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Determination of zoledronic acid in human urine and blood plasma using liquid chromatography/electrospray mass spectrometry

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ABSTRACT

A new method for the analysis of 1-hydroxy-2-imidazol-1-yl-phosphonoethyl phosphoric acid (zoledronic acid) in urine and blood samples has been developed. It consists of a derivatisation of the bisphosphonate with trimethylsilyl diazomethane under multiple methylester formation. The formed derivative can, in contrast to the non-derivatised analyte, easily be separated by reversed phase liquid chromatography due to its reduced polarity. Detection is performed by electrospray tandem mass spectrometry. For calibration purposes, a deuterated internal standard has been synthesised in a three-step synthesis starting with d_4-imidazole. For human urine, the limit of detection (LOD) is 1.2×10^{-7} mol/L, limit of quantification (LOQ) is 3.75×10^{-7} mol/L in the MRM mode. For human blood plasma, a LOD of 1×10^{-7} mol/L and a LOQ of 2.5×10^{-7} mol/L were determined. The linear dynamic range comprised 3.5 decades starting at the limit of quantification. The method was successfully applied for the analysis of spiked urine and blood plasma samples as well as samples from two osteoporosis patients.

1. Introduction

Zoledronic acid, 1-hydroxy-2-imidazol-1-yl-phosphonoethyl phosphonic acid (Compound **1**, see Fig. 1), belongs to the group of geminal *N*-containing bisphosphonates [1,2]. It is a potent inhibitor of osteoclastic bone resorption and is clinically used for the treatment of malignant and benign bone diseases, e.g. osteoporosis, Paget's disease or metastases of solid tumours [3–5].

To quantify the amount of zoledronic acid in urine and blood plasma samples during or after therapy, an easy and reproducible sample preparation as well as a sensitive and reliable analytical method is required.

In the last decade, several sample purification and chromatographic methods for bisphosphonates have been published. For purification, co-precipitation with calcium phosphate under alkaline conditions [6–8] and subsequent removal of calcium ions using various solid-phase extraction columns [9,10] was the most frequently discussed approach. Unfortunately, due to the chemical nature of zoledronic acid, its chromatographic separation is challenging. Since bisphosphonates contain two phosphoric acid groups, they are ionic and highly polar. Thus, they show no retention on conventional reversed phase liquid chromatographic (RP-LC) stationary phases such as RP-8 or RP-18. Furthermore, the ability to complex ubiquitous metal ions such as Ca^{2+} and their tendency to form multiply charged species gives rise to poor peak shape, baseline disturbance and irreproducible chromatograms.

For these reasons, only a limited number of analytical approaches describing the chromatographic determination of bisphosphonates is available in literature. The respective methods are mainly based on ion exchange [11–13] and ion pair chromatography [6,14–16] coupled with UV and fluorescence detection. However, since most bisphosphonates do not contain strong chromophores, a derivatisation is necessary to be compatible with these detectors. For example, post-column derivatisation with copper(II) ions [13], an acidic iron(III) solution [15] as well as thorium(IV)-ethylenediaminetetraacetic acid-xylenol orange [17] are used to convert bisphosphonates into products, which are suitable for UV detection. For fluorescence detection, derivatisation techniques based on (9-fluorenylmethyl)chloroformate (FMOC) [9,16], fluorescamine [7] and 2,3-naphthalene dicarboxyaldehyde-N-acetyl-D-penicillamine [8] have been developed. Furthermore, gas chromatography/mass spectrometry (GC/MS) with pre-column derivatisation was reported: The bisphosphonates were converted into volatile methyl esters [18] or were trimethylsilylated and analysed with capillary GC/MS [19].

In order to develop a method based on liquid chromatography/tandem mass spectrometry (LC/MS/MS), a derivatisation of the zoledronic acid yielding a less polar product is necessary.

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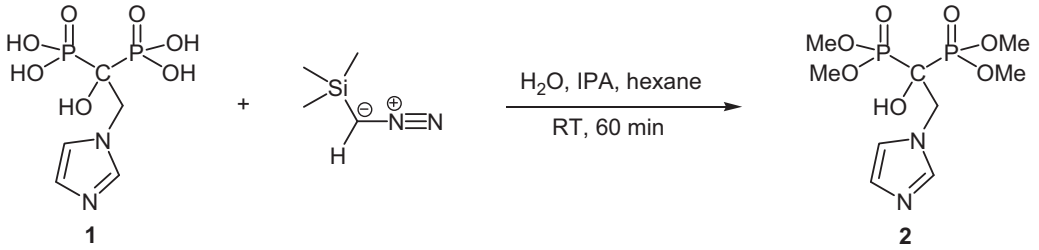


Fig. 1. Derivatisation of zoledronic acid with trimethylsilyl diazomethane.

Tarcomnicu et al. derivatised alendronate, a related geminal *N*-containing bisphosphonate, with the goal to obtain a less polar product [20]. First, the amino group was treated with isobutyl chloroformate, followed by a methylation of the hydroxyl groups as well as a methylation and cyclisation of two adjacent hydroxyl groups with trimethyl orthoacetate. Zhu et al. developed a derivatisation method for risedronic and alendronic acid with diazomethane (DAM) [21]. The hydroxyl groups were methylated to obtain a less polar product, which was then accessible for LC/MS/MS. Unfortunately, DAM is explosive (>90 °C), toxic, highly carcinogenic and irritant. Because of its instability, it has to be generated *in situ* prior to each use. Due to these disadvantages, a more stable, easier to handle alternative with similar properties is required. Ranz et al. found that trimethylsilyl diazomethane (TMS-DAM) is suitable for the derivatisation of different herbicides with carboxylic acid functionalities and that it is a less explosive replacement for DAM [22]. In their assay, they compared the reaction yields of both reagents with each other. While for TMS-DAM, yields of 90–100% were obtained, for DAM only 85–95% were determined. Therefore, TMS-DAM appears to be a promising alternative for the derivatisation of bisphosphonates as well.

