the method is based on the procedure described by Lefevre et al. [20]. Two hundred microliters of sheep anti-rabbit antibody (Sigma, 3631) diluted at 5 μg/ml in 0.1 mol/l of carbonate buffer at pH 9.6 were dispensed into each well of a microtiter plate, and incubated overnight at room temperature. The plates were washed three times with the washing buffer. Two hundred and fifty microliters of PBS containing 0.2% gelatin (Fluka # 48722) (blocking buffer) were added into each well, and incubated for 2 h at room temperature. The microtiter plates were washed, and 200 μl of rabbit anti-zoledronate antiserum diluted 1:10 000 in 0.1 mol/l borate buffer at pH 8 (Merck, Titrisol™ # 9888) (dilution buffer) were added to each well and incubated for 3 h at + 37 °C.

The microtiter plates were washed three times with washing buffer. One hundred microliters of tracer ([^{125}I]CGP 58318) diluted at 80 nCi/ml in dilution buffer were added to each well, with successively, 25 μl of calibration solution, quality control or in vivo samples with unknown concentrations of zoledronic acid and 25 μl of reference biological fluid (i.e. blank plasma or urine). The plates were gently shaken for a few seconds and incubated overnight at room temperature. The microtiter plates were covered by an adhesive plastic film and kept in the dark during all incubation steps.

After washing, the microplate wells were broken apart and put into polypropylene tubes (one well/tube) and the radioactivity of the tracer bound to the anti-zoledronic acid antibodies was measured using a gamma counter (Wizard 1470, EG&G).

The assay was performed using nine calibration points (0.04–40 ng/ml in serum or plasma, 1–1000 ng/ml in urine) in triplicate. Blank samples using drug-free plasma, serum or urine were added. Non specific binding (NSB) was measured in three wells, where no anti-zoledronic acid antibody was added.

2.4. RIA validation

The assay was validated by measuring quality control samples (QC) prepared in human serum, plasma and urine, and rat in plasma and urine. Intra-day reproducibility was evaluated by repeatedly measuring four to seven independent samples of blank serum, plasma and urine obtained from healthy volunteers, spiked with zoledronic acid. Serum and plasma were spiked with 0.4, 1 and 4 ng/ml of zoledronic acid. Urines were spiked with 5, 10, 40, 50 and 100 ng/ml.

Inter-day variation was assessed by performing four independent assays on 4 consecutive days. The samples were analyzed in triplicate. Each sample concentration was calculated from the standard curve established on the corresponding plate.

2.5. Stability of stock solution

The stability of the stock solution of zoledronic acid in water (stock solution at 100 μg/ml) was determined by diluting aliquots at various concentrations in human plasma and performing the RIA on the plasma samples. The concentrations were calculated with a calibration curve established from a freshly prepared stock solution. The stability was tested after 1, 4 or 11 months after preparation.

2.6. Long term storage of patient samples

Following initial analysis, plasma samples from patients who received infusions of zoledronic acid were reanalyzed after 2.5 and 8 months of storage at −20 °C. Urine samples from patients were re-analyzed after 2 weeks, 1, 3 and 6 months of storage at −20 °C

2.7. Freeze–thaw cycles and sample storage stability

Drug-free human serum was spiked with zoledronic acid at three concentration levels. The samples were analyzed and then frozen at −20 °C. They were re-analyzed 12 and 15 days after one or two freeze–thaw cycles.

2.8. Sample dilution

Human plasma samples were analyzed undiluted and diluted 10 or 100 fold in drug free human plasma. Human urines were analyzed after a 10, 100 or 1000 fold dilution in drug free human urine.

2.9. Rat studies

Sprague–Dawley rats received intravenously (iv) a dose of 0.16 mg/kg of [^{14}C]zoledronic acid (specific activity 1.8 MBq/mg (see Fig. 1). Blood samples were collected using heparin anticoagulant at 5, 15 and 30 min post drug application and aliquots were centrifuged for 10 min at 2000 rpm. The plasma was separated and stored frozen at −20 °C. Urine was collected individually on ice up to 24 h post dose.

Total radioactivity was measured in each plasma and urine sample by direct liquid scintillation counting (LSC). The liquid scintillation solution used was Irgasave® (Packard Instr. Co., Meriden, CT, USA). Soluene® 350 (Packard Instr. Co., Meriden, CT, USA) was used for solubilizing the blood. Radiometry was performed using a 2700 TR liquid scintillation systems (Packard Instr. Co., Meriden, CT, USA).

The concentrations of total ^{14}C-radioactivity in blood and plasma were determined in weighed samples and converted from nmol/kg to nmol/l, assuming a density of 1.00 g/ml for blood and plasma.

