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High-throughput HPLC-MS/MS method to determine ibandronate in human plasma for pharmacokinetic applications

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ABSTRACT

A sensitive high-throughput liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of ibandronate in human plasma. In a previous study, we have analyzed alendronate in urine samples of subjects treated at therapeutic dosages, using a derivatization approach; a similar derivatization was adapted and improved to determine ibandronate in plasma. The bisphosphonate was isolated from the biological matrix by liquid–liquid extraction, and derivatized with trimethylsilyldiazomethane prior to separation on a reversed-phase column (Supelco Discovery HSC18) and detection on a quadrupole-linear ion trap mass spectrometer (API 4000 QTrap). Various parameters of extraction and derivatization were optimized in order to get adequate recovery, high derivatization yield and minimal ion suppression; a deuterated analogue, d3-ibandronate, was used as internal standard. The transitions 376.1 → 114.2 and 379.1 → 61.0 were acquired to monitor ibandronate and d3-ibandronate derivatives, respectively. A multiplexing LC system made possible the overlapping of two chromatographic runs, thus the interval between injections being reduced to only 2 min, a very short analysis time for compounds of this class. The method was fully validated over the quantification range 0.2–175.0 ng/ml, allowing an appropriate evaluation of the plasma concentrations of ibandronate, expected at therapeutic dosage, as proved by application to a pharmacokinetic study. A good linearity over the selected range ($r > 0.99$), accuracy and precision within ±15% of the target values and a recovery over 50% were obtained.

1. Introduction

Ibandronate (see chemical formula in Fig. 1) is an aminobisphosphonate drug used in the treatment of osteoporosis [1,2], metastatic bone disease [3–5] and other bone resorption disorders. As a tablet, it is administered once per month, being advantageous to the patient, but therefore leading to very low plasmatic concentrations. This aspect, together with its main chemical features common to other bisphosphonates (high polarity, high water-solubility, no significant chromophore or fluorescent groups in the structure), makes the isolation and quantification of ibandronate from biological samples a difficult and interesting issue.

To enhance both the chromatographic separation and the detection limits, HPLC methods with derivatization, were mainly used to determine aminobisphosphonates in pharmaceutical and biological samples; fluorescence detection was generally preferred as described in literature [6,7]. More sensitive and selective GC-MS methods were also developed to analyze bisphosphonates [8] after the derivatization of the hydroxyl groups, while a HPLC-MS method was only recently described [9]; ibandronate concentrations in biological samples were measured by ELISA or GC-MS [10,11]. Capillary electrophoresis was also explored for the detection and quantification of these drugs or impurities from technical products [12]. A comprehensive review of analytical methods was recently published [13]. With respect to the biological sample pretreatment, the most used techniques are solid-phase extraction on ion-exchange resins and precipitation as calcium salts. Both of them present some drawbacks, such as elevated costs of the solid-phase extraction material, laborious procedures and a high-salt content in the samples purified by calcium precipitation and finally inadequate clean-up for further derivatization reactions.

In a recent paper, our group presented a HPLC-MS/MS method for the quantification of alendronate in human urine, optimizing the derivatization procedure introduced for GC-MS, in order to obtain stable derivatives adequate for reversed-phase HPLC sep-

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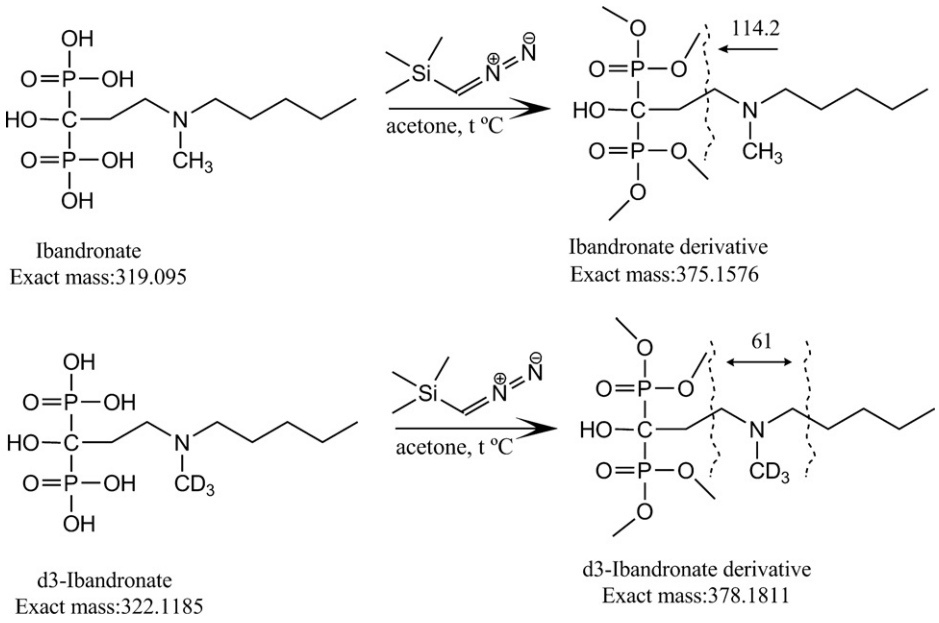

Fig. 1. Chemical structures of ibandronate, d3-ibandronate (left formulas) and their derivatization products obtained with TMSD (right formulas).

aration with MS/MS detection [14]. Stable and safe derivatization reagents were proposed and liquid–liquid extraction was preferred as isolation technique. In that method two derivatization steps were carried out: the first-one was performed with isobutylchloroformate (IBCF) reacting with the amino group of alendronate [14], while for the second-one, the methylation of the phosphonic hydroxyls, trimethyl orthoacetate (TMOA) [14] was selected.

Based on this approach, a new HPLC-MSMS method was developed and fully validated for the quantification of ibandronate in human plasma. Compared to alendronate, ibandronate has already a tertiary amine substituted by methyl and pentyl residues therefore only one derivatization step, the methylation of the phosphonic hydroxyls, was necessary. The bisphosphonate was isolated from the biological matrix by liquid–liquid extraction and, after a series of trials, trimethylsilyldiazomethane (TMSD) was selected as derivatizing agent prior to separation on a reversed-phase column. MS/MS detection in multiple reaction monitoring (MRM) mode, following characteristic transitions for tetramethylated ibandronate and the internal standard trideuteromethyl-ibandronate (d3-ibandronate), was used. Separations were carried out in gradient conditions using an Aria LX-2 multiplexing LC system, overlapping two analytical runs and permitting the injection of one sample at every 2 min, a very short time compared to the classical HPLC methods with fluorescence detection with runs generally of 10–20 min.

