Quantitative Determination of Bisphosphonate Pharmaceuticals and Excipients by Capillary Ion Chromatography Mass Spectrometry

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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 analysis of bisphosphonates 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 quality control, 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 spectrometric detection (MS) would be a sensitive approach but the application to bisphosphonate analysis is limited due to the obvious incompatibility of the ion paring agent used in the mobile phase.[12]

Here, the authors present a quantitative method for the direct analysis of bisphosphonates and excipients in prescription tablets using capillary IC (Cap IC) with conductivity and mass spectrometric detection. A Thermo Scientific Dionex IonPac™ AS18 capillary anion-exchange column was 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 the 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 was used to ensure quantitation accuracy.

Equipment

Thermo Scientific Dionex ICS-5000 capillary ion chromatography (Cap IC) system with Eluent Generation (EG)

Thermo Scientific MSQ Plus™ single quadrupole mass spectrometer

AXP-MS Auxiliary pump (×2)

Thermo Scientific Dionex Chromeleon™ Chromatography Data System (CDS) version 6.8 SR10

Xcalibur™ 2.0.7 with MSQ 2.0 SP1

Chromatographic Conditions

System:	Dionex ICS-5000 Capillary Ion Chromatography with Eluent Generation (EG)
Column:	Dionex IonPac AS18/AG18-Fast (0.4 mm i.d.)
Column Temperature:	40 °C
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:	EGC-KOH (Capillary) Cartridge
Flow Rate:	20 µL/min
Injection:	2 µL
Detection:	1st: Suppressed Conductivity with Thermo Scientific Dionex ACES™300 Anion Capillary Electrolytic Suppressor (external water mode, 50 µL/min deionized water delivered by AXP-MS pump) 2nd: MSQ Plus single quadrupole mass spectrometer

Mass Spectrometric Conditions

System:	MSQ Plus single quadrupole mass spectrometer
Interface:	Capillary Electrospray Ionization (ESI) with negative polarity
Probe Temperature:	300 °C
Needle Voltage:	3500 V
Desolvation Solvent:	20 µL/min acetonitrile delivered by an 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 Details of SIM events are shown in Table 1.

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

Reagents and Chemicals

All chemical standard chemicals were purchased from Sigma-Aldrich unless noted.
Etidronate disodium hydrate (P5248)
Clodronate disodium (D4434)
Tiludronate disodium hydrate (T4580)
Benzoic acid sodium salt (B3375)
p-Hydroxybenzoic acid (H5376)
Citric acid (27788)
Isotope labeled internal standard citric acid-d_4 (C/D/N isotopes, D-3745)
Deionized (DI) water with 18.2 MΩ-cm resistivity (Millipore water station)
Acetonitrile (HPLC or better grade)

Standard Preparation

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

IS stock solution was also prepared at 1000 ppm in DI water and then diluted to 10 ppm to prepare calibration standards and spike unknown samples.

Calibration standards were prepared 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

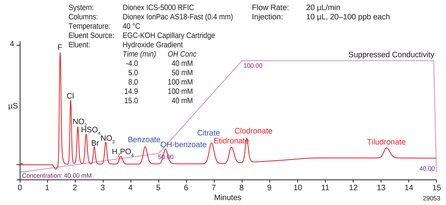
Etidronate Disodium 200 mg Tablets (Tablet Samples) were supplied by a customer. Each tablet was weighed individually and an average was calculated at 0.346 g/tablet. Tablet Samples were ground into fine powder form and three subsamples (10~15 mg each) were weighed to the nearest 0.01 mg and dissolved in DI water to the concentration of 1.0 mg sample per mL DI water. Each solution was sonicated in a water bath at room temperature for 30 min and filtered through a 25 mm 0.2 µm PES syringe filter and 5 µL of each filtrate was diluted to 10 mL with DI water then injected for analysis and quantitation.

Results and Discussion

Chromatography

Chromatographic methods have been used extensively for analysis of bisphosphonates. Among the reported chromatographic methods included are reversed-phase LC, ion-paring LC, IC, Capillary electrophoresis (CE), and GC. IC is an obvious method choice because of its ease of configuration, avoidance of derivatization, sensitivity of detection using suppressed conductivity for charged analytes, and the capability for simultaneous determination of impurities such as phosphate and other anionic species. For these reasons, IC was selected as the chromatography method of choice in this study. A Dionex ICS-5000 Cap IC system was used here because Cap IC offers improved sensitivity with injection of the same or less amount when compared with standard IC,[13] and better sensitivity when coupled with an ESI interface to a mass spectrometric detector.[14] A hydroxide-selective anion-exchange column (Dionex IonPac AS18-Fast) was selected as the separation column 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 seven common anions. The Dionex IonPac AS18-Fast 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 eluted as early peaks, with phosphate being the latest. Phosphate may be a targeted impurity in a regulated environment, e.g. QA/QC laboratories, and can be easily quantified as it is 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.

