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Application Note

LC-MS Analysis of Underivatized Bisphosphonate Drugs Using Mixed-Mode Reversed-Phase/Anion-Exchange Chromatography

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Abstract

Bisphosphonates (BPs) are an important class of drugs used for the treatment of bone density loss from natural aging and from human disease. Many of the methods previously reported for these polar, acidic drugs require derivatization for analysis by LC-MS (liquid chromatography-mass spectrometry). In this application note, a 6-minute reversed-phase LC-MS method was developed for the detection of four bisphosphonate drugs without requiring a derivatization step.

For this work, a mixed-mode reversed-phase/anion-exchange column was employed, specifically the Atlantis™ Premier BEH™ C_{18} AX Column. The analytical data demonstrated good retention and resolution of four bisphosphonate drugs. Peak analytical data was reproducible across 6 replicate injections. Additionally, the column's MaxPeak™ High-Performance Surfaces (HPS) hardware provided higher peak areas when compared to a stainless-steel counterpart by mitigating non-specific adsorption of the analytes to the column hardware.

Benefits

- A fast mixed-mode LC-MS method is reported for bisphosphonates that doesn't require sample derivatization
- The Atlantis Premier BEH C_{18} AX Column resolved most bisphosphonates with good retention and peak shape
- The Atlantis Premier Column with HPS demonstrated higher peak areas and improved area precision compared to a conventional stainless-steel column

Introduction

BPs have played a major role in the mitigation of bone density loss caused by diseases such as osteoporosis, Paget's Disease of bone, and bone complications related to malignancy.[1] Specifically, BPs adsorb to bone surfaces and, once ingested and metabolized by osteoclastic (bone breaking) cells, inhibit their activity by blocking several key enzymatic pathways. Although patents for some bisphosphonates have ended or are about to end, their development as generic drugs is predicted to continue. Additionally, newer generation BPs called designer bisphosphonates are being synthesized with defined properties in terms of mineral affinity and actions on relevant enzymatic pathways.[1] Moreover, exciting new applications in medicine such as the treatment of inflammatory bone loss, orthopedic implant fixation, and anti-parasitic effects are being investigated.

BPs are a challenge to analyze by liquid chromatography. They are highly polar acidic molecules that are not well retained on reversed-phased columns. Most of them are not readily detectable using UV absorbance. Additionally, BPs can react with metal ions to form adducts and multiple-charged substances that are difficult to detect by mass spectrometry.[2] As a result, most HPLC methods for these drugs require a sample derivatization step to improve their retention and detection. This is especially needed for the analysis of BPs in complex biological matrices to help improve method sensitivity.[2]

In this work, a mixed-mode reversed-phase /anion-exchange (RP/AX) LC-MS method was developed to retain and separate bisphosphonate drugs using an Atlantis Premier BEH C_{18} AX Column. This column combines the functionality of a C_{18} stationary phase with anion exchange (AX) capability to retain highly polar acidic compounds. This column also employs an HPS to prevent undesired interaction with the column hardware, increasing sensitivity.

Experimental

Sample Description

Four bisphosphonate drugs were prepared as individual stock standards at a concentration of 1 mg/mL in 90:10 (v/v) water and acetonitrile. They were then combined to create a bisphosphonate standard mix. All standards were stored at 4 ºC in polypropylene containers and were aliquoted into Waters™ QuanRecovery™ Vials for analysis by LC-MS.

Method Conditions

LC Conditions

LC system:	ACQUITY™ UPLC™ H-Class Plus System with HPS[a] tubing
Mobile phase A:	Water
Mobile phase C:	Methanol
Mobile phase D:	200 mM Ammonium Formate, pH 3
Washes/diluent:	90:10 (v/v) Water and Acetonitrile
Gradient:	See below
Flow rate:	0.4 mL/min
Injection volume:	2 µL
Columns:	Atlantis Premier BEH C_{18} AX 1.7 µm, 2.1 x 50 mm (p/n: 186009407)

	Atlantis BEH C_{18} AX 1.7 µm, 2.1 x 50 mm stainless steel hardware (packed in-house)
Column temperature:	40 °C
Cone, capillary voltage:	15 V, 0.8 kV
Scan mode:	ESI negative, SIR

Gradient Table

Time	%A	%C	%D
Initial	100	0	0
3.40	84	10	6
4.50	84	10	6
5.00	100	0	0
6.00	100	0	0

MS Conditions

MS system:	ACQUITY QDa™ Detector
Detection mode:	ESI-
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Scan mode:	ESI negative, SIR

Compound	*m/z*
Alendronic Acid	249.10
Zoledronic Acid	272.09
Risedronic Acid	283.11
Pamidronic Acid	235.07

Table 1. Mass-to-charge ratios for the target bisphosphonates.