2. Experimental

2.1. Chemicals

Ibandronate sodium was provided by Maprimed (Buenos Aires, Argentina), the internal standard, d3-ibandronate sodium, was purchased from SynFine Research (Ontario, Canada) and disodium clodronate tetrahydrate was obtained from Sifavitor (Casaletto Lodigiano, Italy) and from Sigma (Deisenhofen, Germany).

The derivatizing agent TMSD (2 M solution in hexane)—Aldrich, oxalic acid (analytical grade) and formic acid (88%)—Fluka were purchased from Sigma (Deisenhofen, Germany). Acetone (analytical grade), *n*-hexane (analytical grade), hydrochloric acid (analytical grade), acetic acid (analytical grade), calcium chloride and potassium chloride (both analytical grade), methanol and acetonitrile (both gradient grade for LC) were purchased from Merck (Darmstadt, Germany). Diethyl ether (analytical grade) was obtained from Chimopar (Bucharest, Romania) and 2-propanol (gradient grade for LC) from Biosolve (Valkenswaard, Netherlands). Ultra pure water was obtained with a Milli-Q system (Millipore, Molsheim, France).

2.2. Standard solutions

Ibandronate and d3-ibandronate stock solutions, at 1 mg/ml, were prepared in ultra pure water; these solutions were prepared each month and stored at −20 °C. Working dilutions in water or water/methanol were done when needed.

2.3. Derivatization procedure for standard solutions in water

Solutions of ibandronate or its internal standard (d3-ibandronate) in water (0.1 mg/ml) as well samples of distilled water (reaction blank) were derivatized as follows:

Aliquots (0.05 ml) were transferred in 3-ml polypropylene tubes and evaporated to dryness under nitrogen at 60 °C in a Zymark TurboVap evaporator (Hopkinton, MA, USA). The dried extract was redissolved in 0.2 ml trimethylsilyldiazomethane 0.2 M solution in acetone; tubes were then capped and incubated for 30 min at 70 °C in a Techne Dri-Block (Barloworld Scientific Ltd., UK) dry block heater. After cooling down to room temperature, the samples were evaporated to dryness under nitrogen at 37 °C. The final extract was reconstituted with 0.5 ml methanol and further diluted with methanol/water (50/50, *v*/*v*) prior to be used for the optimization of MS parameters and for chromatographic trials.

2.4. Ibandronate isolation from human plasma and derivatization

Plasma samples (0.4 ml aliquots) were transferred with an Eppendorf Research 100–1000 μl pipette (Eppendorf, Hamburg, Germany) in 10 ml polypropylene tubes, spiked with 0.05 ml of internal standard (d3-ibandronate 250 ng/ml in water/methanol 50/50, *v*/*v*), added with 0.57 ml of a mixture consisting of water (0.5 ml), oxalic acid 50 mg/ml in water (0.05 ml) and disodium clodronate 0.2 mg/ml in water (0.02 ml), then acidified with 0.05 ml of hydrochloric acid (3 M). Automatic pipettes Multipette-plus (Eppendorf, Hamburg, Germany) were used for the dispensing

steps. Finally, the extraction solvent (3 ml of diethyl ether/2-propanol, 70/30, *v*/*v*) was added; the tubes were then mixed for 10 min on a rocker-mixer Heidolph Reax II (Schwabach, Germany) and centrifuged for 3 min at 4000 rpm with a centrifuge Sigma model 6K15 (Osteroede, Germany).

Next, the clear supernatant was carefully transferred in 3 ml tubes and evaporated to dryness under nitrogen at 60 °C. The extract was dissolved in 0.2 ml trimethylsilyldiazomethane 0.2 M in acetone by shaking for 3 min on a vortex-mixer IKA Vibrax (Staufen, Germany) and then incubated for 30 min at 70 °C in a dry block heater. Subsequently, 0.2 ml formic acid and 0.4 ml *n*-hexane were added in each tube, followed by vortexing and centrifugation; then 0.3 ml of the clear subnatant were recovered in 1.5 ml polypropylene tubes and evaporated to dryness under nitrogen, at 60 °C. Finally, the dried extracts were reconstituted with 0.15 ml of methanol/water (50/50, *v*/*v*), transferred in 96-well polypropylene autosampler microplates and injected in the LC-MS/MS system.

2.5. Chromatographic separations

Chromatographic separations were carried out with a high-throughput HPLC system model Aria LX-2 developed by Cohesive Technologies (Thermo Fisher Scientific). The Aria multiplexing system with its specific software is integrating through a valve interface module (VIM) a HTS PAL autosampler (CTC Analytics, Switzerland) and two Agilent 1100 binary pumps (Agilent Technologies, Santa Clara, USA). The derivatized products were eluted on a reversed-phase Discovery HSC18 column, 10 cm × 2.1 mm i.d., 5 μm from Supelco (Bellefonte, PA, USA), in gradient conditions at 0.6 ml/min, with a mobile phase composed by water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The initial mobile phase was composed by 100% A. After 0.25 min, the composition was changed to 80% A, 20% B, in a 0.25 min linear gradient step and maintained for 1.5 min, until the elution of the analyte and internal standard. Next, the solvent (B) was increased to 90% in 0.1 min, and kept for 0.25 min, just to push out of the column secondary reaction products and co-extracted matrix. The initial mobile phase composition was restored then in 0.1 min and preserved for 1.67 min. The injection volume was 10 μl.

The two parallel LC systems insured maximum MS performance, permitting one injection every 2 min. Data acquisition was triggered 1.5 min after sample injection and continued for 0.8 min, also avoiding in this way excessive contamination of the interface by salts or other water-soluble components of the sample. While one system was connected to the MS, the other was diverted to the waste, always keeping the source equilibrated with mobile phase.

2.6. Mass spectrometry

Validation procedures were performed on an Applied Biosystems-Sciex (Toronto, Ontario, Canada) quadrupole-linear ion trap mass spectrometer model API 4000 QTrap, equipped with an atmospheric pressure pneumatically assisted electrospray ionization source model turboionspray; the interface was operated in positive ions mode. High purity nitrogen served both as collision-induced dissociation (CAD) gas (simplified setting: medium) and curtain gas (set at 15 psi). The nebulizer and turbo gas were supplied with compressed zero grade air, regulated both at 50 psi. The turboionspray temperature was maintained at 500 °C and the needle voltage was optimized at 3500 V.