In addition to the total radioactivity measurements, urine and plasma samples were analyzed for zoledronic acid concentration using the radioimmunoassay according to the procedure described above.

2.10. HPLC-reverse isotope dilution (RID) analysis of rat specimens

The HPLC analysis of rat urine was performed under the following conditions: Reversed phase column: Phenomenex® Luna 3 μm C8 150 mm length and 3 mm internal diameter; Column temperature: at 20 °C; Column oven: Column chiller model 7955 (OmniLab, 8932 Mettmenstetten,

Switzerland); HPLC pump system: two Jasco PU-980 solvent delivery pumps (OmniLab) driven by a Jasco-Borwin Vers. 1.50 (Build 11A); Autosampler: Jasco AS-950-10 (OmniLab); Radiomonitor: Radioflow detector LB 508, Cell Z200-4 (EG&G Berthold, 8105 Regensdorf, Switzerland); Liquid scintillator: Rialuma (1.2 ml/min) (Lumac LSC B.V. Groningen, Netherlands); UV Detector (230 nm): Jasco AS-970-M (OmniLab); Data acquisition: Winflow evaluation software version 1.21.07 (EG&G Berthold). The analytical runs were performed under isocratic conditions (flow rate: 0.4 ml/min, total run time: 32 min) with a mobile phase containing 7.12 g of hydrogen phosphate dihydrate, 4.52 g of tetrahexylammonium hydrogen sulfate (counter ion), 0.025 mmol of EDTA, 100 ml of acetonitrile and 60 ml of methanol in 840 ml of water.

The column was preconditioned with a solution of di-sodium-3-amino-1-hydroxy-propylidene-1-1-bisphosphonate-pentahydrate (pamidronate) in mobile phase A for 40 min at a flow rate of 0.4 ml/min.

All urine samples were spiked with 5.21 μg non-radiolabeled zoledronic acid per ml urine (25 μl of urine injected). HPLC fractions were collected every 30 s from 0 to 30 min. The radioactivity in the fractions was determined by off-line radioactivity determination (LSC).

2.11. Human studies

Study patients (13 M/10 F, mean age 57 years, range 44–79 years) had histologically confirmed diagnosis of cancer (multiple myeloma $n = 11$, breast $n = 4$, prostate $n = 3$, lung $n = 1$, other and/or unknown etiology $n = 4$) with biopsy and/or radiological evidence of bone metastases. Patients were ineligible if they had received any prior bisphosphonate therapy, or anti-hypercalcemic medications (calcitonin, gallium nitrate, mithramycin, chemotherapy, or investigational drug(s)) within a pre-specified time prior to study entry. Each patient received open-label in random order a single infusion of zoledronic acid (ZOMETA®), 4 mg/15 min ($n = 4$), 4 mg/5 min ($n = 3$), 8 mg/15 min ($n = 8$), or 16 mg/15 min ($n = 8$). Plasma at multiple time points and quantitative urine collections were obtained up to 24 h post dose, with periodic plasma sampling and spot urine collections continuing up to 28 days post dose. Patients were observed throughout the study for adverse events and safety was monitored at baseline and post dose based on clinical laboratory evaluations, vital signs, and physical examinations.

Pharmacokinetic parameters (peak concentration, area under concentration time curve) were derived from the individual plasma concentration versus time curves using non-compartmental methods.

3. Results

3.1. Antiserum preparation

Only one rabbit gave significant levels of polyclonal antibodies against zoledronic acid. This animal was immunized with immunogen 1. A titer significantly different from the control serum was observed at 18 weeks after the first injection. However, a high titer was not observed until 36 weeks after the first injection.

3.2. RIA performance in human serum, plasma and urine

3.2.1. Standard curves and assay performances

The dynamic range of the assay is 0.04–40 ng/ml in plasma or serum, and 1–1000 ng/ml in urine (Figs. 2 and 3). The assay was validated using quality control samples which had been prepared by spiking with a known concentration of zoledronic acid in drug-free plasma, serum or urine (QC samples).

In serum the intra-assay variation was better than 20% for each concentration with a minimum of 12% for the lowest concentration (0.4 ng/ml) and a maximum of 17% for the mid concentration (1 ng/ml). The overall accuracy was in the range 102–118%. In plasma the intra-assay variation was in the range 17–20% with an overall accuracy of 95–103%. In urine the intra-assay variation was between 16 and 22% with an overall accuracy of about 100% (Table 1).