Data Management

Chromatography software:	Empower™ Chromatography Data System (CDS)

Results and Discussion

Considering the structures of the analytes (Figure 1) and the results of preliminary column screening, it was determined that a mixed-mode RP/AX column provides the best retention. These molecules are ionized above about pH 2, as the negative log of their first and second acid dissociation constants (pK_{a1}, pK_{a2}) are generally 2 or lower.[3] While the amine substituents present in most of these drugs are protonated under acidic conditions, the presence of two phosphonate groups means that they mostly exist as anions. Consequently, they benefit from a column with anion-exchange functionality, such as Waters Atlantis Premier BEH C_{18} AX Column. The stationary phase in this column is bonded with both C_{18} and tertiary alkylamine groups, creating a positive surface charge below pH 8.[4] The BEH particles used for this stationary phase have a smaller pore size than other BEH stationary phases (95 Å compared to 130 Å), providing increased retention due to a 50% higher surface area.[4] Additionally, this column is usable over a wide pH range (2–10), so a variety of buffers at various pH values can be tested for method optimization.

The MaxPeak Premier Technology used in Atlantis Premier BEH C_{18} AX Columns was specifically designed to

mitigate unwanted interactions of analytes with the column hardware. These interactions can have a detrimental impact on separations, affecting peak shape and peak areas and causing high variability.[6] This is especially true for acidic analytes that can interact with the oxide layer of the stainless-steel hardware.[7] In LC-MS, an additional issue is that some analytes can form adducts with metal ions released from the hardware, decreasing the intensity of the target ions. It has been demonstrated in the literature that the analytical recovery and thus sensitivity for certain metal-sensitive analytes such as citric acid cycle intermediates can be improved with MaxPeak Premier Technology.[8]

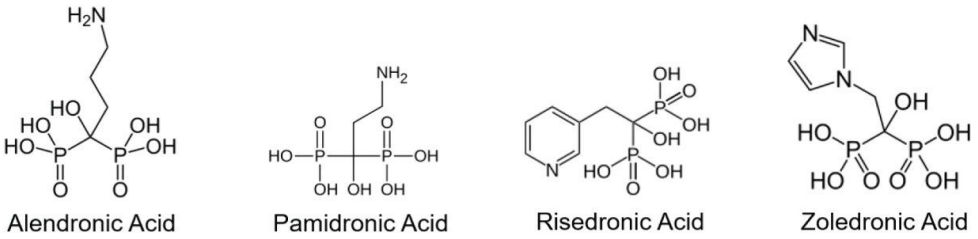

Figure 1. Molecular structures of the bisphosphonate drugs.

The ability of MaxPeak Premier Technology to mitigate non-specific adsorption of the bisphosphonates was evaluated. Six replicate injections of the bisphosphonate standard mix were made on an Atlantis Premier BEH C_{18} AX Column and were compared to the chromatography of 6 injections on a stainless-steel column packed with the same stationary phase (Figure 2). The Atlantis Premier Column showed much higher area counts. For zoledronic acid, for example (component 3), the area count for the first injection on the Atlantis Premier Column is around double the area for the stainless-steel column. Additionally, as shown in Figure 3 and Table 2, the Atlantis Premier Columns gave consistently higher area counts for all four bisphosphonates. It is interesting to

note that the plots show low area counts in the early injections on the stainless-steel column that slowly rise over subsequent injections. This demonstrates the lengthy sample conditioning effect that is not necessary when using the Atlantis Premier Column.

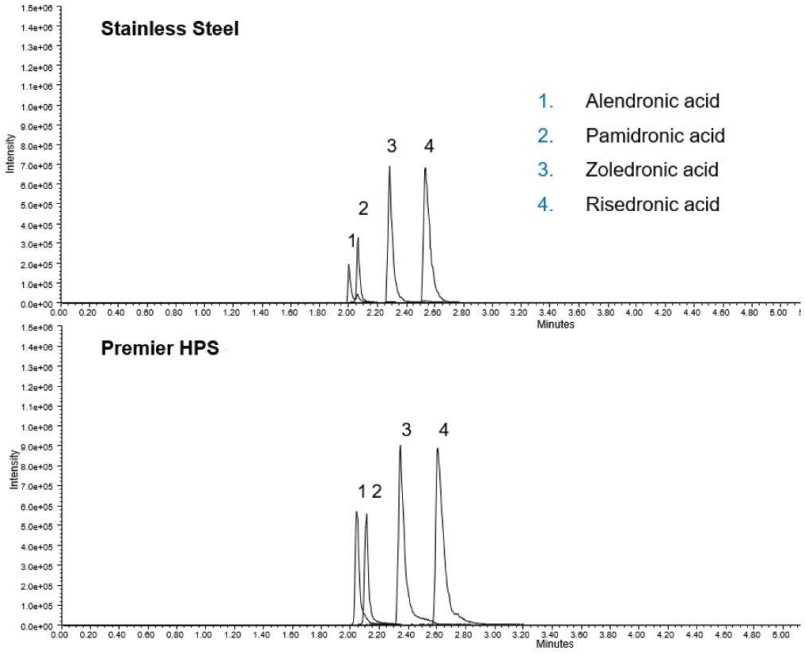

Figure 2. Representative chromatogram of the first injection of the bisphosphonate standard on the stainless steel Atlantis BEH C_{18} AX Column and the Atlantis Premier BEH C_{18} AX Column.