For these reasons, we developed a method for the derivatisation of zoledronic acid with TMS-DAM for subsequent LC/MS detection and a calibration using a stable isotope-labelled internal standard. This is the first approach showing the quantitative analysis of zoledronic acid in urine and blood samples. The respective results are presented within this manuscript.

2. Experimental

2.1. Chemicals

All reagents and chemicals were obtained from Sigma Aldrich (Steinheim, Germany) or Fluka Chemie (Buchs, Switzerland) in the highest quality available and were used without further purification. Water was purified using a Milli-Q Gradient A 10 system and filtered through a 0.22 μM Millipak 40 filter unit (Millipore, Billerica, USA). Methanol (MeOH) for HPLC was obtained in gradient grade quality from Merck (Darmstadt, Germany). Zoledronic acid was provided by Novartis (Basel, Switzerland). Imidazole-d_4 (99.1% atom%D) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Solid phase extraction cartridges (SAX, 3 mL, 60 mg) were obtained from Agilent Technologies (Böblingen, Germany). Trimethylsilyl diazomethane (TMS-DAM, 2.0 mol/L solution in hexane) was purchased from Sigma Aldrich (Steinheim, Germany).

Human urine and blood was provided by healthy volunteers and was collected in house. After receiving informed consent, three urine and three blood samples were collected from two female volunteers. They were diagnosed with osteoporosis and took Zometa®, a zoledronic acid containing medicine, as a single dose of 4 mg.

2.2. Synthesis

Yields refer to chromatographically and spectroscopically pure compounds. All moisture-sensitive reactions were carried out under a dry argon atmosphere.

2.3. Instrumentation

^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded using an AC 200-spectrometer (Bruker, Karlsruhe, Germany) at 200.1 MHz, 50.3 MHz and 81.0 MHz, respectively. Chemical shifts (δ) of the signals are quoted in ppm, using residual solvent peaks or phosphoric acid as external standard. The multiplicity of the signals is abbreviated as s = singlet, t = triplet, m = multiplet. All coupling constants *J* are listed with the numbers of involved bonds in Hertz (Hz). Accurate mass electrospray mass spectra were recorded on a micrOTOF spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionisation source. For preparation of blood samples, a Hettich Universal centrifuge (Hettich, Tuttlingen, Germany) was used.

The LC/MS setup comprised a Shimadzu (Duisburg, Germany) LC system and either an API 2000 or a Q-TRAP 2000 mass spectrometer (Applied Biosystems, Darmstadt, Germany), equipped with a turbo ionspray (pneumatically assisted ESI) source. The LC system consisted of two LC-10ADVP pumps, a DGC-14A degasser, a SIL-HTVP autosampler, a CTO-10AVP column oven, and a SPD-10AVVP UV detector. The software used for controlling LC and MS was Analyst 1.4.1 (Applied Biosystems, Darmstadt, Germany). The analytes were ionised in the ESI interface with an ion spray voltage of 4500 V, using 90 psi nebuliser gas and 90 psi heater gas with a temperature of 450 °C. Fragmentation experiments were performed on an maXis quadrupole-time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an electrospray ionisation source.

2.4. Synthesis of deuterated zoledronic acid (internal standard)

2.4.1. Synthesis of imidazol-1-yl-acetic acid tert-butyl ester-d_4 (**4**)

To a stirred solution of imidazole-d_4 (1.0 g, 15 mmol) in ethyl acetate (16 mL), powdered potassium carbonate (2.9 g, 21 mmol) was added, followed by *tert*-butyl chloroacetate (2.6 mL, 18 mmol) at room temperature. The reaction mixture was stirred under reflux for 16 h and then allowed to cool down to room temperature. After completion of the reaction as indicated by thin layer chromatography (methanol:chloroform = 1:9, I_2 active), the reaction was quenched with water (8 mL, 0 °C) and the ethyl acetate layer was separated. The aqueous layer was extracted with ethyl acetate (2 × 8 mL). The organic phases were combined, washed with brine (5 mL), dried over sodium sulphate, filtered and concentrated under reduced pressure. The resulting solid was purified by column chromatography on silica gel using dichloromethane:methanol (95:5).

