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A new IC-MS/MS approach for the analysis of bisphosphonates in horse plasma

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Keywords

IC-MS/MS, HPIC, TSQ Altis, ICS-6000, Dionex IonPac AS18 4µm Fast Column, horse doping, bisphosphonates

Application benefits

- Significant simplification and method performance improvements over current reference methods
- Derivatization-free protocol with fewer sample preparation steps
- Quantification of all bisphosphonates including *N*-containing bisphosphonates

Introduction

Bisphosphonates are prohibited drugs according to Article 6 of the International Agreement on Breeding, Racing and Wagering of the International Federation of Horseracing Authorities (IFHA).[1] These compounds are used for the treatment of navicular syndrome and related diseases in horses and are divided in two groups: non-nitrogen-containing bisphosphonate drugs (e.g., tiludronic acid) and nitrogen-containing bisphosphonate drugs (e.g., zoledronic acid).

Due to their very polar and strong chelating characteristics, determination of bisphosphonates represents a real analytical challenge for routine screening. The scientific literature reports different approaches to perform bisphosphonates analysis: ion-pairing HPLC with light scattering detection;

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ion chromatography (IC) with UV indirect detection[2]; HPLC with charged aerosol detection[3]; or LC-MS/MS with analyte derivatization[4].

Currently, analysis for such compounds in a complex matrix is based on a solid phase extraction (SPE) with a weak anion exchange sorbent, followed by detection using UHPLC-MS/MS after hydroxyl methylation with trimethyl orthoacetate (TMOA).[5] This approach can provide the required selectivity and sensitivity for the purpose of horse doping control. However, sample preparation remains a very time-consuming step and this derivatization is inefficient for determining *N*-containing bisphosphonates.

To remove the complexity of derivatization, a new approach using IC linked to mass spectrometry is considered. IC using eluent generation and suppressed conductivity detection provides chromatographic selectivity, low chemical noise, and high compatibility with the mass spectrometer. This application note was developed with a Thermo Scientific™ Dionex™ ICS-6000 HPIC™ ion chromatography system and a Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer to improve the detection of bisphosphonates in horse plasma.

Experimental

Equipment

Ion chromatography

- Dionex ICS-6000 HPIC system with Reagent-Free IC (RFIC™) capabilities with a dual pump (one pump used for elution and the other to supply water for the suppressor regeneration)
- Conductivity detector with integrated cell
- Thermo Scientific™ Dionex™ ICS-6000 SP pump (make-up)
- Thermo Scientific™ Dionex™ AS-AP temperature controlled autosampler

Mass spectrometry

- TSQ Altis triple quadrupole mass spectrometer equipped with the Thermo Scientific™ OptaMax™ NG source housing
- Thermo Scientific syringe pump for method optimization