To tune the mass-dependent parameters for the analyte (ibandronate derivative) and internal standard (d3-ibandronate derivative), molecular ions were obtained by direct infusion of the standards solution in water (derivatized as described above) and diluted at 1 μg/ml in water/methanol (50/50, *v*/*v*), then the parameters for the product-ion scan were optimized. A Harvard syringe was used to deliver the solution at 10 μl/min. Quantitative determinations were performed in multiple reaction monitoring scan mode using the following transitions: 376.1 → 114.2 for ibandronate derivative, and 379.1 → 61.0 for d3-ibandronate derivative.

The collision energy (CE) was set at 31 and 59 V, respectively and the collision extraction potential (CXP) at 6 V for both derivatives. For structural confirmation chromatograms in product and precursor-ion scan mode were acquired. Preliminary experiments were also carried out on bisphosphonate derivatives with an Applied Biosystems API 5000 triple quadrupole MS.

To complete the pharmacokinetic (PK) study, involving 10,036 unknown samples from 193 subjects orally treated with ibandronate sodium 150 mg, together with calibration curves and quality control samples according to Food and Drug Administration (FDA) rules, two other Aria high-throughput systems, both equipped with high-pressure Agilent 1200 SL pumps and coupled to an API 5000, respectively an API 4000 triple quadrupole mass-spectrometers, were used.

2.7. Validation procedures

The analytical range to be validated was chosen on the basis of the expected plasma concentrations [10]; the lower quantification limit (LLOQ) was set at 0.2 ng/ml while the upper limit of quantification (ULOQ) was fixed at 175.0 ng/ml. To validate the quantitative determination of ibandronate in plasma samples the 2001 FDA validation guidelines [15] and the FDA/AAPS Crystal City 2007 White Paper recommendations [16] were considered. Spiked plasma samples were prepared for calibration curves (8 points in the interval 0.2–175.0 ng/ml) and quality controls (4 different concentration levels) as detailed in the next chapter. The following validation tests were carried out:

(1) *LLOQ evaluation*: The response (peak area) in each blank plasma sample (8 replicates from different subjects including hemolytic and lipemic plasma) compared with spiked LLOQ samples prepared with plasma from the same volunteers. The peak areas in blank samples cannot exceed 20% of the mean ibandronate (derivatized) LLOQ peak areas; the peak areas precision in replicate LLOQ has to be ≤20%. The precision and mean accuracy of back-calculated LLOQ replicate concentrations must be respectively ≤20% and ±20%.
(2) *Selectivity*: Analyses were performed on 8 blank plasma samples (including a hemolytic and a lipemic plasma) collected from different healthy volunteers without addition of internal standard, then with addition of the internal standard or ibandronate; no peak, interfering with those of ibandronate or the internal standard, must appear.
(3) *Calibration curves*, *regression model*, *precision and accuracy*: A calibration curve was prepared in replicate ($n = 6$) and analyzed. The accepted correlation coefficient (r), obtained using the simplest regression model giving the best fitting in the whole range of tested concentrations, must be >0.99, with precision and accuracy, for the back-calculated concentrations of the calibration points, within ±15%, except for the lower quantification limit.
(4) *Within-run and between-run variability*: In the case of within-run evaluation, a minimum of 9 quality controls at each concentration level were prepared and analyzed in the same sequence. For the between-run, at least 18 quality controls at each concentration levels were prepared and analyzed in at least 3 different sequences and in different days. In both conditions the precision and accuracy for replicated quality controls at various concentrations must be situated within ±15%.

(5) *Samples stability* (*plasma samples and extracts*): According to the clinical protocol the plasma samples were stored below −20 °C. Spiked plasma samples stored in these conditions up to 4 months were analyzed to exclude that ibandronate undergoes a significant degradation. The stability was also tested on spiked plasma samples kept at room temperature up to 8 h; these tests were carried out to evaluate the ibandronate stability during the sample preparation and extraction. Spiked samples were finally evaluated after 3 freeze-thawing cycles in order to verify if they can be re-analyzed, if needed, after repeated freezing without compromising the results. Considering that the injection of a series of samples contained in the autosampler can take 8 h, the stability of final derivatized extract, at 10 °C, was tested up to 24 h. The stability of the dry plasma extract prior to derivatization was evaluated up to 48 h at −20 °C. The stability of ibandronate under transportation conditions (dry ice) was estimated keeping sets of spiked samples at −70 °C for up to one month. Finally, the stock solution stability of ibandronate and the internal standard were tested for 6 h at room temperature and 1 month at −20 °C.

(6) *Extraction recovery*: The extraction recoveries of ibandronate and d3-ibandronate were measured comparing the peak areas of spiked quality control sets at different concentrations, prepared according to the above described protocol, to those of samples containing ibandronate and d3-ibandronate diluted from standards derivatized in mobile phase, at the concentration expected if the recovery would be 100%, and added over extracted blank samples. A recovery of 50% in the whole range of expected plasma concentrations was considered enough to obtain an adequate sensitivity.

(7) *Matrix effect*: In compliance with FDA/AAPS Crystal City 2007 White Paper recommendations, a deuterated internal standard (N-trideuteromethyl-ibandronate, d3-ibandronate) was used to compensate matrix effects. These effects were evaluated by comparing the signal of a solution in mobile phase against the signal of the same mixture over a blank extract.

(8) *Stock solutions stability*: The stock solutions stability of both the analyte and the internal standard have been tested up to 6 h at room temperature and 1 month at −20 °C comparing peak areas to those of freshly prepared solutions.

2.8. Calibration curves and quality control samples preparation

Spiked calibration standards were prepared at the following concentrations: 0.2, 0.6, 1.8, 5.0, 15.0, 45.0, 110.0, and 175.0 ng/ml. In order to prepare the highest concentration standard, CAL8 (175.0 ng/ml), an aliquot (19.3 ml) of pooled human plasma lot from healthy volunteers was spiked with 0.7 ml of ibandronate solution 0.005 mg/ml in plasma diluted from the stock solution of 1 mg/ml in water. The other points of the calibration curve were obtained by sequential dilutions in plasma. Calibration standards in plasma were prepared each week and divided in 0.4 ml aliquots. They were stored at −20 °C. Spiked quality control (QC) samples were prepared at the following concentrations: 0.5, 10.0, 40.0, and 160.0 ng/ml. QC samples were obtained starting from stock solutions of ibandronate separately prepared (other weightings) from those used for the calibration curves. In order to prepare the highest concentration quality control, QC4 (160.0 ng/ml), an aliquot (48.4 ml) of a pooled human plasma batch from healthy volunteers was spiked with 1.6 ml of the ibandronate solution 0.005 mg/ml in plasma. The next three QC concentrations were obtained by sequential dilutions in plasma starting from QC4. QC samples were prepared, except in some cases specified in the next paragraphs, at the beginning of the validation procedures and stored below −20 °C, divided in 0.4 ml aliquots, for all this period.