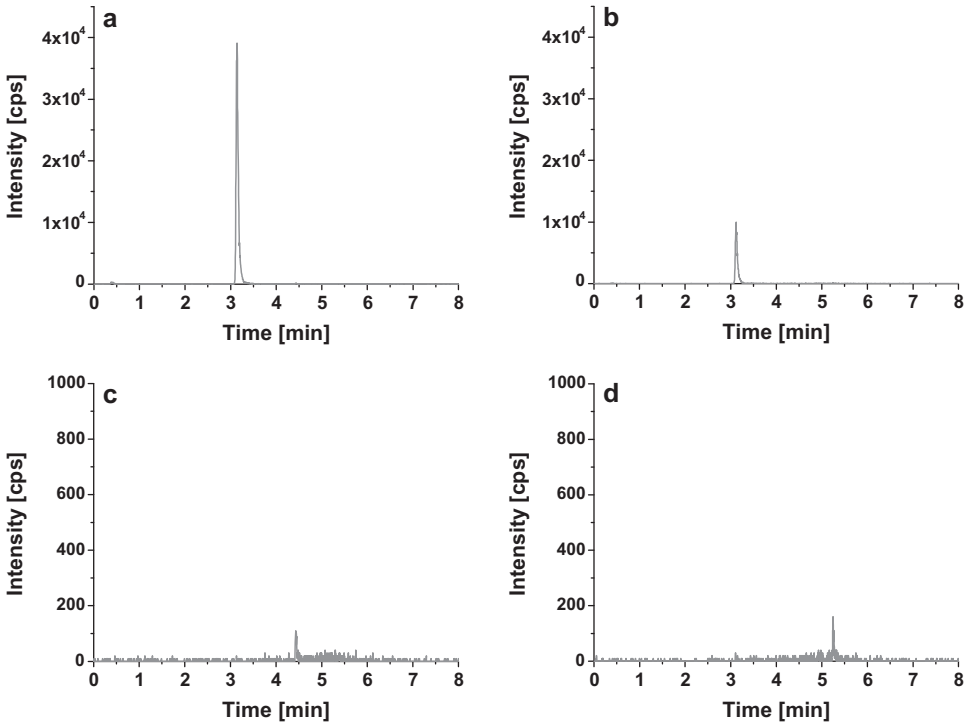


Fig. 2. LC/MS/MS chromatograms of derivatised blood plasma samples: (a) spiked with zoledronic acid *m/z* 329 → 203, (b) spiked with internal standard *m/z* 333 → 206, (c) blank *m/z* 329 → 203 and (d) blank *m/z* 333 → 206.

The solution was concentrated under reduced pressure to obtain **4** as a white solid (0.90 g, 36%) [23].

^{1}H NMR (200.1 MHz, $CDCl_3$): δ [ppm] = 1.46 (s, 9H, O(CH_3)$_3$), 4.62 (s, 1H, NC*H*D), 7.66* (s, *arom H*); ^{13}C{^{1}H} NMR (50.3 MHz, $CDCl_3$): δ [ppm] = 28.1 (s, O(CH_3)$_3$), 83.5 (s, NCDH), 119.8 (t, $^1J_{CD}$ = 28.1 Hz, arom *C*D), 128.5 (t, $^1J_{CD}$ = 28.0 Hz, arom *C*D), 137.6 (t, $^1J_{CD}$ = 26.5 Hz, arom *C*D), 166.4 (s, *C*OO(CH$_3$)$_3$). MS (ESI-EM): *m/z* = 187.1364 [M+H]$^+$ calc. for [$C_9H_{11}D_4N_2O_2$]$^+$ 187.1379.

Signals labelled with an asterisk (*) are caused by aromatic protons. They result from non-quantitative deuteration during the synthesis.

2.4.2. Synthesis of deuterated imidazol-1-yl-acetic acid-d_4 (**5**)

A solution of **4** (0.85 g, 4.6 mmol) in deuterium oxide (20 mL) was stirred under reflux for 22 h. Afterwards, the solvent was evaporated under reduced pressure to give **5** as a white solid (0.56 g, 95%) [24,25].

^{1}H NMR (200.1 MHz, D_2O): δ [ppm] = 4.70 (s, 1H, NC*H*D), 7.39* (s, *arom H*); ^{13}C{^{1}H} NMR (50.3 MHz, $CDCl_3$): δ [ppm] = 51.4 (m, NCDH), 118.9 (t, $^1J_{CD}$ = 30.8 Hz, arom *C*D), 122.5 (t, $^1J_{CD}$ = 31.0 Hz, arom *C*D), 135.0 (t, $^1J_{CD}$ = 33.5 Hz, arom *C*D), 172.4 (s, *C*OO(CH$_3$)$_3$). MS (ESI-EM): *m/z* = 131.0756 [M+H]$^+$ calc. for [$C_5H_3D_4N_2O_2$]$^+$ 131.0753.

2.4.3. Synthesis of zoledronic acid-d_4 (**3**)

A mixture of **5** (0.45 g, 1.6 mmol), phosphorous acid (1.6 g, 20 mmol), and toluene (16 mL) was heated to 80 °C under stirring. After melting of all solids, phosphorus oxychloride (1.8 mL, 19 mmol) was slowly added and the mixture was vigorously stirred at 80 °C for 5 h. Upon cooling of the mixture down to room temperature, the toluene was decanted and deuterated hydrochloric acid (20 wt.% solution in D_2O, 12 mL) was added to the residue. The obtained solution was stirred under reflux for 1 h, filtered and then most of the solvent was removed under reduced pressure. After the addition of isopropanol (100 mL), a white precipitate was formed, which was filtered off and washed with ethanol (5 × 20 mL). After evaporating the solvent under reduced pressure, **3** was obtained as a white solid (0.62 g, 66%). A sample was taken and further purified by recrystallisation from water prior to analysis [26].

^{1}H NMR (200.1 MHz, D_2O): δ [ppm] = 4.73 (m, 1H, NC*H*D), 7.33* (s, *arom H*), 7.48* (s, *arom H*), 8.67* (s, *arom H*); ^{31}P{^{1}H} NMR (81.0 MHz, $CDCl_3$): δ [ppm] = 14.1 (s, *PCP*). MS (ESI-EM): *m/z* = 320.9914 [M−H+2Na]$^+$ calc. for [$C_5H_5D_4N_2O_7P_2Na_2$]$^+$ 320.9926.