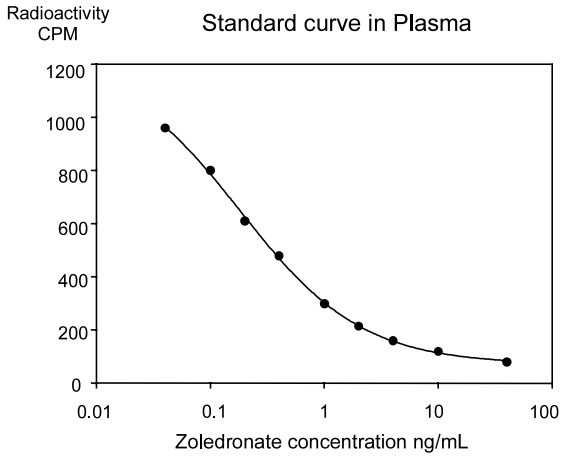

Fig. 2. Standard curve in human plasma. Typical example of a standard curve obtained in human plasma. The curve was fitted using a four parameter logistic algorithm. The concentration range of the curve is between 0.04 and 40 ng/ml.

The inter-assay variation was measured by analyzing the same QC samples in four independent assays performed on 4 different days. The inter assay variation was found in serum between 6.14 and 13.8%, in plasma between 7.1 and 15.3%, and in urine between 6.7 and 8% (Table 2).

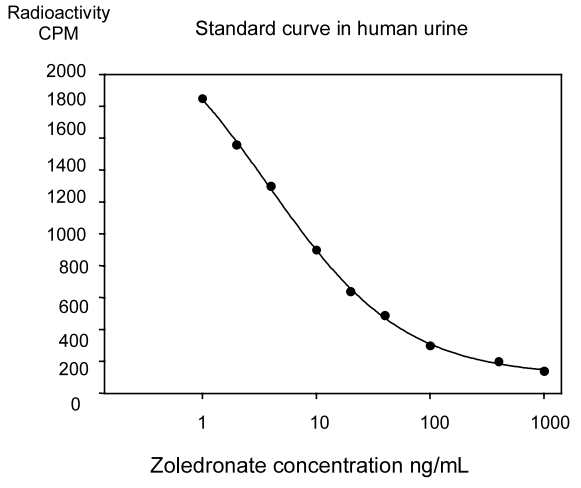

Fig. 3. Standard curve in urine. Typical example of a standard curve obtained in urine. The concentration range is between 1 and 1000 ng/ml.

Table 1
Reproducibility of the zoledronic acid RIA in different human serum, plasma and urine

	Serum ($n = 7$)		
	0.4 ng/ml	1 ng/ml	4 ng/ml
Mean (ng/ml)	0.47	1.11	4.08
SD	0.06	0.19	0.58
CV (%)	12	17	14
Accuracy (%)	118	111	102
	Plasma ($n = 4$)		
	0.4 ng/ml	1 ng/ml	4 ng/ml
Mean (ng/ml)	0.386	1.03	3.8
SD	0.067	0.21	0.72
CV (%)	17	20	19
Accuracy (%)	97	103	95
	Urine ($n = 6$)		
	5 ng/ml	10 ng/ml	100 ng/ml
Mean (ng/ml)	4.91	9.93	100
SD	1.04	1.6	19
CV (%)	21	16	19
Accuracy (%)	98	99	100

Seven different sera, four different plasma and six different urines from healthy volunteers were spiked independently with three concentrations of zoledronic acid. These samples were analyzed with the RIA. The results show reproducibility better than 20%. Accuracy, measured concentration/nominal concentration × 100. SD, standard deviation. CV, coefficient of variation.

3.2.2. Limit of quantitation (LOQ)

The lower limit of quantitation (LLOQ, lowest concentration of a quality control sample measured with a precision better than 30% and an accuracy between 70 and 130%) in serum and plasma was estimated to be 0.4 ng/ml. The upper limit of quantitation (ULOQ, highest concentration of a quality control sample measured with a precision better than 30% and an accuracy between 70 and 130%) in both matrices was set at 4 ng/ml.

Based on the same criteria, the lower limit of quantitation in urine was 5 ng/ml, and the upper limit of quantification was 100 ng/ml.

Table 2
Validation of zoledronic acid RIA in human serum, plasma and urine

	Nominal concentration (ng/ml)	Day 1		Day 2		Day 3		Day 4		Inter-assay variation (%)
		Mean	Accuracy (%)	Mean	Accuracy (%)	Mean	Accuracy (%)	Mean	Accuracy (%)	
Serum	0.4	0.47	119	0.36	91	0.38	96	0.36	89	13.83
	1	1.11	111	0.98	98	0.94	94	0.92	92	8.75
	4	4.08	102	3.62	91	3.87	97	4.16	104	6.14
Plasma	0.4	0.43	107	0.37	94	0.36	91	0.39	98	7.11
	1	1.03	103	0.98	98	0.91	91	1.03	103	5.86
	4	4.5	113	4.2	105	3.12	78	3.8	95	15.28
Urine	5	5.36	107	4.8	96			4.77	95	6.68
	10	10.5	105	8.8	88	9.04	90	9.67	97	8.03
	100	95	95	109	109	92	92	103	103	7.74

Three quality control samples were analyzed in four independent analyses performed on 4 different days.