2.9. Plasma samples from pharmacokinetic study

A group of 193 volunteers took part in a bioavailability study, duly approved by regulatory authorities and ethical Committee, performed in accordance with good clinical practice (GCP) norms; ibandronate was orally administered (fasting conditions) as a single dose of 150 mg. Blood samples (5 ml each) were collected, using lithium heparin as anticoagulant, before dosing (0.0) then 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0, 12.0, 24.0, 36.0, 48.0, 72.0, 96.0, 120.0, 144.0, 168.0, 192.0, 216.0, and 240.0 h post dose. Plasma was immediately separated by centrifugation (5 min at 2000 rpm) and aliquots (1–2 ml in 5 ml polypropylene tubes) were kept frozen at −20 °C until analyzed.

3. Results and discussion

3.1. MS parameters

The positive ions MS spectra recorded during infusion of derivatized (TMSD) ibandronate and its internal standard (d3-ibandronate) at 1 μg/ml in water/methanol (50/50, *v*/*v*) show the protonated molecular ions with *m*/*z* 376.1 and 379.1, confirming the tetramethylation of the bisphosphonate groups for both compounds; chemical formula are presented in Fig. 1.

Daughter positive ions spectra obtained by collision-induced dissociation of the protonated molecular ions of TMSD ibandronate (*m*/*z* 376.0) and d3-ibandronate (*m*/*z* 379.1) derivatives are shown in Fig. 2.

The most relevant ibandronate derivative ion fragments have *m*/*z* 58.0, 114.2 and 250.4, corresponding to N-methyl-N-methylenemethanaminium, N-methyl-N-methylenepenthan-1-aminium or N-methyl-N-penthylidenemethanaminium and (E)-methyl hydrogen 1-hydroxy-3-(methyl(pentyl)amino)prop-1-enylphosphonate radicals. In case of the internal standard (d3-ibandronate) the most relevant ions are shifted, as expected, of 3 mass units more: *m*/*z* 61.2, 117.2 and 253.2.

3.2. Derivatization

In an early stage of method development the methylation of ibandronate with TMOA was evaluated; however, due to the unsatisfactory derivatization yield in case of plasma extracts, it was rapidly abandoned in favor of TMSD.

TMSD has proven to be highly reactive also in the presence of plasma extracts, as already studied by another group [17], and it presents, in comparison to diazomethane, the advantage of being safe and stable enough to avoid the need of dangerous daily laboratory synthesis. Various parameters were explored in order to get the highest yield of the tetramethylated ibandronate; as a first the reaction solvent was evaluated and, after initial experiments with a 1:10 dilution of TMSD with *n*-hexane, a dilution 1:10 with acetone was preferred for the final method. The influence of acidic (5% acetic acid) or basic (1% diisopropylamine) reaction conditions as well different amounts of reagent were then tested without improving the derivatization yield.

The incubation time was another important factor as estimated from the peak areas of samples incubated (at 70 °C) from a minimum of 5 min up to 120 min; an almost linear growth of yield was observed up to 60 min while at 120 min a significant decrease took place, finally 60 min were considered optimal. The reaction temperature at 70 °C was chosen as optimal after carrying out experiments in the range 30–100 °C; at lower temperatures the derivatization reaction proceeded slowly while at higher temperature problems of sample evaporation and sample

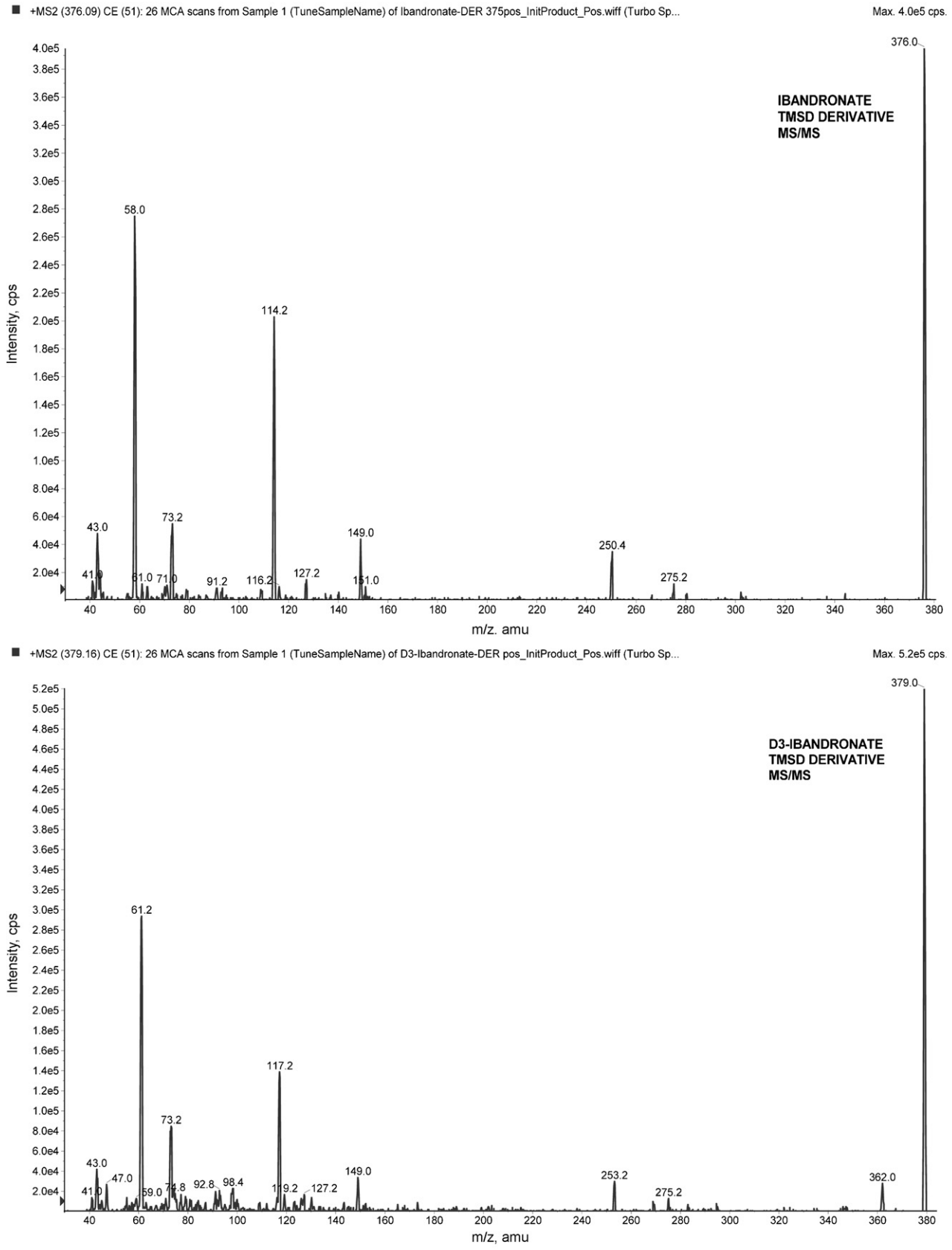

Fig. 2. Positive ions product-ion scan spectra recorded from ibandronate (top) and d3-ibandronate (bottom) reacted with TMSD; samples diluted at 1 μg/ml were infused at 10 μl/min.