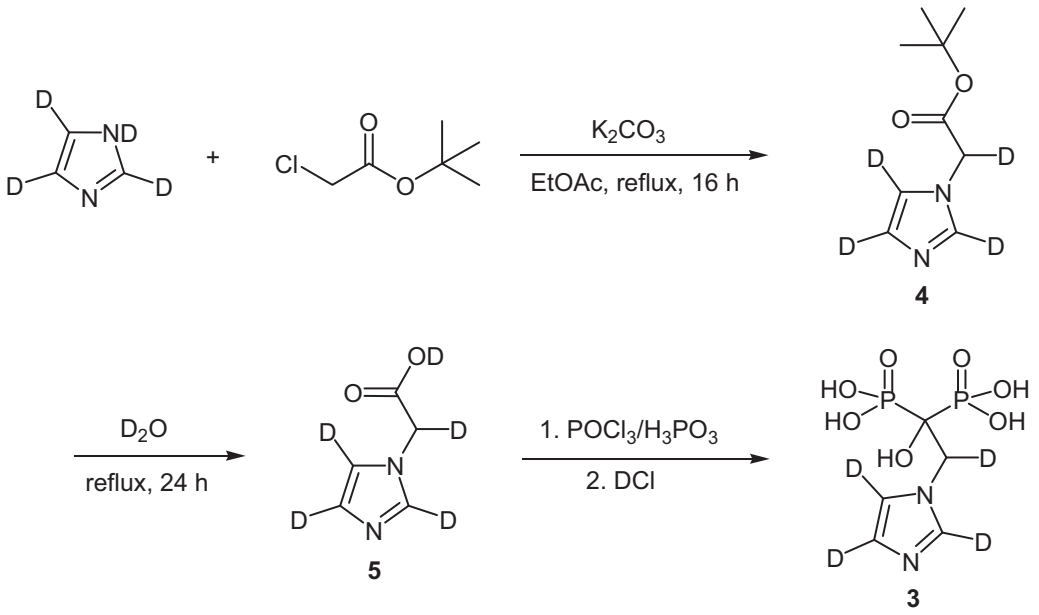
2.5. Analytical procedures

2.5.1. Preparation of zoledronic acid and internal standard stock solutions, calibration and quality control standards

2.5.1.1. Urine samples. Stock solutions of zoledronic acid and deuterated zoledronic acid in water (1×10^{-2} mol/L, each) were prepared. Twelve calibration standards of 7.5×10^{-4}, 3.75×10^{-4}, 7.5×10^{-5}, …, 3.75×10^{-9} mol/L were formed by serial dilution in blank human urine. The internal standard has been diluted to a concentration of 3×10^{-4} mol/L in human urine. Quality control samples were prepared by spiking blank human urine samples with zoledronic acid to give concentrations of 7.5×10^{-4}, 3.75×10^{-5} and 7.5×10^{-7} mol/L. All samples were stored at 4 °C.

2.5.1.2. Blood plasma samples. Stock solutions of zoledronic acid and deuterated zoledronic acid in water (1×10^{-2} mol/L, each) were prepared. Ten zoledronic acid standard solutions of 5×10^{-3}, 1×10^{-3}, 5×10^{-4}, …, 1×10^{-7} mol/L were made by appropriate dilution of the stock solution in water. The internal standard was diluted to a concentration of 1×10^{-3} mol/L of in water.

Eleven calibration standards of 5×10^{-4}, 2.5×10^{-4}, 5×10^{-5}, …, 5×10^{-9} mol/L were prepared by dilution of zoledronic acid and internal standard solutions (50 μL, each) in drug-free human blood (900 μL). Quality control standards of 2.5×10^{-4}, 5×10^{-6} and 2.5×10^{-7} mol/L were made similar to calibration standards. All samples were centrifuged at 4400 U/min for 15 min to separate



Scheme 1. Synthesis of deuterated zoledronic acid in a three-step approach.

plasma from red blood cells. Before further treatment, the plasma samples were stored at 4 °C.

2.5.2. Mass spectrometric optimisation on the basis of derivatised analytes

Derivatised zoledronic acid and deuterated internal standard solutions (10^{-4} mol/L in water) were prepared for the optimisation of the mass spectrometer settings as follows: First, the analyte or the internal standard solution (100 μL) was diluted in isopropanol (400 μL). Then, trimethylsilyl diazomethane (TMS-DAM, 2.0 mol/L solution in hexane, 50 μL) was added. After 1 h, the samples were evaporated to dryness under nitrogen at 40 °C and were subsequently redissolved in methanol:water (1:9, 500 μL) prior to injection (see Fig. 1).

2.5.3. Preparation of urine and blood plasma samples

The sample preparation was optimised based on the method published by Zhu et al. [21]. Blank urine or analytical samples (200 μL) were diluted in ammonium bicarbonate solution (400 μL, 10 mmol/L). Internal standard (50 μL, 3×10^{-4} mol/L in urine) was added, followed again by the addition of ammonium bicarbonate solution (400 μL, 10 mmol/L). Blank blood plasma or analytical samples – already containing internal standard, see Section 2.5.2- (250 μL) were diluted in 800 μL ammonium bicarbonate solution (10 mmol/L). An aliquot of the obtained solutions (700 μL) was transferred onto a SPE cartridge, which was previously conditioned with methanol (3 mL) and water (3 mL). After the addition of the sample, the cartridge was washed with water (2 mL) and with methanol (1.5 mL). Directly after the addition of TMS-DAM (100 μL), the flow was stopped by closing the cartridge and methanol (750 μL) was added. After 1 h, the tube was opened and the solution was collected. The cartridge was subsequently rinsed with methanol (500 μL) and the obtained solution was evaporated under nitrogen at 40 °C. The dried samples were reconstituted in a methanol:water mixture (1:9, 500 μL).