Table 3
Validation of the zoledronic acid RIA in rat plasma and urine

QC	Plasma determinations			Urine determinations		
	4 ng/ml	10 ng/ml	40 ng/ml	10 ng/ml	50 ng/ml	100 ng/ml
ng/ml (mean value)	4.25 ($n = 9$)	10.2 ($n = 10$)	40.2 ($n = 10$)	7.21 ($n = 2$)	43.5 ($n = 3$)	99.6 ($n = 3$)
Accuracy (%)	106	102	100	72.1	87	99.6
CV (%)	18	16	11	22	7	9.9

3.3. RIA performances in rat plasma and urine

The precision and the accuracy of the zoledronic acid RIA in rat plasma and urine are summarized in Table 3. The performance of the assay was similar to that in human plasma and urine.

3.4. Stability of zoledronic acid

The recovery of the stock solution concentration tested 1, 4 and 11 months after preparation, was 94, 97 and 90%, respectively.

3.5. Freeze–thaw cycles and sample storage stability

No decrease of zoledronic acid concentrations was observed, demonstrating stability of drug in plasma after two freeze–thaw cycles.

3.6. Long term storage of patient samples

No decrease of zoledronic acid in plasma was observed, showing that zoledronate is stable after 8 months of storage.

No decrease of zoledronic acid in urine samples was observed after a storage of 1 month. However a decrease was observed after a storage of 3 and 6 months.

3.7. Dilution of human samples

Comparison between analyses of plasma or urine samples at different dilutions showed correlation coefficients of 0.99 and slopes of 1.02 and 0.97 for plasma and urine, respectively, demonstrating linearity of dilution (Figs. 4 and 5).

3.8. Zoledronic acid in rat plasma and urine

The ^{14}C concentrations in plasma were similar to concentrations of zoledronic acid measured by RIA at all time points and in all animals (Fig. 8, Table 4). Zoledronic acid concentrations calculated from ^{14}C total radioactivity and measured by RIA in the 0–24 h urine fractions were similar. The radioactivity recovered in the urine represented 17–34% of the administered dose (Fig. 8, Table 4).

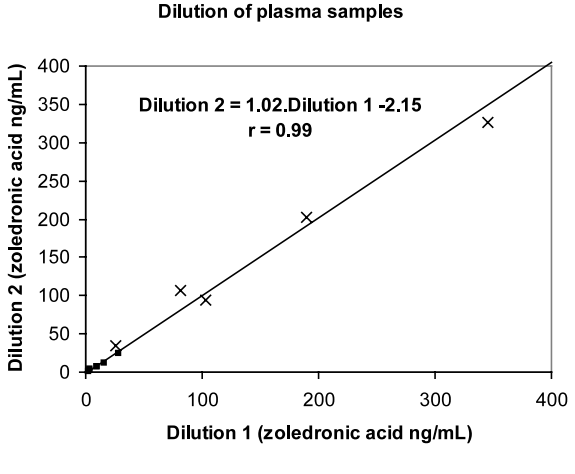

Fig. 4. Dilution of patient plasma samples. Plasma patient samples were analyzed after different dilutions in drug free human plasma. The graph shows the comparison between two dilutions: undiluted/diluted 10 times (■) or diluted 10 times/diluted 100 times (×). The comparison of zoledronic acid concentration in samples after various dilution gives a coefficient of correlation of 0.99. No bias was observed (slope = 1.02).

