# Quantitative Determination of Bisphosphonate Pharmaceuticals and Excipients by Capillary IC-MS

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## Key Words

MSQ Plus, Chromeleon, Etidronate, Clodronate, Tiludronate, Excipients, ICS-5000

## Goal

To develop and demonstrate a simple, fast, sensitive, and robust analytical method to quantify bisphosphonate pharmaceuticals and common excipients.

## Introduction

Bisphosphonates are a group of compounds that are used as active pharmaceutical ingredients (APIs) to treat bone disorders including osteoporosis, Paget's disease, and hypercalcemia.[1,2] Typical methods for bisphosphonates analysis include liquid chromatography (LC) with derivatization and/or ion pairing,[3,4,5] ion chromatography (IC),[6,7] capillary electrophoresis (CE),[8,9] and gas chromatography (GC) with derivatization.[10,11] The reported analytical methodologies for bisphosphonates were summarized and compared in a review article published in 2008.[12] This article concluded that for pharmaceutical purpose quality control (QC), IC with conductivity is "an obvious solution," offering "simplicity, avoidance of derivatization steps, adequate sensitivity and simultaneous separation of ionic impurities." The authors also indicated that mass spectrometry (MS) would be a sensitive approach, but the application to bisphosphonate analysis is limited due to the obvious incompatibility of the ion-pairing agent used in the mobile phase.

Here we present a quantitative method for the direct analysis of bisphosphonates and excipients in pharmaceuticals using capillary IC with suppressed conductivity and mass spectrometric detection. A Thermo Scientific™ Dionex™ IonPac™ AS18-Fast Capillary anion-exchange column is used to achieve chromatographic retention and resolution for target analytes, and the elimination of derivatization steps simplifies the workflow and improves method throughput. The detection by suppressed conductivity provides sufficient sensitivity for QC analysis and MS offers additional selectivity and sensitivity for bisphosphonates in complex matrices, such as biological fluids. An isotope labeled internal standard (IS) citric acid-$d_4$ is used to ensure quantitation accuracy.

## Equipment

- Thermo Scientific™ Dionex™ ICS-5000 Capillary IC* system with eluent generation
- Thermo Scientific™ MSQ™ Plus Mass Spectrometer (single quadrupole)
- Thermo Scientific™ Dionex™ AXP-MS Auxiliary pump (×2)
- Thermo Scientific™ Chromeleon™ Chromatography Data System software 6.8 SR11
- Thermo Scientific™ Xcalibur 2.0.7 with MSQ™ 2.0 SP1

*A Thermo Scientific™ Dionex™ ICS-6000 HPIC system or Thermo Scientific™ Dionex™ ICS-4000 HPIC system can be used for equivalent results

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## Reagents and Chemicals

- All chemical standard chemicals were purchased from Sigma-Aldrich unless noted.
- Etidronate disodium hydrate (PN P5248)
- Clodronate disodium (PN D4434)
- Tiludronate disodium hydrate (PN T4580)
- Benzoic acid sodium salt (PN B3375)
- *p*-Hydroxybenzoic acid (PN H5376)
- Citric acid (PN 27788)
- Isotope labeled internal standard citric acid-$d_4$ (C/D/N Isotopes, Inc., PN D-3745)
- Deionized (DI) water with 18.2 MΩ-cm resistivity
- Acetonitrile (LC/MS grade, Fisher Scientific or equivalent)

## Standard Preparation

Prepare individual stock solutions at 1000 µg/mL [parts per million (ppm)] by weighing each pure chemical to the nearest 0.1 mg, and dissolving in DI water. Prepare working standards containing six target analytes (etidronate, clodronate, tiludronate, benzoate, *p*-hydroxybenzoate, and citrate) from individual stock solutions at 10 ppm. Dilute working standard solutions to 1 ppm and 100 parts per billion (ppb) to prepare calibration standards.