2.5.4. HPLC/MS/MS

A binary gradient consisting of mobile phases A and B (A: 95% ammonium acetate and 5% MeOH (10 mM, pH 7), B: 95% MeOH and 5% ammonium acetate (10 mM, pH 7)) was applied for the chromatographic separation. The stationary phase employed for the HPLC separation was a ProntoSIL C18 UHC 330 column (Bischoff, Leonberg, Germany) with a particle size of 3 μm, a pore diameter of 120 Å, a length of 30 mm, and an inner diameter of 3.0 mm. The injection volume was 10 μL. As flow rate, 0.6 mL/min was selected. The derivatives were eluted with the following gradient profile: 0% B for 1 min, followed by a 3 min linear gradient to 90% B and finally a 1 min period at 90% B. The column was reequilibrated to the initial conditions with a 1 min linear gradient to 0% B and an isocratic period of 2 min.

The analytes were detected in the positive ion mode using tandem mass spectrometry in the multiple reaction monitoring (MRM) mode with the collision activated dissociation gas set to high. Declustering potential (DP), entrance potential (EP), and cell exit potential (CXP) as well as collision energy (CE) were optimised under HPLC conditions and were used for all LC/MS measurements. MRM was used to monitor transitions at *m/z* 329 → 203 (*m/z* 333 → 206 for the internal standard).

2.5.5. Determination of creatinine

Creatinine was determined by use of an HPLC–UV method reported elsewhere with some modifications [27,28]. A volume of 10 μL of urine was diluted with 990 μL of the mobile phase and of this solution, an aliquot of 10 μL was injected on the HPLC column (Nucleodur RP, Macherey Nagel, Düren, Germany; 4.6 mm × 150 mm, 5 μm particle size). The mobile phase consisted of water and ACN (95/5 v/v) containing 10 mM sodium 1-octanesulphonate with the pH adjusted to 3.2 with orthophosphoric acid. The flow rate was 1.0 mL/min, and creatinine was detected by UV absorbance at 236 nm.

2.5.6. Method validation

The range of quantification was defined as the range between the lowest and the highest concentrations on the calibration function. All data outside this range were considered as invalid. The method selectivity was demonstrated for blank urine and blood samples obtained from healthy volunteers: no interfering peaks appeared in the chromatograms. Specificity was determined by the absence of significant peaks in the chromatograms at the retention time of methylated zoledronic acid and methylated internal standard.

Intra-batch and inter-batch mean values, coefficient of variation (CV) and bias were determined 5-fold at three concentration levels. To determine the long-term stability, blood plasma and urine samples were spiked to relevant concentrations, deep frozen at −25 °C and analysed by LC–MS/MS one month later. Freeze–thaw stability was determined in blood plasma and urine samples immediately after spiking and after one, two and three freeze–thaw cycles. Short-term temperature stability was tested at room temperature for at least 24 h.

3. Results and discussion

3.1. Deuterated internal standard

Deuterated zoledronic acid **3** was prepared starting from imidazole-d_4. The synthesis involved three steps: First, imidazole-d_4 was reacted with *tert*-butyl chloroacetate in a condensation reaction under the removal of hydrochloric acid. Subsequently, the *tert*-butyl protective group was cleaved to obtain the free acid **5**. In the last step, the intermediate **5** was mixed with phosphorus oxychloride and phosphorous acid to obtain the above mentioned product **3** (see Scheme 1).

During the synthesis, two intermediates (**4**, **5**) containing a keto group were formed. Under acidic conditions, a keto-enol-tautomerism may occur, which may lead to the exchange of hydrogen by deuterium at the methylene group. Thus, a mixture of intermediates being three-, four- or fivefold deuterated was obtained. This variation of isotope ratios was also found for zoledronic acid. After integration of the peak areas determined by accurate mass analysis, it was found, that 21.5% of the threefold, 48.3% of the fourfold and 30.2% of the fivefold deuterated isomer was formed. Fortunately, zoledronic acid shows no keto-enol-tautomerism and the deuterium ratio remained unchanged after storage in water. Thus, it can perfectly be used as internal standard. In addition, long-term stability was proven by repeated measurements over several months. Structural characterisation was performed by means of ^{1}H, ^{13}C and ^{31}P NMR and accurate mass spectrometric measurements.

3.2. HPLC/MS/MS of zoledronic acid derivatised with TMS-DAM

To allow the HPLC/MS/MS analysis of zoledronic acid, a derivatisation with TMS-DAM was carried out. This reaction leads to a methylation of hydroxyl groups in acidic functionalities, while alcoholic hydroxyl groups remain non-modified [22]. Therefore, the derivatisation can be performed in presence of methanol or isopropanol, as described in Sections 2.5.2 and 2.5.3. Finally, a less polar product was obtained (Compound 2, see Fig. 1) that was retained on a RP column.

For quantification, the synthesised deuterated zoledronic acid was added as internal standard to the sample solution. The fourfold derivatised products were retained sufficiently with reversed phase liquid chromatography. Fig. 2 shows typical chromatograms of the derivatised products in blood plasma samples. Additionally, in Fig. 3 LC/MS/MS chromatgrams of a sample at the LOD and a patient blood sample were given. The selectivity of the method was confirmed by the absence of significant peaks at the characteristic retention time of zoledronic acid and internal standard, which were detected in the MRM mode based on the transitions m/z 329 → 203 and m/z 333 → 206 for analyte and internal standard, respectively. Regarding the internal standard, it should be taken into account that while three reaction products were formed, only the MRM transition of the most abundant fourfold deuterated derivative was analysed. However, this led to a lower signal intensity compared to zoledronic acid. The relative response factors between both substances were determined over the complete linear dynamic range (n = 8), and it was shown that they were constant (0.2239 ± 0.0013 for urine samples). Furthermore, the value showed no significant change after storage of the internal standard in methanol:water (1:9) at room temperature for one month (0.2274 ± 0.0024) as well as for two months (0.2245 ± 0.0029). For blood plasma samples, similar results have been found.