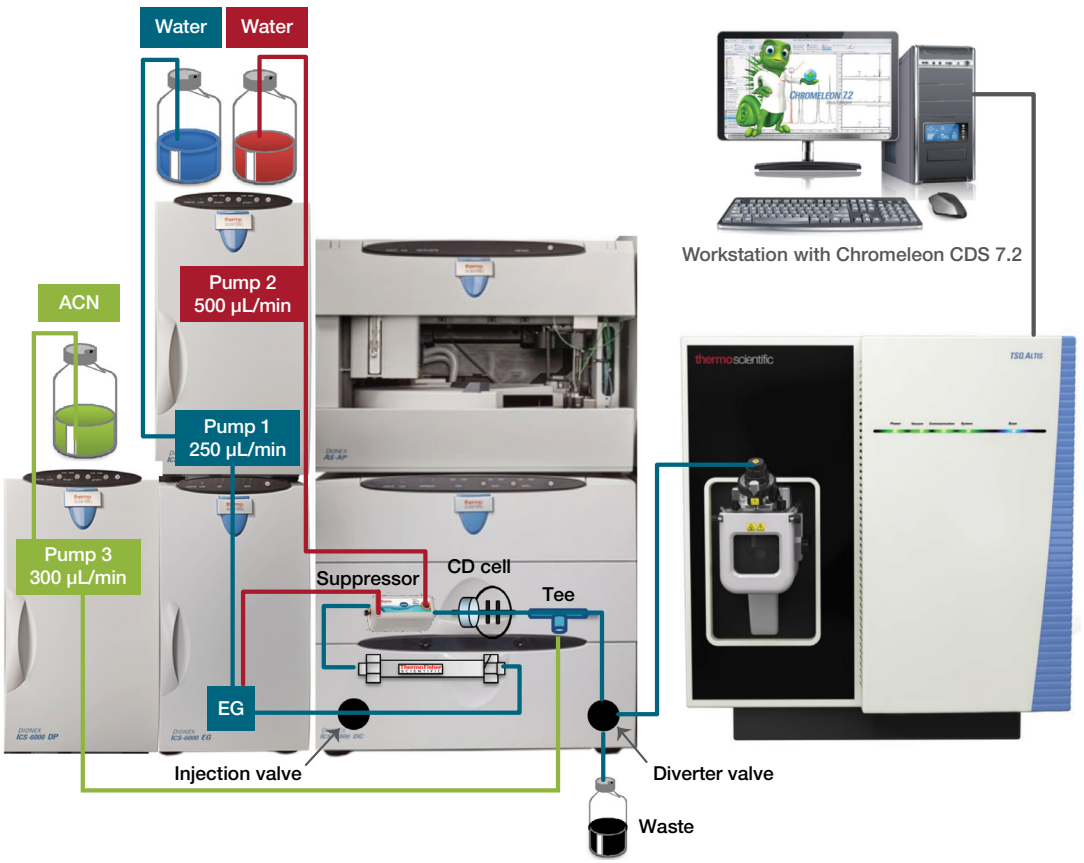


Figure 1. Schematic flow path for the IC-MS/MS setup

Software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), 7.2.9 version

Method

Table 1. IC setup

Column:	Thermo Scientific Dionex™ IonPac™ AS18-Fast-4 µm IC Column 150 × 2 mm (P/N 076036)
Eluent:	KOH gradient from 35 to 100 mM generated by Thermo Scientific™ Dionex™ ICS-6000 EG Eluent Generator (for details, see Table 2)
Eluent source:	Thermo Scientific™ Dionex™ EGC 500 KOH cartridge (P/N 075778), Thermo Scientific™ Dionex™ CR-ATC 600 trap column (P/N 088662), high pressure degasser module
Flow rate:	0.25 mL/min
Injection volume:	50 µL
Detection:	Suppressed conductivity, Thermo Scientific™ Dionex™ AERS™ 500e 2 mm suppressor (P/N 302662), external water mode at 0.5 mL/min
Typical conductance background:	<1 µS/cm

Table 2. KOH gradient generated by the Dionex ICS-6000 EG

Time (min)	Concentration (mM)
0	35
2	35
2	100
7	100
7	35
14	35

Table 3. Mass spectrometer setup

Parameter	Setting
Run time:	14 min
Ion source:	H-ESI
Spray voltage:	3500 V
Sheath gas:	50
Auxiliary gas:	10
Sweep gas:	1
Ion transfer tube temperature:	325 °C
Vaporizer temperature:	325 °C
Make up:	0.3 mL/min ACN
Experiment type:	Selected reaction monitoring (SRM)
Cycle time:	1.2 s
Chromatography peak width:	10 s
Collision gas pressure:	1.5 mTorr
Q1 resolution:	0.7 FHMW
Q3 resolution:	1.2 FHMW

Table 4. SRM parameters for each bisphosphonate

Compound	Start Time (min	End Time (min)	Polarity	Precursor Ion (*m/z*)	Quantification Ion (*m/z*)	Collision Energy (V)	Confirmation Ion n° 1 (*m/z*) Ion n° 2 (*m/z*)	Collision Energy (V)	RF Lens (V)
Pamidronic acid	4.6	6.6	Neg	233.95	152	18	198	15	49
							216	12	49
Alendronic acid	4.7	6.7	Neg	248.00	148	20	166	16	50
							194	16	50
Clodronic acid	4.9	6.9	Neg	242.83	79	26	143	15	58
							171	13	58
Zoledronic acid	4.9	6.9	Neg	270.95	127	25	189	18	57
							235	15	57
Risedronic acid	4.9	6.9	Neg	282.01	63	30	264	12	55
							200	17	55
Tiludronic acid	7.9	9.9	Neg	316.96	299	16	79	29	63
							281	20	63
Internal Standard*	8.8	10.8	Neg	350.95	79	31	333	17	69