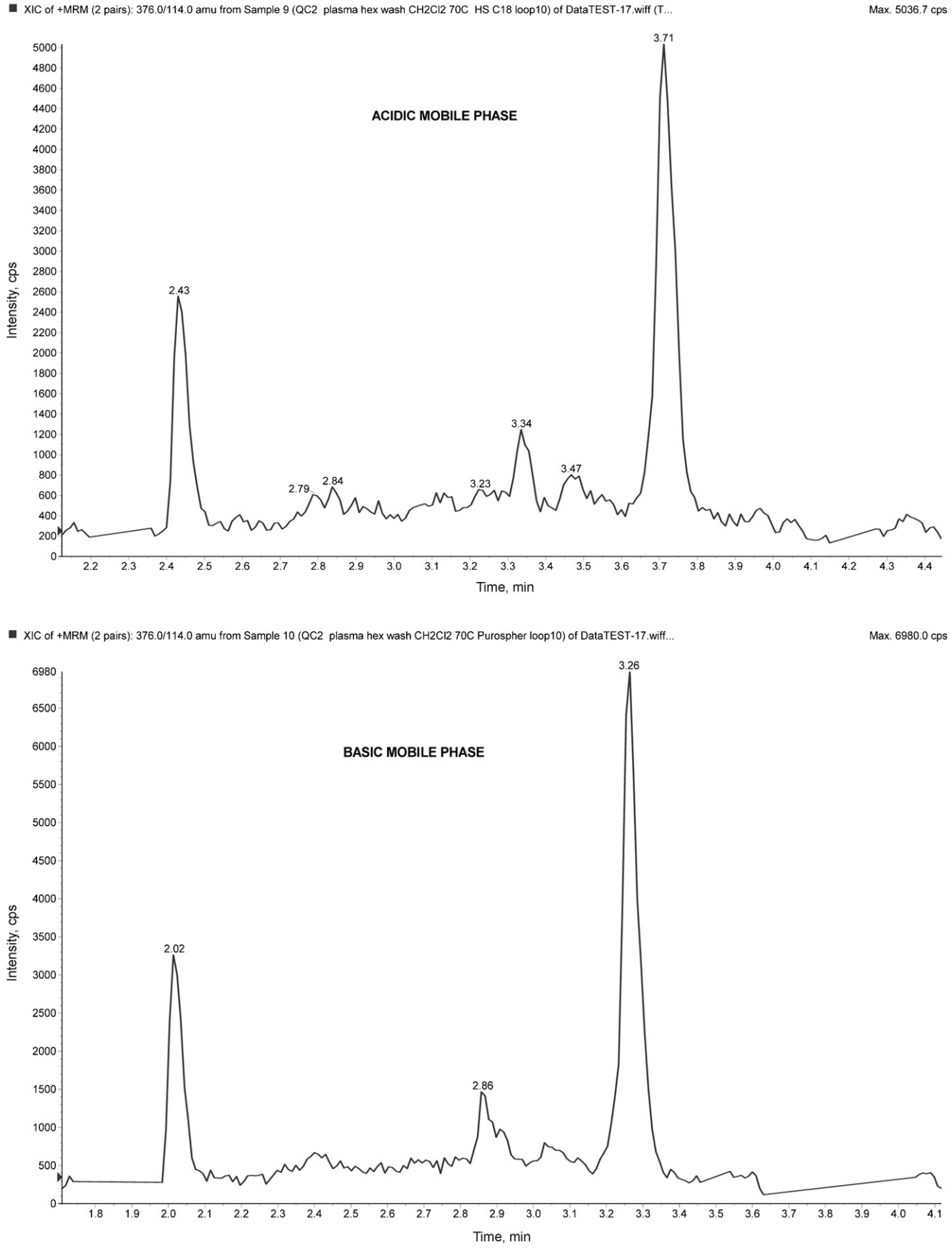

Fig. 3. Chromatograms of ibandronate derivative recorded in multiple reaction monitoring after the injection of plasma extracts. Top data were obtained with a column Discovery HSC18 (10 cm × 2.1 mm, 5 μm) and a mobile phase gradient of formic acid 0.1% in water/acetonitrile. Bottom data were recorded using a column Purospher Star