Prepare IS stock solution at 1000 ppm in DI water and then dilute to 10 ppm to prepare calibration standards and spike unknown samples.

Prepare calibration standards at 6 levels with each of the target analytes (3 bisphosphonates, 3 excipients) at 5 ppb, 10 ppb, 50 ppb, 100 ppb, 200 ppb, and 500 ppb with IS spiked at 100 ppb in each level.

## Sample Preparation

Etidronate disodium 200 mg tablets were supplied by a customer and analyzed in this laboratory. Weigh each tablet individually and calculate the average weight (0.346 g /tablet). Grind tablet samples into fine powder form and weigh three subsamples (10–15 mg each ) to the nearest 0.01 mg. Dissolve each subsample in DI water to the concentration of 1.0 mg sample per mL DI water. Sonicate each solution in a water bath at room temperature for 30 min and filter through a 25 mm 0.2 µm PES syringe filter (PALL Life Science, PN 4583T). Dilute 5 µL of each filtrate to 10 mL with DI water and then inject for analysis and quantitation.

## Conditions

### Chromatographic Conditions

| | |
|---|---|
| System: | Dionex ICS-5000 capillary IC system with eluent generation |
| Column: | Thermo Scientific™ Dionex™IonPac™ AS18-Fast Capillary Column (0.4 × 150 mm, PN 072062)<br>Dionex IonPac AG18-Fast Capillary Guard Column (0.4 × 35 mm, PN 072063) |
| Eluent: | Hydroxide gradient |

| Time (min) | Concentration (mM) |
|---|---|
| -4.0 | 40 |
| 0.0 | 40 |
| 5.0 | 50 |
| 8.0 | 100 |
| 13.9 | 100 |
| 14.0 | 40 |

| | |
|---|---|
| Eluent Source: | Dionex EGC-KOH (Capillary) Cartridge (PN 072076) |
| Flow Rate: | 20 µL/min |
| Injection: | 2 µL |
| Temperature: | 40 °C |
| Detection: | 1) Suppressed conductivity with Thermo Scientific Dionex ACES 300 Anion Capillary Electrolytic Suppressor (external water mode, 30 µL/min DI water delivered by AXP-MS pump)<br>2) MSQ Plus single quadrupole mass spectrometer |

### Mass Spectrometric Conditions

| | |
|---|---|
| System: | MSQ Plus mass spectrometer, single quadrupole |
| Interface: | Capillary low-flow electrospray ionization (ESI) negative polarity |
| Probe: | MSQ Plus ESI probe with low-flow option (PN 078996) |
| Probe Temperature: | 300 °C |
| Needle Voltage: | 3500 V |
| Desolvation Solvent: | 20 µL/min acetonitrile delivered by a Dionex AXP-MS pump |
| Nebulizer Gas: | Nitrogen at 65 psi |
| Acquisition: | Selected ion monitoring (SIM) with cone voltage set at 55 V for each SIM with 0.3 amu span<br>See Table 1 for SIM events details |

Table 1. Timed SIM scan events

| Analyte | $t_R$ (min) | SIM (*m/z*) | Timed Event (min) | Scan Time (s) |
|---|---|---|---|---|
| Benzoate | 3.9 | 121 | 3.6–5.2 | 0.2 |
| *p*-Hydroxybenzoate | 4.4 | 137 | 3.6–5.2 | 0.2 |
| Citrate | 5.8 | 191 | 5.2–10.0 | 0.2 |
| IS (citrate-$d_4$) | 5.8 | 195 | 5.2–10.0 | 0.2 |
| Etidronate | 6.6 | 205 | 5.2–10.0 | 0.4 |
| Clodronate | 7.3 | 243 | 5.2–10.0 | 0.4 |
| Tiludronate | 12.1 | 317 | 10.0–14.0 | 1.0 |

## Results and Discussion

### Chromatography

Chromatographic methods have been used extensively for bisphosphonate analysis. Among the reported chromatographic methods included are reversed-phase LC, ion-paring LC, IC, CE, and GC. IC is an obvious method choice because of its ease of configuration, avoidance of derivatization, sensitive detection via suppressed conductivity for charged analytes, and also the capacity for simultaneous determination of impurities such as phosphate and other anionic species. Thus IC was selected as the chromatography method of choice in this study.