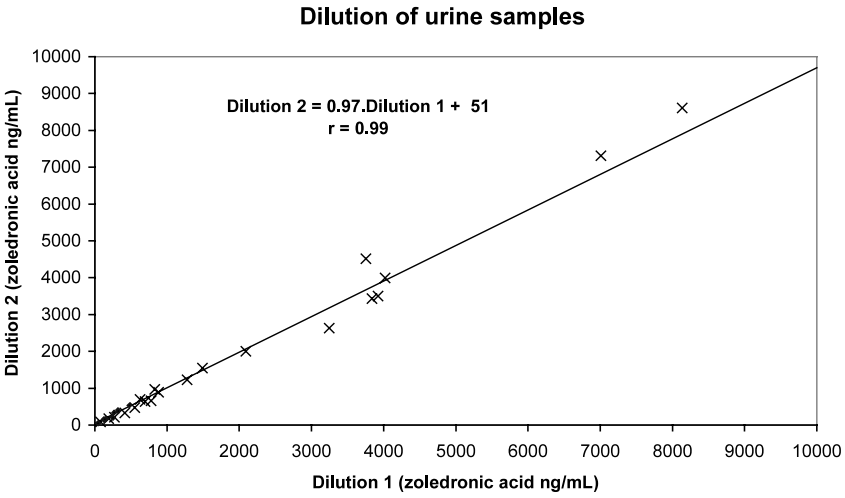

Fig. 5. Dilution of patient urine samples. Urine patient samples were analyzed after different dilutions in drug free human urine. The graph shows the comparison between two dilutions: diluted 10 times/diluted 100 times (■) or diluted 100 times/diluted 1000 times (×). The comparison of zoledronic acid concentration in samples after various dilution give a coefficient of correlation of 0.99. No bias was observed (slope = 0.97).

3.9. Investigation of zoledronic acid metabolites in rat urine

Since the radioactive ^{14}C dose administered to the rats was predominantly excreted renally, the 0–24-h urine samples of the rats were selected for metabolism investigation using the HPLC method.

The off-line radiochromatograms (Fig. 7) demonstrate that only unchanged zoledronic acid (retention time 12 min) could be detected in the 0–24 h urines. The trace amounts of radioactivity detected as peaks with shorter retention times are not biotransformation products but represent impurities ($< 1\%$) of the [^{14}C]zoledronic acid, as these peaks were also seen in the HPLC radiochromatograms of the drug solution used for iv dosing (Fig. 6) and represent radioactive impurities.

3.10. Human studies

Of the 23 patients enrolled, two patients withdrew from the study on days 14 and 28 post dose prior to completion of all scheduled pharmacokinetic sampling due to disease progression. All serious adverse events reported during the study ($n = 5$ events in five patients) were considered to be disease, not drug related. The mean plasma concentration versus time curves for the four treatment groups are shown in Fig. 9. Zoledronic acid concentrations were above the limit of quantitation of the method (0.4 ng/ml) for at least 24 h post-dose. There was an initial rapid decline of concentrations, to on average $< 10\%$ of peak concentration by 4 h, and $< 1\%$ of peak concentration by 24 h. Day 28 post-dose, concentrations of the 8 and 16 mg dose groups were 0.1% of peak concentration, representing the limit of quantitation, with the concentrations of the 4 mg dose groups not being measurable at this time point. Peak concentrations occurred at the end of infusion with immediate steep declines in concentrations thereafter. The mean cumulative urinary excretion of zoledronic acid for the three dose groups is shown in Fig. 10. Table 5 summarizes the mean pharmacokinetic parameters for the treatment groups.

4. Discussion

4.1. Antiserum production

The failure of all animals to respond to the immunogenic challenge of zoledronic acid–protein conjugates 2, 3, 4, and 5 could be due to the low immunogenicity of these conjugates. The response of only a single animal to conjugate 1, which did not provide measurable antiserum titers until week 18 and high titer until week 36 following multiple immunizations, is unusual and attests to the low immunogenicity of the bisphosphonate structure, possibly related to an interaction with calcium, as discussed below.

4.2. Standard curves in human plasma and urine

The standard curve concentration range is lower in plasma/serum than in urine (0.04–40 ng/ml in plasma/serum, 1–1000 ng/ml in urine), in part due to the additional dilution required in urine samples. The goal of this dilution step, involving addition of a constant amount of blank urine to each sample, was to reduce the high variability observed in undiluted urine samples from different sources. Additionally, the conditions of the immunological reaction between the free zoledronic acid, the tracer and the antibody differ for plasma/serum and urine due to differences in protein concentration in the incubation mixture.

4.3. Validation of the RIA in human plasma/serum and urine

The high variability initially observed in the immunological reaction between the antibody, tracer and free zoledronic acid necessitated an analysis of the causes of variability and several steps for assay optimization.