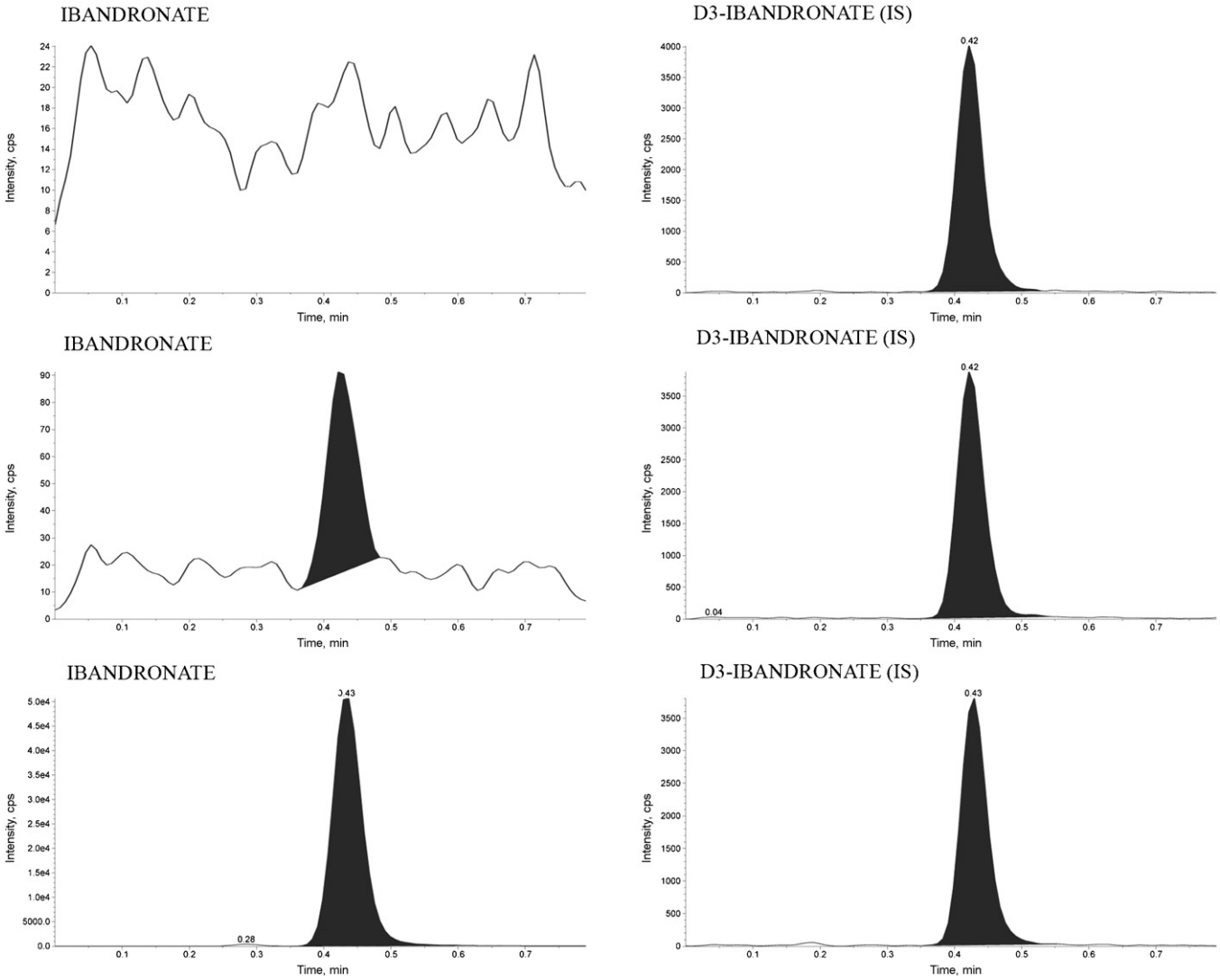

Fig. 4. HPLC traces of the MRM transitions selected for ibandronate (left) and d3-ibandronate (right) recorded on a blank plasma sample (top chromatograms), plasma spiked with ibandronate for CAL1—0.2 ng/ml (middle chromatograms) and CAL8—175.0 ng/ml (bottom chromatograms) concentrations. *Note*: The retention time shown in the figure is relative, as the analyses were performed in overlapping mode; 1.5 min must be added to get the total retention time.

handling, without real improvement of the reaction, were encountered.

3.3. Extraction

Considering the first stage of the sample preparation, the analyte extraction from plasma, a liquid–liquid organic solvent extraction approach was investigated based on the good results obtained in the past with alendronate in urine [8,14]. A solvent mixture consisting of diethylether/isopropanol in strongly acidic pH was used after adding a solution of disodium clodronate–oxalic acid to the sample in order to prevent analyte absorption on the tubes and to promote displacement from proteins. During method development a cleanup of the samples after derivatization proved to be very important; the redissolved samples (after derivatization) indeed were quite turbid and gave relevant chromatographic interferences. For this cleanup, washing of the derivatized extracts with dichloromethane, diethyl ether or *n*-hexane was tested; the best results were obtained with this last solvent.

3.4. HPLC separation

The derivatization step successfully reduced the polarity of ibandronate, thus allowing the separation by reversed-phase LC, using a Discovery HSC18 column (10 cm × 2.1 mm i.d., 5 μm particle size) and a mobile phase composed by formic acid 0.1% in water and formic acid 0.1% in acetonitrile, with a composition gradient. An alternative satisfactory approach using alkaline mobile phase (ammonium hydroxide 0.05% in water/ammonium hydroxide 0.05% in acetonitrile) on a Purospher Star RP-18e (3 cm × 2.1 mm, 2.7 μm) was also studied; good peak intensities, similar to the ones with acidic mobile phase, were achieved and examples can be seen in Fig. 3 (top—acidic mobile phase, bottom—basic mobile phase).

RP-18e (3 cm × 2.1 mm, 2.7 μm) with a mobile phase gradient of ammonium hydroxide 0.05% in water/acetonitrile; the injection volume was 10 μl in both cases. *Note*: The retention time shown in the figure is relative, as the analyses were performed in overlapping mode; 1 min must be added to get the total retention time.

Table 1
Within-run ($n = 9$ per concentration level) and between-run ($n = 60$ per concentration level in 3 days, 8 analytical runs) precision and accuracy measured on quality control samples at four different concentration levels.

	QC 1 (0.5 ng/ml)	QC 2 (10.0 ng/ml)	QC 3 (40.0 ng/ml)	QC 4 (160.0 ng/ml)
Within-run mean accuracy (%)	101.9	99.9	98.7	98.0
Within-run precision (RSD)	6.6	3.4	1.6	1.9
Within-run concentration range (ng/ml)	$(464.0–574.0) \times 10^{-3}$	$(9515.0–10535.0) \times 10^{-3}$	$(38104.0–40508.0) \times 10^{-3}$	$(151319.0–161427.0) \times 10^{-3}$
Within-run QC within limit	9.0 of 9.0	9.0 of 9.0	9.0 of 9.0	9.0 of 9.0
Between-run mean accuracy (%)	104.9	100.9	100.5	101.6
Between-run precision (RSD)	11.8	3.8	3.5	8.1
Between-run concentration range (ng/ml)	$(381.0–674.0) \times 10^{-3}$	$(9445.0–11261.0) \times 10^{-3}$	$(37805.0–44209.0) \times 10^{-3}$	$(151502.0–106961.0) \times 10^{-3}$
Between-run QC within limit	46.0 of 60.0	60.0 of 60.0	60.0 of 60.0	59.0 of 60.0

The chromatography with formic acid was selected for the method validation due to a better background when analyzing plasma samples.

3.5. Validation procedure

(1) *LLOQ evaluation*: Chromatographic traces of the MRM transitions selected for ibandronate and d3-ibandronate derivatives, recorded on plasma samples blank or spiked at the lowest limit of quantification (CAL1, 0.2 ng/ml) and upper limit of quantification (CAL8, 175.0 ng/ml) concentrations are presented in Fig. 4 from top to bottom. No interfering peak was found in blank samples at the retention time of ibandronate while an evident peak is present in the sample prepared at CAL1 (0.2 ng/ml) concentration.