Table 1
Intra-batch and inter-batch precision and accuracy of zoledronic acid quality control samples in human urine.

Concentration (mol/L)	7.5×10^{-4}	3.75×10^{-5}	7.5×10^{-7}
Intra-batch mean (mol/L)	8.17×10^{-4}	3.79×10^{-5}	7.22×10^{-7}
Intra-batch CV (%)	1.39	1.34	19.8
Intra-batch bias (%)	8.90	1.01	−1.06
Inter-batch mean (mol/L)	8.13×10^{-4}	3.76×10^{-5}	6.59×10^{-7}
Inter-batch CV (%)	1.98	3.05	20.1
Inter-batch bias (%)	8.45	0.16	−13.1

Furthermore, the product ion spectra of zoledronic acid and internal standard at different collision energies were recorded (Fig. 4). The chemical structures of all observed fragments are presented in Fig. 5. The main fragment (m/z 203.0583 for the analyte and 206.0768 for the internal standard) was obtained under the loss of doubly methylated phosphoric acid. Fragments with the mass-to-charge ratio of 135.0207 (136.0266 IS) and 171.0316 (174.0501 IS) were formed starting from the main fragmentation product via the loss of imidazole and methanol.

3.3. Urine and blood plasma sample preparation

Samples containing urine or blood plasma, zoledronic acid as well as internal standard were transferred onto an anion exchange SPE column. This strategy was followed to remove matrix salts or proteins from blood plasma samples and to concentrate the zoledronic acid. Then, the derivatisation was carried on column and the analytes were subsequently removed easily by addition of methanol. This combination of derivatisation and extraction on the SPE cartridge provides the advantage of purification and derivatisation in one step.

3.4. Determination of analytical figures of merit

The linear dynamic range of zoledronic acid in human urine was determined to extend from 3.75×10^{-7} to 7.5×10^{-4} mol/L. For this calibration, regression coefficients between 0.9987 and 0.9999 were observed. The LOD for zoledronic acid was 1.2×10^{-7} mol/L with a signal-to-noise ratio of 3:1 and the LOQ was 3.75×10^{-7} mol/L with a signal-to-noise ratio of 10:1. The linear calibration range for human blood plasma ranged from 2.5×10^{-7} to 5×10^{-4} mol/L. Within this range, regression coefficients of 0.9998 to 0.9999 for zoledronic acid were observed. The LOD was 1×10^{-7} mol/L and the LOQ was 2.5×10^{-7} mol/L. Intra-batch and inter-batch mean values, coefficients of variation (CVs) and bias of the quality control samples in urine with concentra-

Table 2
Intra-batch and inter-batch precision and accuracy of zoledronic acid quality control samples in human blood plasma.

Concentration (mol/L)	2.5×10^{-4}	5×10^{-6}	2.5×10^{-7}
Intra-batch mean (mol/L)	2.47×10^{-4}	5.22×10^{-6}	2.47×10^{-7}
Intra-batch CV (%)	1.82	2.44	4.47
Intra-batch bias (%)	−1.10	4.38	−1.37
Inter-batch mean (mol/L)	2.61×10^{-4}	5.22×10^{-6}	2.33×10^{-7}
Inter-batch CV (%)	5.81	1.99	9.51
Inter-batch bias (%)	4.51	4.40	−6.63