A Dionex ICS-5000 capillary IC system was used in this study because capillary IC offers improved sensitivity with injection of the same or less amount when compared to standard IC,[13] and better sensitivity when coupled with a capillary ESI interface to a mass spectrometric detector.[14] A Dionex IonPac AS18-Fast Capillary hydroxide selective anion-exchange column was selected for separation because it offers total resolution of three targeted bisphosphonates (clodronate, etidronate and tiludronate) and the three excipients (citrate, benzoate and *p*-hydroxybenzoate), as well as the seven commonly seen anions. The Dionex IonPac AS18-Fast Capillary column is also a shorter format (150 mm length) than regular 250 mm columns, thus improving method throughput while still offering sufficient chromatographic resolution. The optimized separation is shown in Figure 1: anionic impurities such as commonly seen anions were eluted as early peaks, with phosphate being the latest eluter. Phosphate may be a targeted impurity in a regulated environment, e.g., QC laboratories, and could be easily quantified since it was well separated from other anions. All bisphosphonates and excipient compounds were well separated from anionic species and from each other within a 14 min analytical run, thus allowing simultaneous accurate quantitation of each individual compound.

### Mass Spectrometry

ESI is the most commonly used interface to couple IC-MS as it is more suitabile for polar and charged analytes than other atmospheric pressure ionization techniques, i.e., atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI). Standard ESI interfaces are generally optimized for analytical flow (100 µL to several mL/min) or nanoflow (<1µL/min) ranges. The capillary IC features a flow rate in the range from 10 to 50 µL/min, thus requiring modification and reoptimization of existing ESI interfaces.[15] Here, a standard MSQ Plus ESI probe with low flow option was used, showing significant improvement for low-flow applications,[15] and thus was used for the rest of the study.

The optimization of interface parameters such as probe temperature, nebulizer gas, needle voltage, type of desolvation solvent and the flow rate plays a critical role in establishing instrument sensitivity.[15] The observed optimum conditions are related to specific analytes and applications, thus optimization of interface parameters is highly recommended during method development. A general condition which serves as a starting point for optimization is recommended. When capillary IC is operating at 10 to 20 µL/min, set the probe temperature at 300 °C, needle voltage at 3 KV, nebulizer gas at 65 psi, and use acetonitrile as a desolvation solvent for anionic applications (isopropyl alcohol for cationic applications) at the same flow rate as the capillary IC. For this application, the source parameters are optimized for the best sensitivity of bisphosphonates, and are listed as follows: probe temperature at 300 °C, needle voltage at 3.5 KV, nitrogen gas at 65 psi, and acetonitrile at 20 µL/min.