In accordance with the results displayed here, analyzing 8 plasma samples from different subjects (including one hemolytic and another lipemic) spiked at LLOQ concentration (0.2 ng/ml), and comparing them with the same blank plasma, no interfering peak was observed. The precision and accuracy of the LLOQ samples were within validation limits (respectively ≤20% and ±20%), considering the analyte concentrations and the peak areas as well.

(2) *Selectivity*: Also the selectivity tests on blank plasma samples or the same-one spiked with ibandronate or internal standard did not show other analytical interferences.

(3) *Calibration curves*, *regression model*, *precision and accuracy*: The calibration curve computed from replicate analyses ($n = 6$) of each calibrator gave the best fit (Pearson correlation coefficient $r = 0.9997$, coefficient of determination $r^2 = 0.9994$) using a linear regression (the simplest model as recommended in FDA guidelines validation [15]), with $1/x$ weighting. The slope of the six curves graph was 0.0733, and the intercept 0.00036. A test of intercept significance was performed and it was not significant ($p = 0.830$). However, a regression model not through origin was preferred in order to may compensate, in sequences from real samples, the presence of a curve offset due to an unexpected interferent noise signal. The accuracy and reproducibility of the back-calculated ibandronate concentrations in calibrators were within the requested limits (±15% except LLOQ with a range of ±20%).

(4) *Within-run and between-run variability*: When validating the method, the intra-day precision and accuracy were assessed by analyzing 36 QC samples (9 samples of each of the four concentration levels) in the same analytical run. The between-run precision and accuracy were calculated on 240 QC samples (60 at each of the four concentration levels), analyzed with the parallel LC system in 8 runs, on three separate days. As summarized in Table 1, adequate reproducibility results, according to FDA parameters, were obtained in all cases.

(5) *Samples stability* (*plasma samples and extracts*): The results of the stability tests carried out during validation are shown in Table 2.

The experiments were carried out for shorter and longer time intervals; only the results for the longer stability are included. As it can be seen, ibandronate was stable in human plasma samples up to 8 h at room temperature and up to 4 months when stored at −20 °C; repeated (3×) freeze-thawing treatments also do not compromise the samples. The experiments proved that methylated derivatives are stable after dissolution in mobile phase up to 24 h. Dried extracts, without derivatization, were stable up to 48 h at −20 °C; thus allowing to split in 2 phases the sample preparation procedures. Samples were also stable in the transportation conditions (−70 °C) and the sampling tubes did not alter the results. A dilution test was performed to find out if samples of patients with concentration above the ULOQ can be analyzed after a 1:4 dilution with blank plasma; the calculations were within accepted limits. All the stability tests were performed with plasma spiked at four QC concentration levels.

Table 2
Results of the stability tests (percent ratios vs. control) carried out on quality control samples at four concentrations (middle column) in different storage conditions (left column).

Different storage conditions	Stability % (comparison vs. control)	
Plasma stability 8 h room temperature	QC1	92.2
	QC2	103.5
	QC3	92.5
	QC4	102.3
Plasma stability 4 months −20 °C	QC1	100.1
	QC2	99.2
	QC3	99.8
	QC4	101.5
Plasma stability 1 months −70 °C	QC1	104.0
	QC2	104.8
	QC3	102.9
	QC4	101.3
Plasma stability freeze-thaw 3 cycles	QC1	103.6
	QC2	101.0
	QC3	101.4
	QC4	100.5
Recipient test	QC1	100.5
	QC2	100.2
	QC3	93.6
	QC4	100.3
Plasma final extract stability 24 h 10 °C	QC1	119.7
	QC2	99.4
	QC3	101.3
	QC4	94.3
Plasma extract stability prior to derivatization 48 h −20 °C	QC1	93.9
	QC2	96.3
	QC3	100.1
	QC4	100.9
Ibandronate stock solution stability	6 h room temperature	100.3
	1 month −20 °C	100.1
d3-ibandronate stock solution stability	6 h room temperature	100.1
	1 month −20 °C	100.2