The main cause of variability was identified as the interaction between calcium and bisphosphonate [11]. This interaction has two possible impacts:

Table 4
Zoledronic acid concentration measured in rat plasma and urine with total radioactivity and RIA

Rat no.	Plasma concentration (nmol/l)					
	5 min		15 min		30 min	
	Tot RA	R/A	Tot RA	R/A	Tot RA	R/A
7	4096	5452	ni	ni	1884	1597
8	3867	4493	ni	ni	1966	2300
9	4249	4917	ni	ni	1700	1645
10	4716	4879	3130	3772	2007	2141
11	4720	4997	3273	3052	2084	2100
12	5184	5228	3497	3883	1818	1603
Mean	4472	4994	3300	3569	1910	1898
CV%	11	6.5	5.6	13	7.3	17
Rat no.	Urine concentration (μg/ml)		% of the dose			
	Tot RA	R/A	Tot RA	R/A		
7	0.62	0.75	28.1	34.1		
8	0.44	0.47	21	22.2		
9	0.59	0.56	18.4	17.4		

A single intravenous dose of 0.16 mg/kg of [^{14}C]zoledronic acid was applied to male rats. The drug concentration in serum and urine was measured either by total radioactivity determination (Tot RA) or analyzed by the RIA method. The results show similar levels.
ni: not investigated

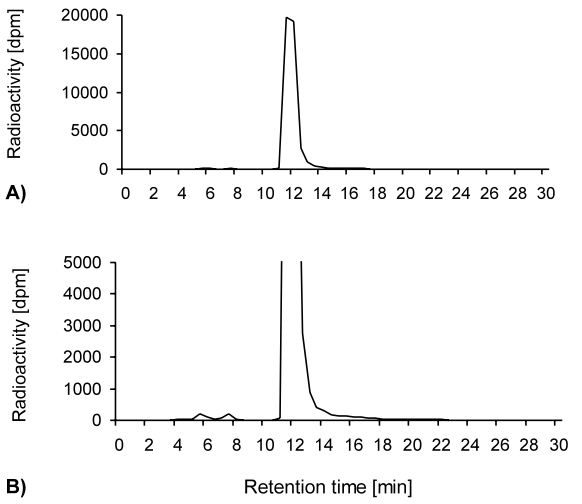

Fig. 6. Radio chromatogram of the zoledronic acid application solution. HPLC radio-chromatogram of the application solution containing [^{14}C]zoledronic acid used for iv administration of rats. The application solution was analyzed on a Phenomenex® Luna column by ion-pair chromatography with tetrahexyl ammonium hydrogene sulfate. The radioactivity in the fractions was determined by LSC analysis. The main peak corresponds to zoledronic acid. The lower panel (B), an enlargement of the upper panel (A), illustrates the presence of radioactive impurities derived from the radiosynthesis of [^{14}C]zoledronic acid.

The extent of the zoledronic acid interaction with calcium can modify the binding to plasma proteins [11]. The equilibrium free/bound could be affected by the calcium concentration, with direct consequences on the equilibrium of the immunological reaction. All variations of these reactions must have a direct impact on the assay variation.

The binding of calcium to zoledronic acid alters the 3-dimensional structure of the molecule. For instance, zoledronic acid in a solution free of calcium is not recognized by the antibody from conjugate 1. Possibly because the zoledronic acid moiety in conjugate 1 was complexed to calcium and the antibodies generated are specific to this complex and do not recognize zoledronic acid not complexed to calcium. Any variation in the formation of this complex has a direct impact on the assay's variability. One direct consequence is that plasma prepared with EDTA is not usable. EDTA would chelate calcium and could dissociate the zoledronic acid/calcium complex, with the resulting free zoledronic acid not being recognized by the antibody, as observed with all plasma samples prepared with EDTA.

The principal objective of assay optimization, to determine the conditions ensuring acceptable reproducibility, was achieved by addition of the same amount of blank matrix (25 μl of plasma, serum or urine used to prepare the calibrators) to all incubation mixtures.

The interaction of the zoledronic acid/calcium complex with anions may induce co-precipitation of the complex. Therefore, any variation of the assay conditions impacting the complex e.g. use of buffers containing phosphate or citrate could adversely affect assay variability. Therefore, only borate buffer was used for all dilution and incubation steps.