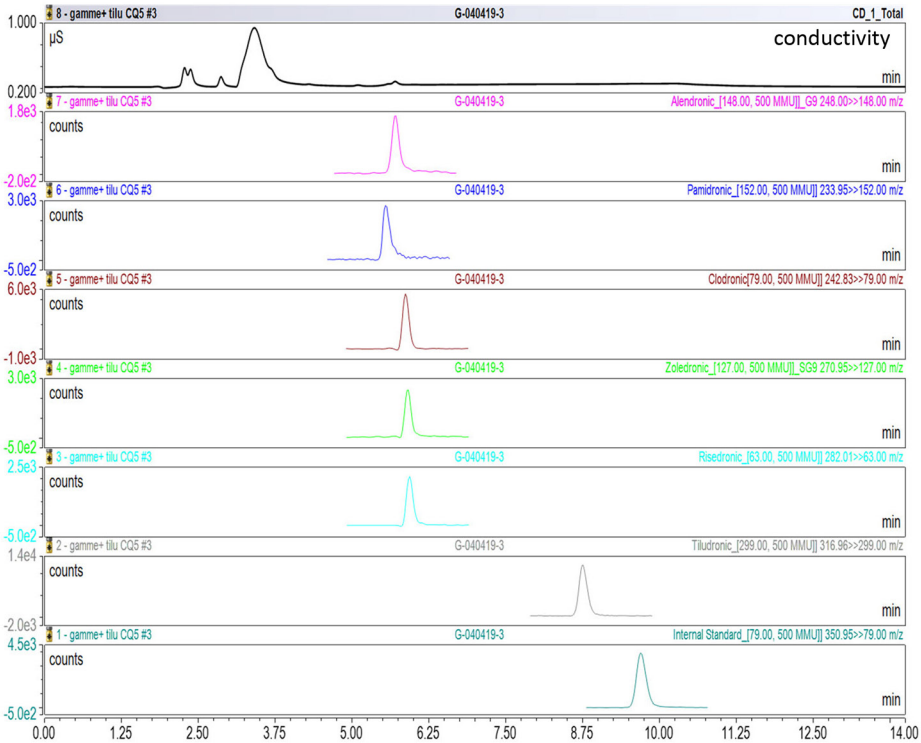
*Internal Standard: (3-Trifluoromethylphenyl) thiomethylene biphosphonic acid

Sample preparation

(3-Trifluoromethylphenyl) thiomethylene biphosphonic acid internal standard (IS) was added to 200 µL of horse plasma. The sample was deproteinized in acidic conditions and water was added before solid phase extraction (SPE) using 96-well plates. SPE was conducted in two steps: firstly on HLB polymeric sorbent to clean samples by filtration; secondly on a sorbent with weak anionic exchange (WAX) properties. Bisphosphonates were eluted using a mixture of water/methanol/ammonium hydroxide. The eluent was evaporated under dried air stream at 50 °C. The extract was dissolved in 100 µL of water. Then, 50 µL were injected into the IC-MS/MS system.

Results and discussion

Separation of *N*-containing and other bisphosphonates was achieved within 14 min. Figure 2 shows the detection of each bisphosphonate in standard solution using the method described in this application note. The selectivity of the mass spectrometer is useful for this application because the chromatogram with suppressed conductivity detection (top trace) shows co-elution of three compounds (clodronic, risedronic, and zoledronic acids). Extraction of each SRM trace allows easier and better detection for each compound. The IC-MS/MS setup provides exceptional selectivity for polar analytes in a complex matrix. Product ion chromatograms show the high specificity of this technique (Figure 3). Each targeted bisphosphonate can be confirmed using at least two confirmatory ions.