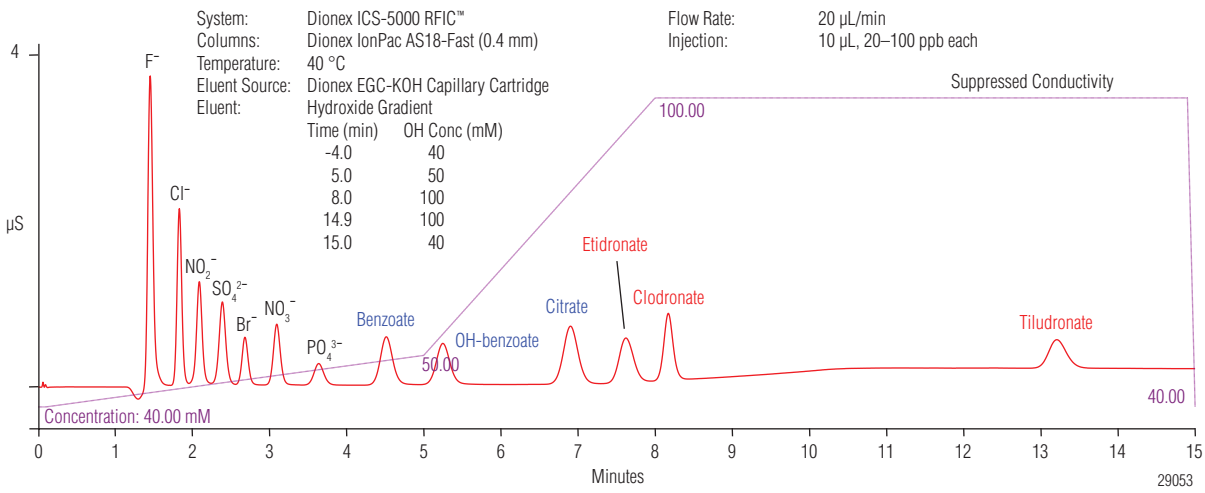



Figure 1. Total resolution of bisphosphonates, excipients and anions

All target analytes predominately show deprotonated molecular ions $[M-H]^-$ in negative polarity, and the respective deprotonated molecular ions were used in the SIM scans for quantitation. As shown in the full scan spectra in Figure 2, the observed pseudomolecular ions for etidronate, clodronate and tiludronate were 205, 243 and 317 *m/z*, respectively. Figure 2 also shows the observed isotopic peaks for clodronate and tiludronate. Matching the observed and theoretical isotope patterns can assist in compound identification or confirmation. The cone voltage of SIM scans was optimized and set at 55 volts, and each SIM scan had a span of 0.3 amu. The details of timed SIM scan events are shown in Table 1. Figure 3 shows the SIM chromatograms of target analytes under optimized conditions, each analyte selectively detected as seen by the single peak in each monitored SIM channel.