FIGURE 1. Total resolution of bisphosphonates, excipients and anions.



Mass Spectrometry

ESI is the most commonly used interface to couple IC-MS due to its suitability for polar and charged analytes over other atmospheric pressure ionization techniques such as 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 nano flow (<1 µL/min) ranges. The Cap IC system features a flow rate range from 10 to 50 µL/min thus requiring modification and re-optimization of existing ESI interfaces.[15] Here, a standard Thermo Scientific MSQ Plus ESI probe was modified using customized parts including a smaller 34 gauge internal diameter (i.d.) metal capillary needle (304SS, 34 gauge, 104.5 mm, Hamilton Company), and a tubing sleeve (F-328, 0.007 inch ID, 1/16 inch i.d., IDEX) to replace the supplied PEEK® tube insert. An ESI probe with the low-flow parts was assembled following standard ESI probe rebuild procedures. The modified ESI interface showed 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. The authors recommend a general condition which serves as a starting point for optimization: when Cap IC is operating at 10~20 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 (use isopropyl alcohol for cationic applications) at the same flow rate as the Cap 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.

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 pseudo-molecular 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 was selectively detected, seen as the single peak in each monitored SIM channel.

FIGURE 2. MS Spectra of three bisphosphonate pharmaceuticals.

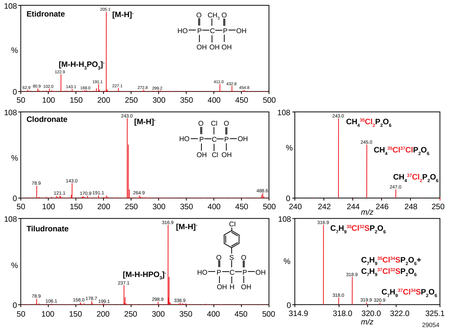
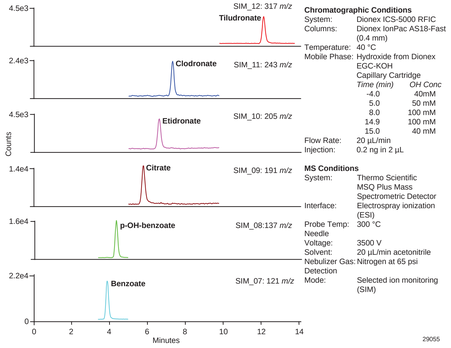


FIGURE 3. SIM chromatograms of bisphosphonates and excipients.



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*
-4.0	40mM
5.0	50 mM
8.0	100 mM
14.9	100 mM
15.0	40 mM

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)

Method Performance

Method performance was evaluated against quality parameters such as calibration range, correlation of determination, precision, accuracy, detection limits, and recovery. In addition, 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 × $t_{99\%,\ n=5}$ where S is the standard deviation and *t* is the Student's *t* at 99% confidence interval. The standard deviation was obtained from 5 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 3 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).

Table 2. Calibration and 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	15.5
Tiludronate	50–500	0.9957	Quadratic	4.51	7.19

[a] %RSD was calculated based on 20 pg injection for benzoate, *p*-hydroxybenzoate and citrate; and 100 pg injection for bisphosphonates.
[b] MDL was calculated by MDL = S × $t_{99\%,\ n=5}$ where S is the standard deviation and *t* is the Student's *t* at 99% confidence interval.

This method was applied to the determination of etidronate in a prescription 200 mg etidronate disodium tablet. The sample preparation procedure was as described in that section. 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 in this study which was treated as anhydrous standard. This tablet sample was used to evaluate method recovery by spiking with 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 effects on the observed MS responses for IS and target analyte. This deviation may 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 3. Accuracy, Precision and Recovery

Analyte	50 ppb (n=3)			200 ppb (n=3)			Original	Observed	% Recovery[c]
	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 this table: ppb.
[c]Recovery was calculated based on (observed amount (original sample+100 ppb spiked each analyte) – original amount)/100 × 100%

Conclusion

This work describes a Cap 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 as 5 ppb level for excipients and 50 ppb for bisphosphonates using SIM acquisition within a 14 minute 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.

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