Columns:	Dionex IonPac AS18-Fast-4µm, 150 x 2 mm
Eluent:	KOH gradient (Table 2)
Eluent Source:	Dionex EGC 500 KOH cartridge, Dionex CR-ATC 600 trap column
Flow Rate:	0.25 mL/min
Inj. Volume:	50 µL
Oven Temp.:	30 °C
Detector 1:	Suppressed conductivity, Dionex AERS 500 suppressor, 124 mA, 15 °C external water mode, 0.5 mL/min
Detector 2:	TSQ Altis, -ESI, SRM (Table 3)
Sample:	1 pg/µL of each compound in water

Peaks:	SRM Q1→Q3
Pamidronic acid	233.95 → 152
Alendronic acid	248 → 148
Risedronic acid	282.01 → 63
Zoledronic acid	270.95 → 127
Clodronic acid	242.83 → 79
Tiludronic acid	316.91 → 79
Internal Standard	350.95 → 79

Figure 2. SRM extraction for each bisphosphonate and comparison with conductivity detection (standard solution at 1 pg/µL)

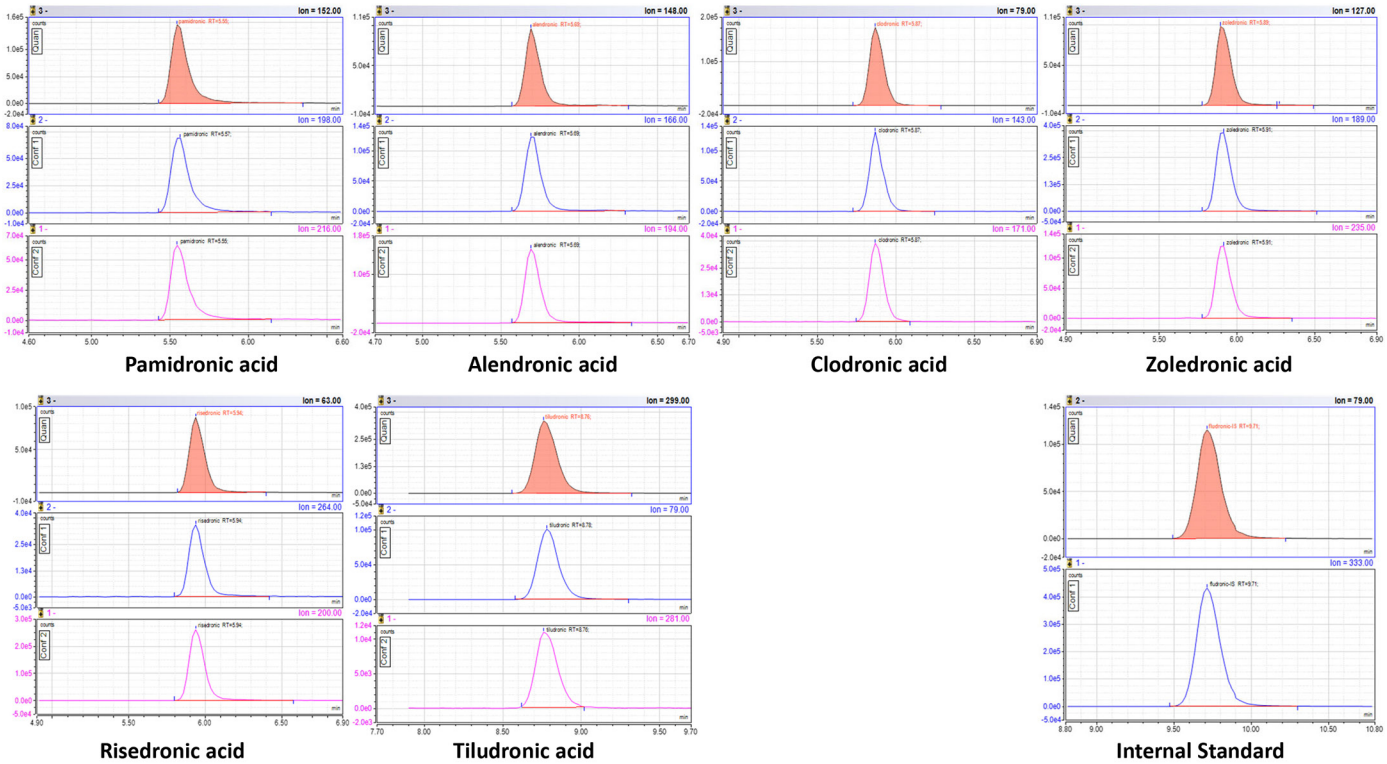


Figure 3. SRM chromatographic representation of seven bisphosphonates monitored with the TSQ Altis MS (standard solution at 1 pg/µL)