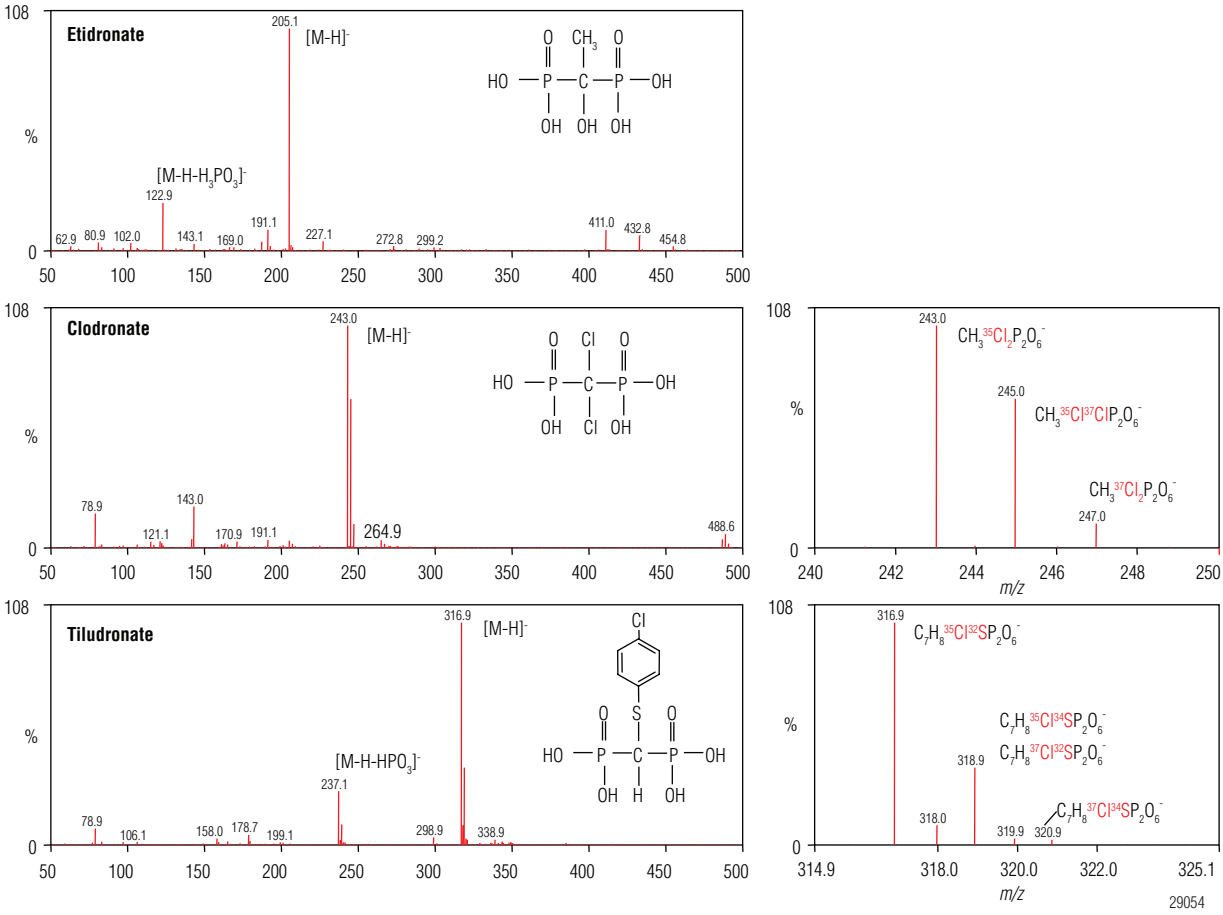
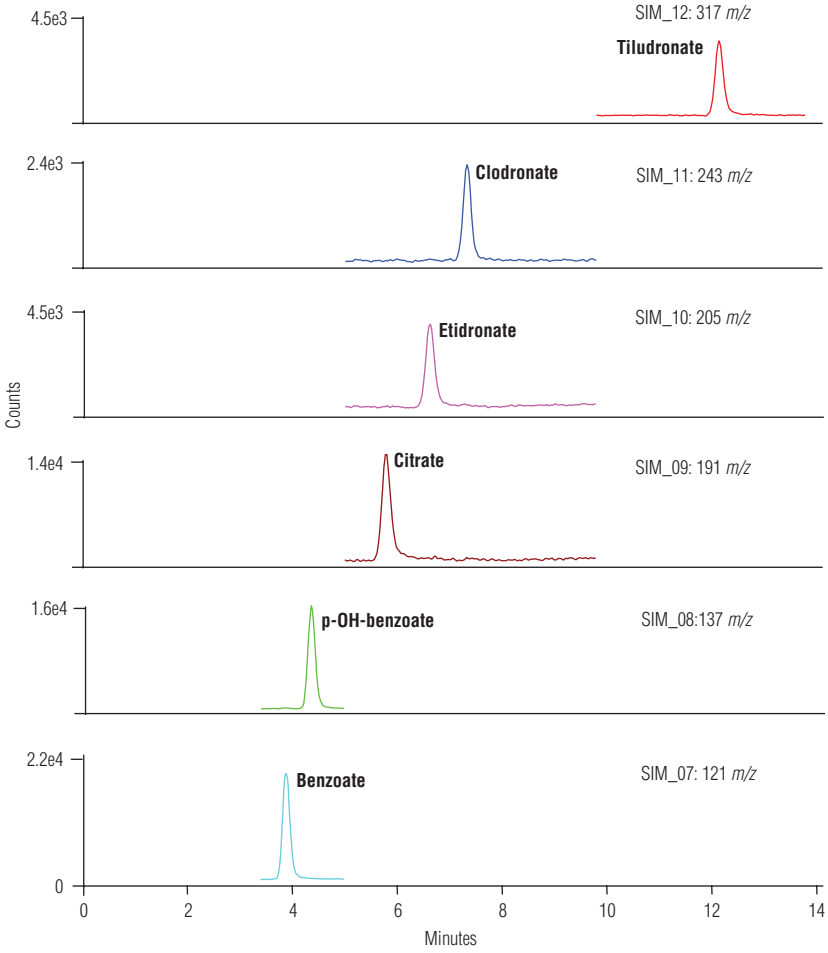