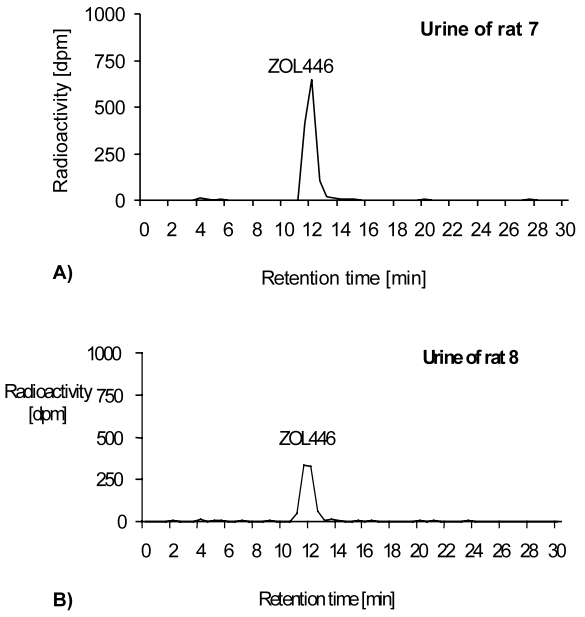

Fig. 7. Radio chromatogram of zoledronic acid in urine of two iv dosed rats. HPLC radio-chromatogram of 0–24 h urine of two rats dosed intravenously with 0.16 mg/kg [^{14}C]zoledronic acid. The urine samples were analyzed on a Phenomenex® Luna column by ion-pair chromatography with tetrahexyl ammonium hydrogene sulfate. The radioactivity in the fractions was determined by LSC analysis. The profiles of the chromatograms is identical to those obtained with the zoledronic acid solution.

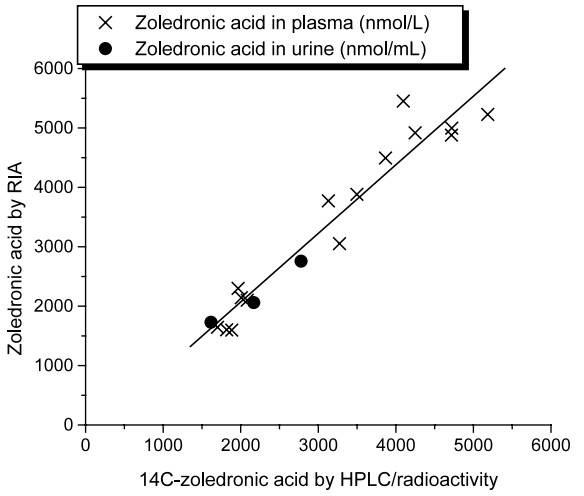

Fig. 8. Comparison RIA with total radioactivity. Zoledronic acid concentrations were measured by RIA and by total radioactivity in plasma and urine of rates after a single infusion of [^{14}C]zoledronic acid (RIA = 1.15 × ^{14}C −239; r^2 = 0.93, $P < 0.0001$).

The acceptability criteria were based on the recent publication of Findlay et al. [21], which provides updated criteria for immunoassay validation based on the original recommendations of Shah et al. [22]. The results of the zoledronic acid RIA validation showed acceptable reproducibility for QC samples prepared from various blank serum, plasma, and urine samples obtained from healthy volunteer subjects.

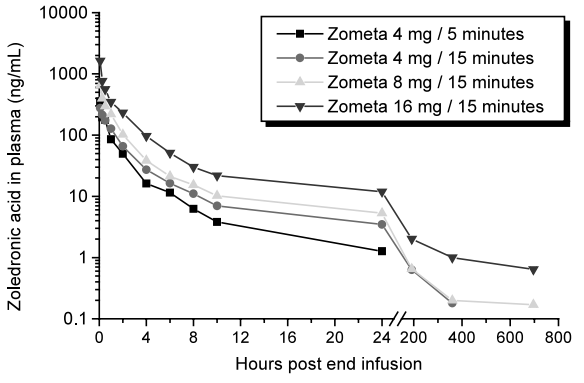

Fig. 9. Pharmacokinetics profiles of zoledronic acid in patients. Mean plasma concentrations of zoledronic acid after administration of a single infusion of ZOMETA® to cancer patients with bone metastases.

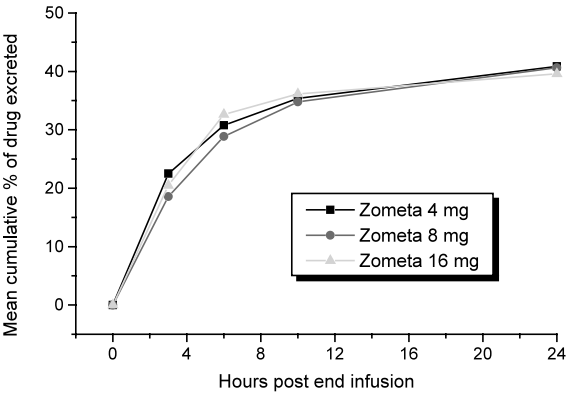

Fig. 10. Zoledronic acid concentration in patient urine. Mean cumulative urinary excretion of zoledronic acid after administration of a single infusion of ZOMETA® to cancer patients with bone metastases.

The high correlation obtained for the comparison of samples after different dilutions demonstrate the absence of matrix effect for the analysis of human plasma as well as human urine.

4.4. Zoledronic acid concentrations in rats

In all plasma and urine samples of rats obtained after an intravenous dose of 0.16 mg/kg [^{14}C]zoledronic acid, the concentrations of drug (measured by RIA) corresponded to the ^{14}C concentrations. The chromatographic analyses performed in this study showed no evidence of any metabolites in urine, attesting to the stability of zoledronic acid to biotransformation. This finding is expected for the class of bisphosphonate compounds as recently reviewed by Lin [15].