Calibration curves generated for each bisphosphonate are shown in Figure 4. This methodology provides an LOD, determined experimentally by injection of standard solution, of 50 fg/µL or less for standard solutions. The MS response remains linear between 0.050 and 10 pg/µL. Figure 5 reports a product ion chromatogram for tiludronic acid at 5 ng/mL in horse plasma. Specific sample preparation described above for horse plasma and specific chromatographic conditions allow elimination of interfering molecules that could induce lower signal than expected. This work has led to a sensitive and specific method for tiludronic acid in horse plasma. In this sample spiked with 5 ng/mL of tiludronic acid, characteristic peaks used for detection generate a signal-to-noise ratio of 150 (Figure 5).

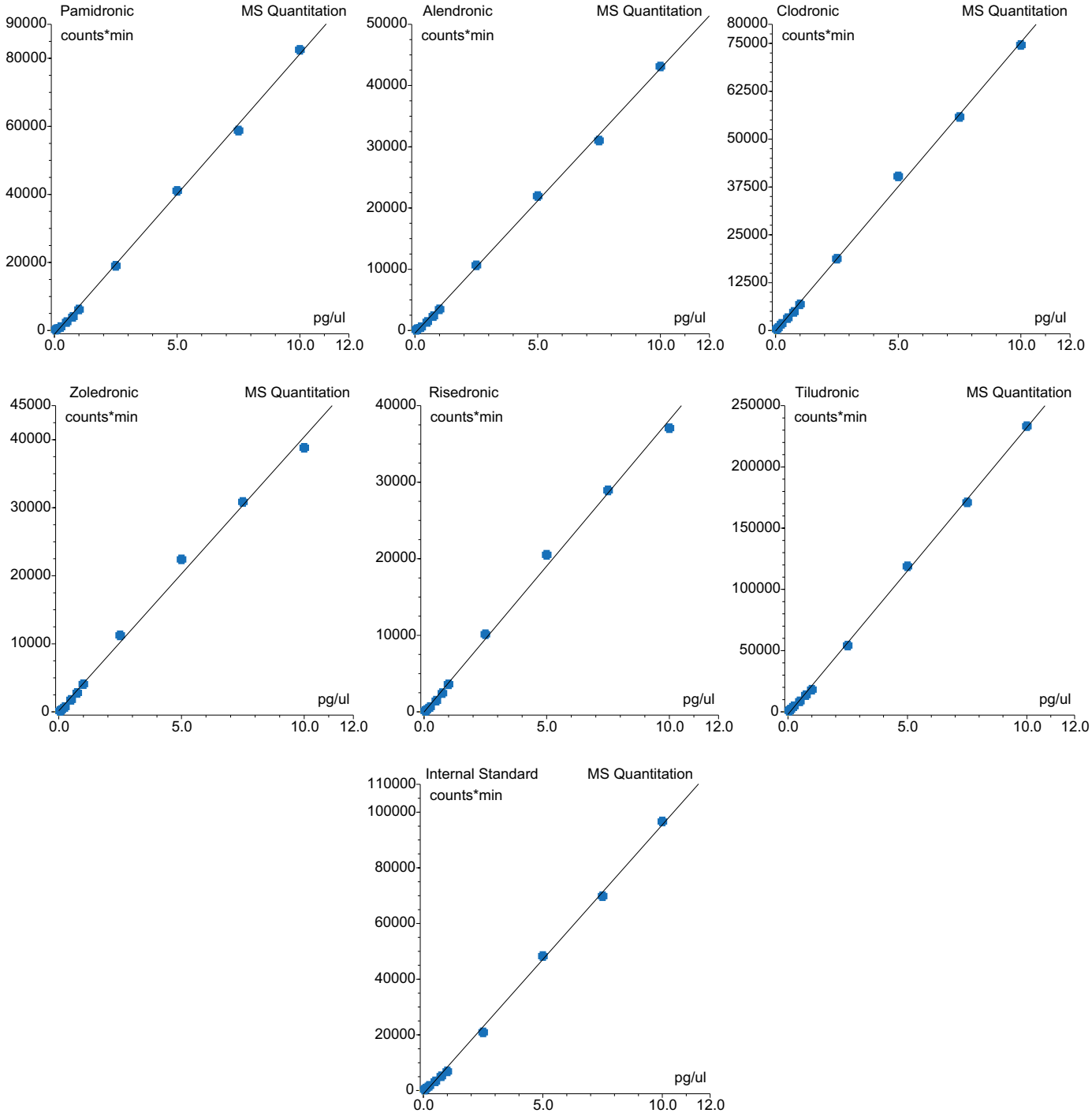


Figure 4. Calibration curves for seven bisphosphonates. Concentration ranges from 0.050 to 10 pg/µL in standard solutions.