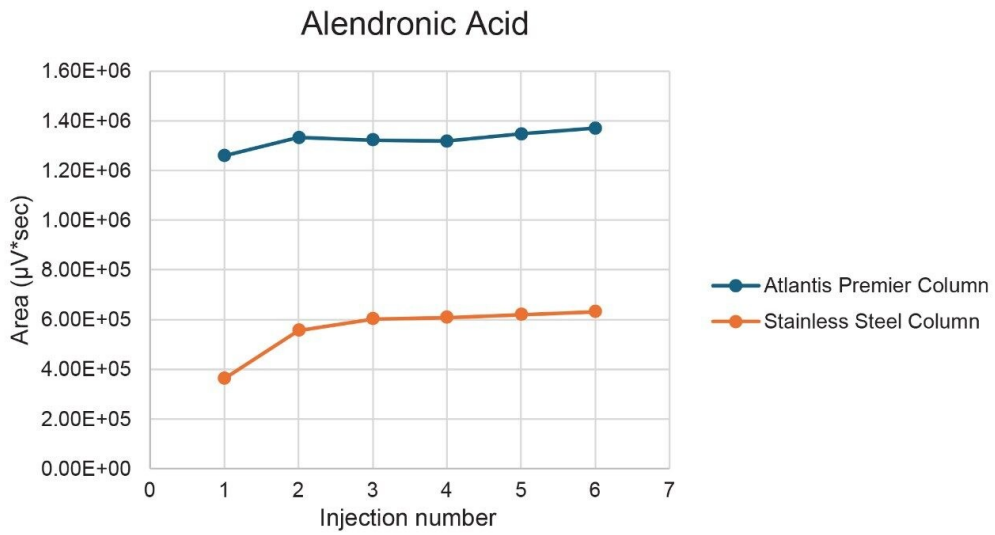

Figure 3. Area count comparison for the Atlantis Premier BEH C_{18} AX Column versus the stainless steel Atlantis BEH C_{18} AX Column.

Lastly, there is also a marked difference in area count precision, with lower %RSDs for the column with the high-performance surface (Table 2). This provides evidence for decreased interactions of the analytes with the column hardware and removes uncertainty in the data.

Area Counts (µV*sec)								
	Alendronic Acid		Pamidronic Acid		Zoledronic Acid		Risedronic Acid	
Replicate	HPS	SS	HPS	SS	HPS	SS	HPS	SS
1	1.26E+06	3.62E+05	1.14E+06	5.24E+05	2.88E+06	1.58E+06	3.64E+06	2.22E+06
2	1.33E+06	5.57E+05	1.15E+06	6.50E+05	2.90E+06	1.69E+06	3.72E+06	2.35E+06
3	1.32E+06	6.02E+05	1.14E+06	7.00E+05	2.82E+06	1.77E+06	3.70E+06	2.48E+06
4	1.32E+06	6.08E+05	1.15E+06	7.19E+05	2.88E+06	1.82E+06	3.69E+06	2.58E+06
5	1.35E+06	6.20E+05	1.16E+06	7.11E+05	2.90E+06	1.89E+06	3.73E+06	2.65E+06
6	1.37E+06	6.31E+05	1.17E+06	7.41E+05	2.89E+06	1.81E+06	3.83E+06	2.72E+06
Mean	1.33E+06	5.63E+05	1.15E+06	6.74E+05	2.88E+06	1.76E+06	3.72E+06	2.50E+06
%RSD	2.82	18.08	0.84	11.78	1.05	6.17	1.73	7.56

Table 2. Area count precision data for 6 replicate injections of the bisphosphonate standard. HPS = Atlantis Premier Column, SS = stainless steel column, %RSD = percent relative standard deviation.

Conclusion

Bisphosphonates can be challenging to analyze chromatographically due to their polar acidic nature. Many of the analytical methods for these drugs reported in the literature require a sample derivatization step. Mixed-mode reversed-phase/anion-exchange HPLC provides an approach for retaining and separating these analytes without derivatization. It also eliminates the need for ion-pairing agents and is compatible with mass spectrometry detection.

In this work, the development of a fast mixed-mode RP/AX LC-MS method for the analysis of four common bisphosphonate drugs is described that provides good retention and separation. The MaxPeak HPS Technology used in Atlantis Premier BEH C_{18} AX Columns was shown to successfully mitigate non-specific adsorption of the bisphosphonates on the column hardware, providing higher analyte peak areas and improved peak area precision compared to a stainless-steel version. This eliminates the need for lengthy column conditioning steps such as the use of chelators in the mobile phase, ion pairing agents, or sacrificial analyte injections.

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