Figure 2. MS Spectra of three bisphosphonate pharmaceuticals




**Chromatographic Conditions**

| | |
|---|---|
| System: | Dionex ICS-5000 RFIC |
| Columns: | Dionex IonPac AS18-Fast (0.4 mm) |
| Temperature: | 40 °C |
| Mobile Phase: | Hydroxide from Dionex EGC-KOH Capillary Cartridge |

| Time (min) | OH Conc (mM) |
|---|---|
| -4.0 | 40 |
| 5.0 | 50 |
| 8.0 | 100 |
| 14.9 | 100 |
| 15.0 | 40 |

| | |
|---|---|
| Flow Rate: | 20 µL/min |
| Injection: | 0.2 ng in 2 µL |

**MS Conditions**

| | |
|---|---|
| System: | Thermo Scientific MSQ Plus Mass Spectrometric Detector |
| Interface: | Electrospray ionization (ESI) |
| Probe Temp: | 300 °C |
| Needle Voltage: | 3500 V |
| Solvent: | 20 µL/min acetonitrile |
| Nebulizer Gas: | Nitrogen at 65 psi |
| Detection Mode: | Selected ion monitoring (SIM) |

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Figure 3. SIM chromatograms of bisphosphonates and excipients

## Method Performance

Method performance was evaluated against quality parameters such as calibration range, correlation of determination, precision, accuracy, detection limits, and recovery. Additionally, this method was also used to quantify the target analytes in prescription tablets.

Calibration curves were generated from calibration standards with concentration from lower limit of quantification (LLOQ) to 500 ppb. The LLOQ was determined as the lowest concentration in prepared calibration standards that consistently demonstrated a signal-to-noise ratio (S/N) greater than 10 and within 20% bias of quantitation precision and accuracy. The LLOQ of the three excipients was observed at 5 ppb (10 pg injection) and at 50 ppb (100 pg injection) for the three bisphosphonates. The coefficient of determination ($r^2$) for each analyte was observed at greater than 0.99 with linear or quadratic fit and 1/x weighting factor. The method detection limit (MDL) was calculated by $MDL = S \times t_{99\%,\, n=5}$ where $S$ is the standard deviation and $t$ is the Student's $t$ at 99% confidence interval. The standard deviation was obtained from five replicate injections of 10 ppb (excipients) or 50 ppb standard (bisphosphonates). The MDL was observed in the range from 1.20 ppb (*p*-hydroxybenzoate) to 15.5 ppb (clodronate). Results for above evaluations are listed in Table 2. The precision and accuracy were evaluated at 50 ppb and 500 ppb and the results are listed in Table 3. The precision was addressed by % RSD of three replicate assays and was observed in the range from 0.76 (citrate at 500 ppb) to 7.07 (*p*-hydroxybenzoate at 500 ppb). The accuracy was calculated by *Observed Amount/Specified Amount* × *100%* and was observed in the range from 83% (tiludronate at 50 ppb) to 108% (benzoate at 50 ppb).

This method was applied to the determination of etidronate in a prescription 200 mg etidronate disodium tablet. The sample preparation procedure was as described above. The tablets were quantified at 273 mg/tablet and the deviation was caused by the unknown number of water molecules in etidronate disodium hydrate standard used here, which was treated as anhydrous standard. This tablet sample was used to evaluate method recovery by spiking 100 ppb of each target analyte, and the result is listed in Table 3. The recovery was observed in the range from 89.5% (benzoate) to 134% (clodronate). The deviation of recovery from 100% can be explained by the different extent of matrix effect on the observed MS responses for IS and target analyte. This deviation could be corrected by using isotope-labeled analogues of each target analyte, as excellent recovery was observed for citrate due to the use of citrate-$d_4$ as internal standard.