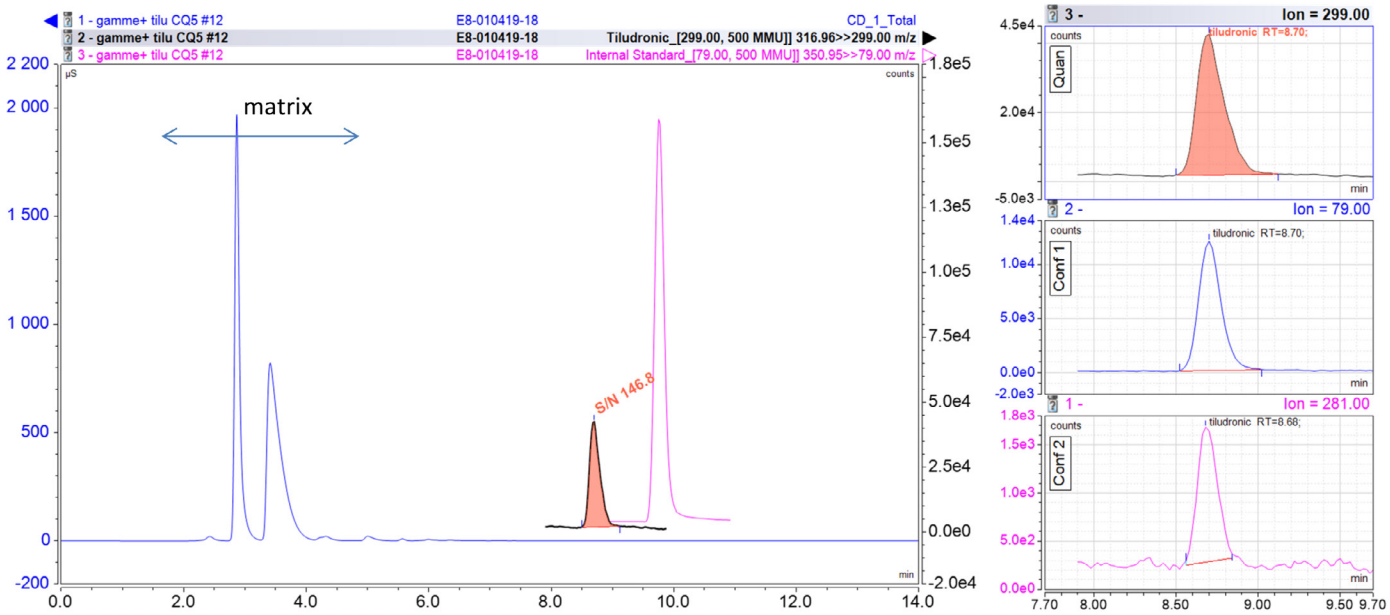


Figure 5. Comparison of chromatograms related to MS signal of tiludronic acid, internal standard, and conductimetric signal in horse plasma spiked with 5 ng/mL of tiludronic acid. Extraction of 3 confirmatory ions for tiludronic acid.

Conclusion

Results demonstrate excellent method performance for the detection and confirmatory analysis of all studied bisphosphonates including nitrogen-containing bisphosphonates. This new analytical approach can allow a significant simplification of sample preparation without derivatization. Matrix interference was quite negligible at the expected retention times of the target transitions.

The applicability of the method was demonstrated by analyzing low levels of tiludronic acid spiked in equine plasma. Results could be reported easily and in a very short time. This new approach could allow screening and confirmatory analysis of bisphosphonates in doping control by IC-MS/MS using an ICS-6000 HPIC system coupled to a TSQ Altis triple quadrupole mass spectrometer. In addition, this innovative system can be extended to other polar molecules for which improvements in selectivity and sensitivity are desired.

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