The observation that the ^{14}C concentrations in all urine samples decreased by 6–28% upon 2 months storage at −20 °C can be explained by a precipitation and/or adsorption of [^{14}C]zoledronic acid at the surface of the sampling tubes. Due to the physico-chemical properties of bisphosphonates (high binding of calcium and precipitation with calcium salts) [23], precipitation of [^{14}C]zoledronic acid in presence of cations e.g. Ca^{2+} or Fe^{3+} could occur. The observations that the radioactive precipitate could be dissolved with the divalent cation chelator EDTA, and HPLC identification of [^{14}C]zoledronic acid in the precip-

itate, suggest that the precipitate is a salt (presumably calcium and/or magnesium salt) of zoledronic acid. The possibility of precipitation of drug out of urine samples mandates strict storage and analysis criteria for pharmacokinetic studies of bisphosphonates in urine.

4.5. Zoledronic acid concentrations in patients

The pharmacokinetic data for zoledronic acid obtained with the newly developed radioimmunoassay demonstrate that following intravenous administration the drug is rapidly eliminated from plasma. The method provides sufficient sensitivity to measure to about 0.1% of peak concentration of the clinically relevant 8 mg dose. The pattern of an early rapid decline, followed by a period of sustained, low drug concentrations in plasma is typical of bisphosphonates [15]. It is thought to result from the parallel processes of clearance by kidney and uptake by bone, with drug being released from bone very slowly at a rate presumably reflecting the physiological bone resorption processes. The pattern of urinary excretion, with only 39–46% of administered drug released into urine at 24 h post dose, suggests that, as has been observed for other bisphosphonates, the majority of administered zoledronic acid is taken up by bone. It remains to be established if, and to what degree, the presence of bone metastases, the occurrence of hypercalcemia, hydration status, renal function, and other clinical variables may affect the uptake of drug by bone, its kinetics in plasma, and the rate and percentage of zoledronic acid excretion in urine.

5. Conclusions

The radioimmunoassay for zoledronic acid is a simple, fast, and reproducible method to analyze drug concentration in patients. This assay allows quantification of concentrations of zoledronic acid as low as 400 pg/ml in human plasma and serum, and as low as 5 ng/ml in human urine, using a small sample volume of 25 μl, providing adequate sensitivity to define the plasma concentration versus time curves and urinary excretion of the drug after clinical doses to 1% or less of peak concentration.

This new analytical tool will allow detailed pharmacokinetics investigations of the disposition of zoledronic acid in patients.

Table 5
Pharmacokinetic parameters (mean ± S.D.) of zoledronic acid in cancer patients with bone metastases

Dose/infusion time/no. patients in dose group	Plasma AUC_{0-24h} (ng/h/ml)	Plasma C_{max} (ng/ml)	Cumulative urinary excretion, Ae_{0-24h} (% of dose)	Renal clearance Ae_{0-24h}/AUC_{0-24h} (l/h)
4 mg/5 min/$n = 3$	411 ± 107	393 ± 100	39.5 ± 8.1	4.1 ± 1.8
4 mg/15 min/$n = 4$	496 ± 212	267 ± 41	39.4 ± 14.3	3.5 ± 2.0
4 mg pooled/$n = 7$	460 ± 168	321 ± 93	39.5 ± 10.4	3.8 ± 1.7
8 mg/15 min/$n = 8$	816 ± 297	528 ± 165	40.6 ± 17.1	4.7 ± 2.9
16 mg/15 min/$n = 8$	2000 ± 468	1717 ± 958	46.2 ± 18.7	4.1 ± 2.5

There was a statistically significantly positive dose response of the plasma pharmacokinetic parameters with dose; the relationship was not inconsistent with dose proportionality. There was no dose response for the renal clearance and the cumulative urinary excretion of drug. However, urinary excretion was incomplete, on average 39.4–46.2% of the administered dose at 24 h post dose, with only trace amounts of drug being excreted in the urine thereafter. Renal clearance was 90 ± 38% of the creatinine clearance (calculated from predose serum creatinine using Cockcroft–Gault equation. Accurate determination of the terminal elimination phase of drug was not possible given the observed very low but prolonged concentrations up to 28 days post dose, but was likely to be much greater than 150 h, with the early phases of rapid decline of plasma concentrations being associated with very short half-lives, of <2 h. Decreasing the infusion time from 5 to 15 min produced an expected, approximately 30% decline in C_{max}, but there was no statistically significant change in the AUC.

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