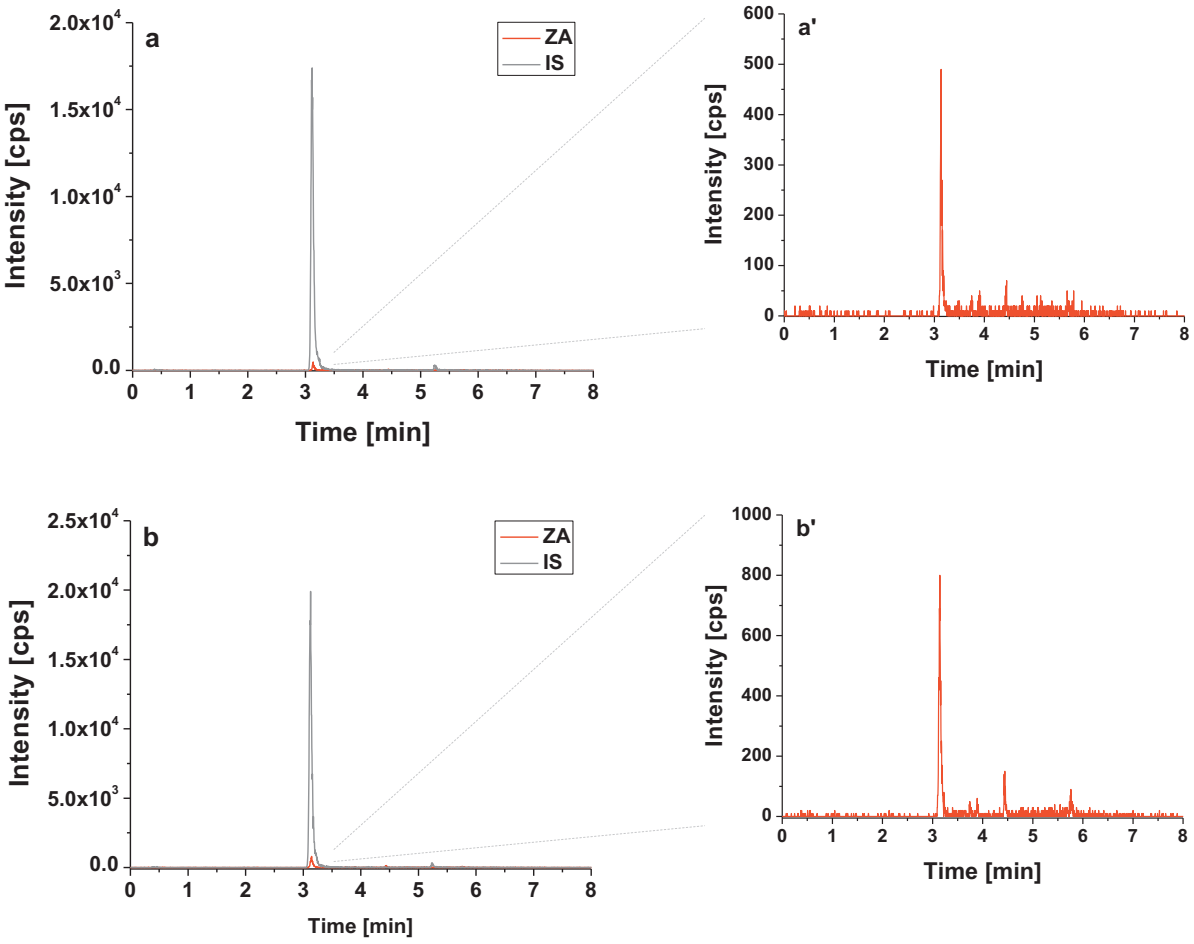


Fig. 3. LC/MS/MS chromatograms of derivatised blood plasma samples: (a) spiked with zoledronic acid (ZA) at LOD with *m/z* 329 → 203 and *m/z* 333 → 206 for the internal standard (IS), (b) patient sample with *m/z* 329 → 203 and *m/z* 333 → 206, (a′) and (b′) show only the transition of *m/z* 329 → 203 for zoledronic acid.

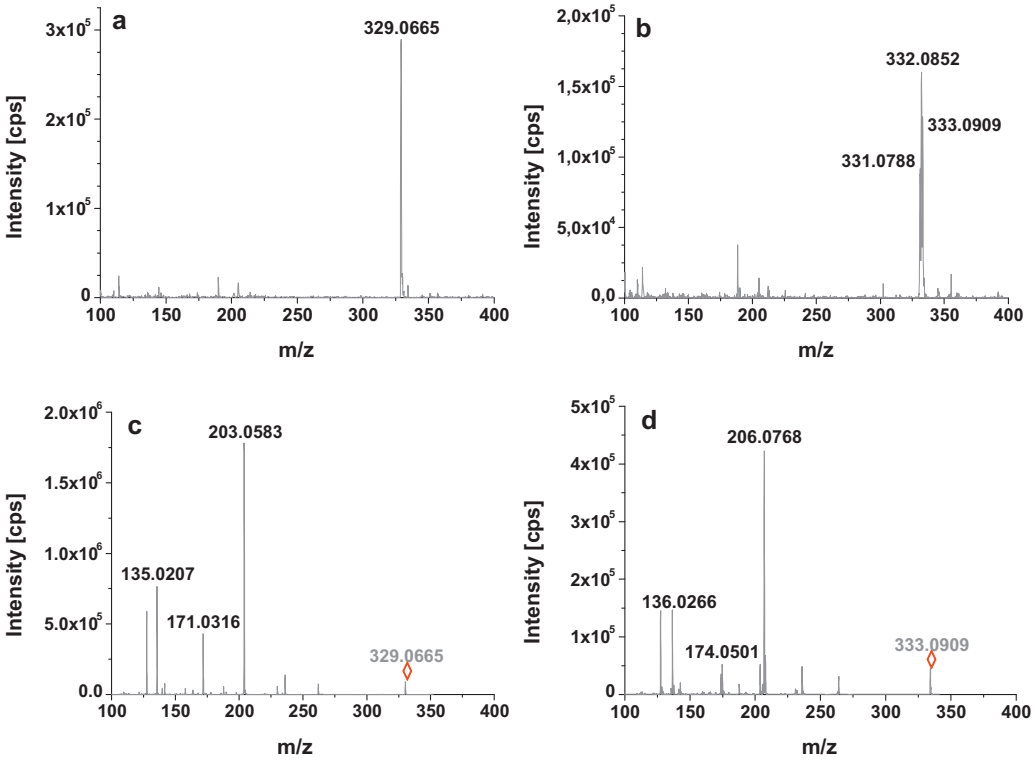
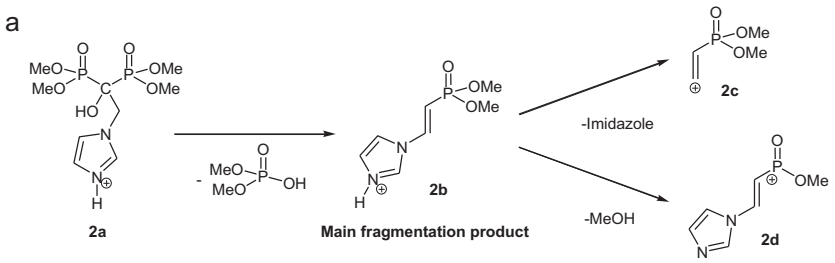