Table 2. Calibration range, precision, and MDL

| Analyte | Calibration Range | $r^2$ | Fit | % RSD[a] (n = 5) | MDL[b] |
|---|---|---|---|---|---|
| Benzoate | 5–500 | 0.9994 | Quadratic | 8.00 | 2.48 |
| p-Hydroxybenzoate | 5–500 | 0.9998 | Quadratic | 5.31 | 1.20 |
| Citrate | 5–500 | 0.9997 | Linear | 3.82 | 1.31 |
| Etidronate | 50–500 | 0.9978 | Quadratic | 5.33 | 9.36 |
| Clodronate | 50–500 | 0.9970 | Quadratic | 10.28 | 15.50 |
| Tiludronate | 50–500 | 0.9957 | Quadratic | 4.51 | 7.19 |

a: % RSD calculated based on 20 pg injection for benzoate, p-hydroxybenzoate and citrate; 100 pg injection for bisphosphonates

b: Calculated as MDL = S × $t_{99\%, n=5}$ where S is the standard deviation and t is the Student's *t* at 99% confidence interval

Table 3. Accuracy, precision and recovery

| Analyte | 50 ppb (n = 3) | | | 200 ppb (n = 3) | | | Original | Observed | % Recovery* |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | % RSD | % Accuracy | Mean | % RSD | % Accuracy | | | |
| Benzoate | 54.2 | 2.75 | 108 | 499 | 4.71 | 99.9 | ND | 89.5 | 89.5 |
| p-Hydroxybenzoate | 52.4 | 1.53 | 105 | 499 | 7.07 | 99.9 | ND | 93.5 | 93.5 |
| Citrate | 49.4 | 2.58 | 98.7 | 497 | 0.76 | 99.5 | ND | 102 | 102 |
| Etidronate | 43.4 | 4.27 | 86.8 | 498 | 2.18 | 99.5 | 424 | 542 | 117 |
| Clodronate | 44.8 | 4.30 | 89.7 | 498 | 1.05 | 99.6 | ND | 134 | 134 |
| Tiludronate | 41.5 | 3.26 | 83.0 | 497 | 1.28 | 99.4 | ND | 121 | 121 |

Unit shown in ppb

* Recovery calculated based on [observed amount (original sample + 100 ppb spiked each analyte) – original amount]/100 × 100%

## Conclusion

This study described a capillary IC-MS method for the simultaneous quantitation of three bisphosphonate pharmaceuticals (etidronate, clodronate and tiludronate) and three commonly used excipients (benzoate, hydroxybenzoate and citrate). Sensitive and selective quantitation can be achieved at as low a level as 5 ppb for excipients and 50 ppb for bisphosphonates using SIM acquisition within a 14 min run time. This configuration also provides confirmative information, such as molecular ions and isotope patterns for identity confirmation. This method was successfully applied for the analysis of etidronate disodium tablet samples.