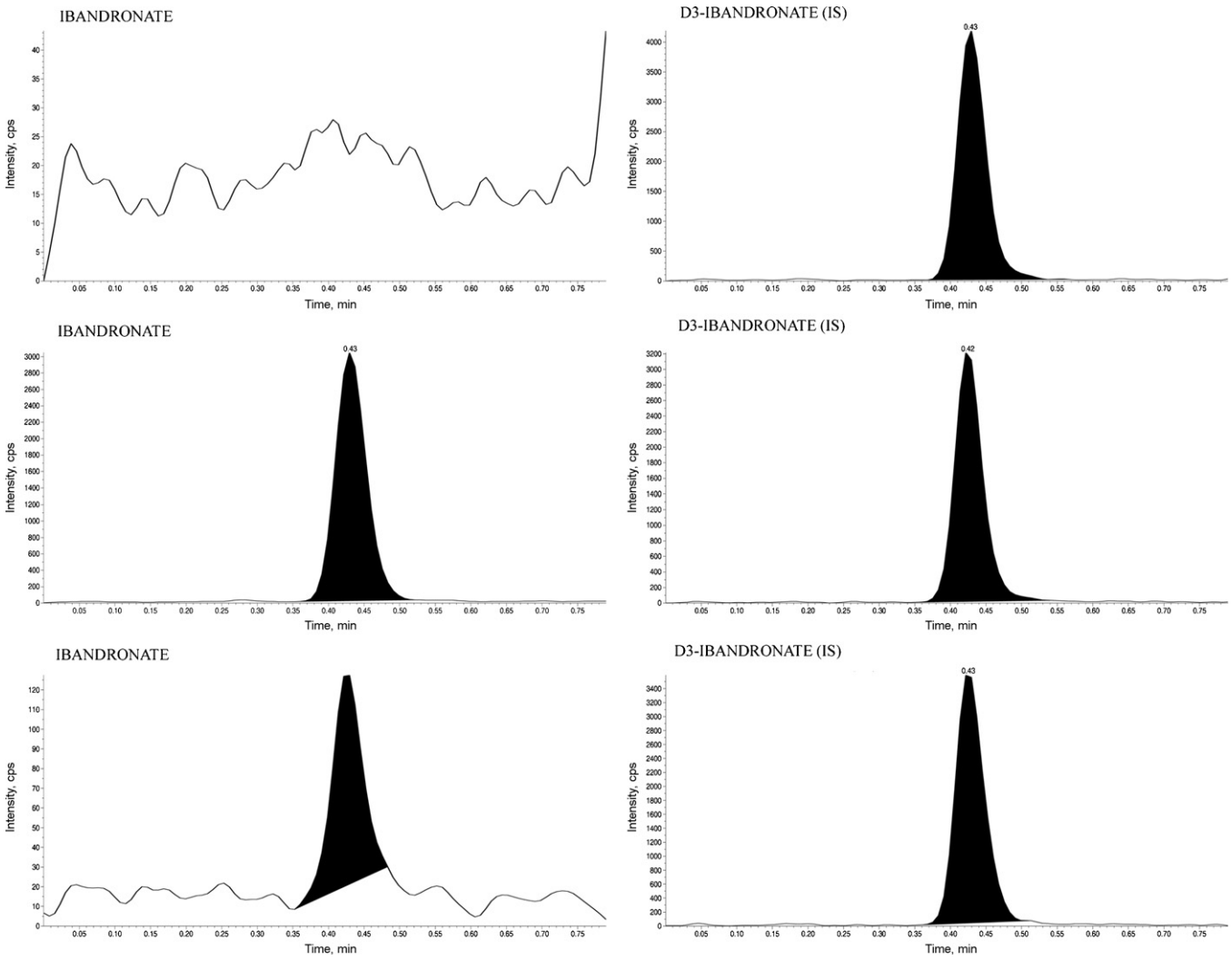

Fig. 5. Chromatograms of ibandronate (left) and d3-ibandronate (right) derivatives recorded in three plasma samples of a subject treated orally with ibandronate 150 mg; the data, from top to bottom, were collected 1 h before drug administration, 0.5 and 24 h after dosing. *Note*: The retention time shown in the figure is relative, as the analyses were performed in overlapping mode; 1.5 min must be added to get the total retention time.

(6) *Extraction recovery*: Extraction recoveries of 54.3% at the quality control 1 level, 79.2% at the quality control 2 level, 67.8% at the quality control 3 level, and 58.2% at the 4 level, with a coefficient of variation (CV) of 12.8%, 4.8%, 4.3%, and 7.5%, respectively obtained for the ibandronate derivative. The results with d3-ibandronate were similar (65.3% with a CV 5.7%). It must be noticed that this test includes the real recovery of the analytes by extraction and also the efficiency of derivatization. With QC level 1 the lowest mean extraction result was obtained (54.3% recovery); however, the data at the other QC levels did not show a clear concentration recovery correlation. A higher influence of the matrix on the derivatization yield is a plausible explanation.

(7) *Matrix effect*: A clear matrix effect, due to the plasma extract, was observed being the mean ibandronate response (peak area) 46.2% with plasma extract when compared to mobile phase dilutions; a similar effect, 46.4%, was determined for d3-ibandronate, This matrix effect was reproducible (coefficient of variation < 15%) when tested in different plasma samples and, in any case, a deuterated internal standard was used in order to guarantee an optimal matrix effect correction.

(8) *Stock solutions stability*: The stability results of ibandronate and d3-ibandronate stock solutions were adequate both at room temperature and at −20 °C (comparisons vs. the fresh solutions)

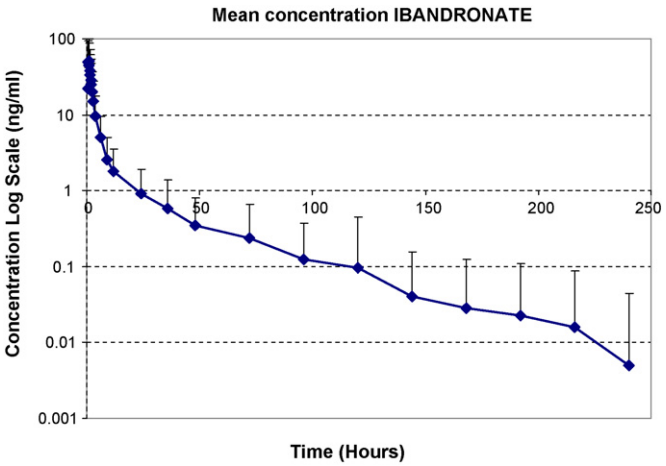

Fig. 6. Mean ibandronate pharmacokinetic curve obtained on 193 volunteers in a bioavailability study with ibandronate 150 mg orally administered (semilogarithmic plot with standard deviation bars).

3.6. Application

Fig. 5 shows the ibandronate and internal standard chromatograms recorded in 3 plasma samples of subjects treated orally with ibandronate 150 mg; the data, from top to bottom, were obtained from plasma samples collected 1 h before drug administration, then 0.5 h (the time with the maximum concentration for this volunteer) and 24 h after dosing. As it can be seen, no significant peak was present in the pretreatment samples while relevant peaks are visible thereafter; also at 24 h, late in the elimination curve, a clear peak is detectable.

The mean ibandronate pharmacokinetic curve (semilogarithmic plot with standard deviation bars) obtained in a bioavailability study with ibandronate 150 mg orally administered is presented in Fig. 6. The measured concentrations were very close to results already published [10,18,19] and adequate pharmacokinetic calculation could be performed.

4. Conclusions

The analytical method described above for the determination of ibandronate from human plasma was fully validated and applied to a pharmacokinetic study. The quantification limits (from 0.2 to 175.0 ng/ml) were suitable for the evaluation of the plasmatic concentrations of patients treated orally with ibandronate 150 mg. During method development, ibandronate isolation from plasma and derivatization with TMSD were key steps. The sample preparation (extraction and derivatization parameters) was optimized in order to get the highest recovery and an efficient derivatization keeping procedures as simple as possible. TMSD proved to react fast and effectively therefore it was chosen as a safe alternative to diazomethane widely employed in methylation reactions for GC-MS analysis [8]. The time-consuming coprecipitation with calcium phosphate, often used in the analysis of bisphosphonates [6,7] was avoided, and replaced by an effective liquid–liquid solvent extraction. All the sample preparation procedures were performed in a timely fashion with the help of semi-automated lab-equipment and they can also be adapted to complete automation with robotic liquid handlers.

The chromatographic separation of the derivatized ibandronate and its internal standard, d3-ibandronate, was explored in reversed-phase conditions, with acidic or basic mobile phase. Elution at acidic pH was preferred for this study due to better background, but the basic mobile phase can be also considered.

A short analytical run was obtained with a simple composition gradient; the use of an integrated multiplexing LC system (Aria LX-2) with two columns in parallel and overlapping two samples resulted in an interval between two injections of 2.1 min. This high-throughput set-up allowed the fast and operative analysis of a very large number (approx. 10,000) of unknown samples for a bioequivalence study.

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