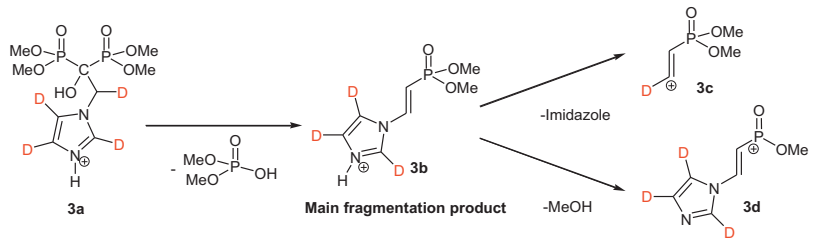
Fig. 4. Positive ion electrospray MS spectra for zoledronic acid and for deuterated zoledronic acid (a and b). MS/MS product ion spectra (enhanced product ion mode) of *m/z* = 329.1 for zoledronic acid (c) and of *m/z* = 333.1 for deuterated zoledronic acid (d).

a



	Molecular Formula	m/z calculated $[M+H]^+$	m/z determined	Deviation [ppm]
2a	$C_9H_{19}N_2O_7P_2$	329.0662	329.0665	−0.9
2b	$C_7H_{12}N_2O_3P$	203.0580	203.0583	−1.4
2c	$C_4H_8O_3P$	135.0206	135.0207	−0.8
2d	$C_6H_8N_2O_2P$	171.0318	171.0316	1.3

b



	Molecular Formula	m/z calculated $[M+H]^+$	m/z determined	Deviation [ppm]
3a	$C_9H_{15}D_4N_2O_7P_2$	333.0913	333.0909	1.1
3b	$C_7H_9D_3N_2O_3P$	206.0768	206.0768	0.0
3c	$C_4H_7DO_3P$	136.0268	136.0266	2.0
3d	$C_6H_5D_3N_2O_2P$	174.0506	174.0501	3.2

Fig. 5. (a) Chemical structures of fragments of zoledronic acid. Additionally, molecular formulae of all fragments derived from accurate mass measurements are provided. (b) Chemical structures of fragments of deuterated zoledronic acid. Additionally, molecular formulae of all fragments derived from accurate mass measurements are provided.

Table 3
Concentration of zoledronic acid in blood and urine samples from two female volunteers, determined at several times after a single i. v. dose of 4 mg Zometa®.

Blood				
Time after administration (h)	Patient A		Patient B	
	Concentration of zoledronic acid (mol/L)	CV (%)	Concentration of zoledronic acid (mol/L)	CV (%)
1	1.49×10^{-6}	4.70	1.50×10^{-6}	5.21
4	3.28×10^{-7}	8.95	3.92×10^{-7}	7.33
24	2.28×10^{-8}	13.2	2.64×10^{-8}	12.9

Urine				
Patient A Time after administration (h)	Concentration of zoledronic acid (mol/L)	CV (%)	Concentration of creatinine (mmol/L)	[ZA]/[creatinine] × 1000
5	7.74×10^{-5}	4.77	8.04	9.63
11	1.17×10^{-5}	3.97	8.31	1.40
30	3.52×10^{-6}	8.35	13.9	0.68

tions of 7.5×10^{-4}, 3.75×10^{-5} and 7.5×10^{-7} mol/L are presented in Table 1. In Table 2 the validation parameters for the QC samples in blood plasma at 2.5×10^{-4}, 5×10^{-6} and 2.5×10^{-7} mol/L are reported.

CVs from 1.34% to 20.1% for urine and 1.82% to 9.51% for blood plasma samples were observed. A bias from −13.1% up to a maximum of 8.90% for urine and from −6.63% to 4.51% for blood plasma was determined. To sum up, the CVs and the bias were acceptable for each tested concentration level.

Furthermore, no decrease of zoledronic acid concentrations in urine and blood plasma was observed after storage at −25 °C for one month, after storage at room temperature for 24 h, as well as after one, two and three freeze–thaw cycles. These data clearly demonstrate the applicability of the developed method for the determination of zoledronic acid in urine and blood plasma samples.

3.5. Analysis of patient urine and blood plasma samples

The determined zoledronic acid concentrations in urine were normalised to creatinine. Creatinine is an endogenous metabolite and is excreted by the kidneys, predominantly by glomerular filtration. As the renal excretion of creatinine is almost constant, its urinary concentration reflects the dietary dilution of the urine sample and is thus frequently used in clinical chemistry as pseudo-internal standard. Normalising the concentration of a particular analyte to the creatinine concentration compensates for the dilution of the urine samples.

For blood plasma samples, a zoledronic acid concentration of 1.5×10^{-6} mol/L has been determined 1 h after administration. Only 24% of the analyte were determined after 4 h compared to the measurements after 1 h and after 24 h, the concentration was only 5% of the initial value (compare Table 3). For urine samples, a similar decrease was determined. After 5 h, a concentration ratio of zoledronic acid to creatinine of 9.6×10^{-3} was found, after 11 h only 15% and after 30 h only 7% of the 5-h-value were found (compare Table 3).

4. Conclusion

The determination of zoledronic acid with LC/MS/MS after derivatisation with TMS-DAM proved to be a simple and reproducible analytical method. Furthermore, the synthesis of deuterated zoledronic acid as internal standard was carried out and the product was successfully used for the quantification of zoledronic acid in human urine and blood plasma samples in the concentration range between 3.75×10^{-7} to 7.5×10^{-4} mol/L and 2.5×10^{-7} to 5×10^{-4} mol/L, respectively. To investigate the decay of zoledronic acid and to determine concentrations in blood plasma and urine samples after single dose administration, three urine and three blood plasma samples of two volunteers were analysed successfully.

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