## References


1. Elomaa, I.; Blomqvist, C.; Porkka, L.; Holmstrom, T.; Taube, T.; Lamberg-Allardt, C.; Borgström, G. H. Diphosphonates for Osteolytic Metastases. *Lancet* **1985,** *325* (8438), 1155–1156.
2. Stevenson, M.; Jones, M. L.; Nigri, E. D.; Brewer, N.; Davis, S.; Oakley, J. A Systematic Review and Economic Evaluation of Alendronate, Etidronate, Risedronate, Raloxifene and Teriparatide for the Prevention and Treatment of Postmenopausal Osteoporosis. *Health Tech. Assess.* **2005,** *9* (22), 1–160.
3. Kline, W. F.; Matuszewski, B. K.; Bayne, W. F. Determination of 4-Amino-1-Hydroxybutane-1,1-Bisphosphonic Acid in Urine by Automated Pre-Column Derivatization with 2,3-Naphthalene Dicarboxyaldehyde and High-Performance Liquid Chromatography with Fluorescence Detection. *J. Chromatogr., B: Biomed. Sci. Appl.* **1990,** *534,* 139–149.
4. Tarcomnicu, I.; Silvestro, L.; Savu, S. R.; Gherase, A.; Dulea, C. Development and Application of a High-Performance Liquid Chromatography-Mass Spectrometry Method to Determine Alendronate in Human Urine. *J. Chromatogr., A* **2007,** *1160* (1–2), 21–33.
5. Jiang, Y.; Xie, Z. Determination of Ibandronate and Its Degradation Products by Ion-Pair RP LC with Evaporative Light-Scattering Detection. *Chromatographia* **2005,** *62* (5–6), 257–261.
6. Taylor, G. E. Determination of Impurities in Clodronic Acid by Anion-Exchange Chromatography. *J. Chromatogr., A* **1997,** *770* (1–2), 261–271.
7. Tsai, E. W.; Ip, D. P.; Brooks, M. A. Determination of Alendronate in Pharmaceutical Dosage Formulations by Ion Chromatography with Conductivity Detection. *J. Chromatogr.* **1992,** *596* (2), 217–224.
8. Sirén, H.; Määttänen, A.; Riekkola, M.-L. Determination of Small Anions by Capillary Electrophoresis Using Indirect UV Detection with Sulphonated Nitrosonaphthol Dyes. *J. Chromatogr., A* **1997,** *767* (1–2), 293–301.
9. Perjesi, P.; Kim, T.; Zharikova, A. D.; Li, X. X.; Ramesh, T.; Ramasubbu, J.; Prokai, L. Determination of Clodronate Content in Liposomal Formulation by Capillary Zone Electrophoresis. *J. Pharm. Biomed. Anal.* **2003,** *31* (5), 929–935.
10. Muntoni, E.; Canaparo, R.; Della Pepa, C.; Serpe, L.; Casale, F.; Barbera, S.; Romano, P.; Zara, G. P.; Eandi, M. Determination of Disodium Clodronate In Human Plasma and Urine Using Gas-Chromatography-Nitrogen-Phosphorous Detections: Validation and Application in Pharmacokinetic Study. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2004,** *799* (1), 133–139.
11. Leis, H. J.; Fauler, G.; Windischhofer, W. Use of 18O3-Clodronate as an Internal Standard for the Quantitative Analysis of Clodronate in Human Plasma by Gas Chromatography/Electron Ionisation Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2004,** *18* (23), 2781–2784.
12. Zacharis, C.; Tzanavaras, P. Determination of Bisphosphonate Active Pharmaceutical Ingredients in Pharmaceuticals and Biological Material: A Review of Analytical Methods. *J. Pharm. Biomed. Anal.* **2008,** *48* (3), 483–496.
13. Thermo Scientific Technical Note 90: Mass Sensitivity of Capillary IC Systems Explained. LPN 2649, Sunnyvale, CA, 2011. [Online] https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/TN-90-Mass-Sensitivity-of-Capillary-IC-Systems-Explained.pdf (accessed Nov. 10, 2017).
14. Abian, J.; Oosterkamp, A. J.; Gelpí, E. Comparison of Conventional, Narrow-Bore and Capillary Liquid Chromatography/Mass Spectrometry for Electrospray Ionization Mass Spectrometry: Practical Considerations. *J. Mass Spectrom.* **1999,** *34* (4), 244–254.
15. Wang, J.; Schnute, W. C. Capillary Ion Chromatography Mass Spectrometry: Instrumentation and Applications. 59th ASMS Conference on Mass Spectrometry and Allied Topics, Denver, CO, 2011.


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