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Insights into the Mechanism of Separation of Bisphosphonates by Zwitterionic Hydrophilic Interaction Liquid Chromatography: Application to the Quantitation of Risedronate in Pharmaceuticals

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Abstract: Bisphosphonates are used to treat various skeletal disorders, as they modulate bone metabolism by inhibition of the osteoclast-mediated bone resorption. These compounds are both polar and ionic, and therefore, by using reversed phase liquid chromatography are eluted rapidly. Hydrophilic interaction liquid chromatography (HILIC) is an advantageous technique for the separation and analysis of polar molecules. As the elution order in HILIC is reversed to reversed phase liquid chromatography, a reasonable retention and selectivity for polar compounds is expected. In this work the retention mechanism of three bisphosponates, namely risedronate, tiludronate and zoledronate, was investigated under zwitterionic HILIC conditions. The key factors influencing the retention of the analytes on a zwitterionic ZIC®-pHILIC column (150.0 × 2.1 mm i.d., 200 Å, 3.5 μm) have been systematically investigated. It was found that apart from partition, electrostatic repulsions play an important role in the retention of bisphosphonates. Peak tailing of risedronate and zoledronate was improved by the addition of sodium pyrophosphate in the mobile phase. A zwitterionic hydrophilic interaction liquid chromatography-photodiode array (HILIC-PDA) method was further optimized and fully validated to quantitate risedronate in commercial film-coated tablets. The calibration curves for risedronate showed good linearity ($r \geq 0.9991$) within the calibration range tested. The intra- and inter-day coefficient of variation (CV) values was less than 0.6%, while the relative percentage error (%Er) was less than −2.3%. Accelerated stability studies of risedronate conducted under several degradation conditions including hydrolysis, oxidation and heat demonstrated the selectivity of the procedure. A short-run analysis of not more than 6 min allowed the analysis of large samples per day. The applicability of the method for the quantitation of risedronate was demonstrated via the analysis of commercial tablets containing this compound.

Keywords: bisphosphonates; risedronate; zoledronate; tiludronate; ZIC-HILIC; PDA; quantitation; tablets

1. Introduction

Bisphosphonates belong to a unique class of drugs which are chemically stable analogues of the inorganic pyrophosphate anion, a secondary product of various biochemical processes.

The concentration levels of pyrophosphate anion in blood are associated with the mechanism of bone calcification [1]. Like pyrophosphate, bisphosphonates have high affinity for bone mineral and bind strongly to hydroxyapatite calcium in the bone. The skeletal accumulation of bisphosphonates (on the skeleton) depends highly on the disposal of hydroxyapatite binding sites. Non-nitrogen containing bisphosphonates are accumulated into newly formed adenosine triphosphate (ATP) analogues and inhibit ATP-dependent processes, leading to osteoclast apoptosis [2]. Conversely, nitrogen containing bisphosphonates inhibit the action of the enzyme farnesyl pyrophosphate synthase (FPPS) enzyme, which is involved in the mevalonate pathway [3]. These drugs have become the therapy of choice for the management of various skeletal disorders such as several types of osteoporosis, hypercalcemia, Paget disease and malignancy metastatic to bone [4]. However, despite the well-recognized benefits of bisphosphonates, these drugs may cause also osteonecrosis of the jaw [5,6].

Bisphosphonates are both polar and ionic compounds and by using reversed phase liquid chromatography are eluted rapidly. The development of a chromatographic method for the analysis of bisphosphonates is a challenge for the analysts due to their high hydrophilicity. In addition to this, the lack of chromophores in some bisphosphonates structures necessitates the use of tedious and time-consuming derivatization procedures for their detection. A literature survey revealed that in most publications adequate retention of bisphosphonates is achieved by using ion-pairing agents in the mobile phase [7–10], anion-exchange chromatography [11] and in some cases pre-column [12] or post-column [13] derivatization procedures [14]. A fused core Ascentis Express HILIC column has been used to quantitate risedronate sodium in pharmaceuticals with PDA and tandem mass spectrometric detection [15]. Bisphosphonates in biological matrices have been quantified after their methylation with trimethylsilyl diazomethane by the use of liquid chromatography–mass spectrometric methods [16–21].

During the last twenty years, HILIC has been proved to be a promising technique for the analysis of polar substances. The separation mechanism in HILIC involves multiple factors, such as partitioning, normal phase/adsorption interactions, hydrogen bonding, reversed-phase and electrostatic interactions [22]. The significance of each of these mechanisms depends on the type of mobile phase and stationary phase that will be used. HILIC requires the use of highly organic mobile phases that contain an aprotic solvent (mainly acetonitrile) in combination with at least 3% of an aqueous salt solution and polar stationary phases so to facilitate chromatographic separation [23]. The major factors affecting retention in HILIC are the type of the stationary phase, the percentage content of water which is the strongest eluent, along with the concentration, pH and type of the aqueous solution of the salt [24]. Studies on the retention mechanism of compounds in HILIC are of great interest, since it is difficult to predict the effect of the operation parameters on the retention of substances. Bisphosphonates, due to their increased polarity, are perfect candidates to study their retention mechanism in HILIC. Up to now, only a limited number of publications have been reported for the analysis of bisphosphonates in HILIC [15]. This paper describes studies on the retention mechanism of two nitrogen-containing bisphosphonates, namely risedronate and zoledronate, and one non-nitrogen-containing bisphosphonate, namely tiludronate, on a polymeric zwitterion ZIC®-pHILIC column. It is the first time that zwitterionic hydrophilic interaction liquid chromatography is used to study the retention of bisphosphonates. The zwitterionic hydrophilic interaction liquid chromatography used in this work is a unique form of HILIC, which involved the use of substrates containing zwitterionic functional groups. The key factors influencing the chromatography of these analytes were systematically investigated. A HILIC stability-indicating assay method coupled with photodiode array detection was further optimized and validated to quantitate risedronate in commercial film-coated tablets. Accelerated stability studies of risedronate were also conducted under stress conditions to demonstrate the selectivity of the procedure. The applicability of the method for the quantitation of risedronate was finally proven via the analysis of commercial film-coated tablets containing risedronate as the active ingredient.

2. Materials and Methods

2.1. Chemicals and Reagents

Risedronate sodium salt, hydroxy-(1-hydroxy-1-phosphono-2-pyridin-3-ylethyl)phosphinate; sodium and tiludronate disodium salt, [(4-chlorophenyl)sulfanyl-[hydroxy(oxido) phosphoryl]methyl]-hydroxyphosphinate; disodium, were obtained from Sigma-Aldrich, Germany. Zoledronic acid monohydrate, (1-hydroxy-2-imidazol-1-yl-1-phosphonoethyl)phosphonic acid; hydrate, was obtained from TGI Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). All bisphosphonates were of pharmaceutical purity grade. Solvents of HPLC grade were obtained from E. Merck, Germany. Ammonium formate, ammonium acetate, acetic acid and sodium pyrophosphate were purchased from Acros Organics part of Thermo Fischer Scientific (Geel, Belgium). Water was deionized and further purified by means of a Merck Millipore Synergy UV system (Darmstadt, Germany). Kinesis KX hydrophilic polytetrafluoroethylene (PTFE) syringe filters (diameter 13 mm, pore size 0.22 μm) were purchased from Kinesis Ltd, Cambridge shire, UK.

Commercial film-coated tablet labelled to contain 35 mg risedronate sodium (equivalent to 32.5 mg risedronic acid). Inactive ingredients of the tablet core consist of crospovidone A, cellulose microcrystalline, magnesium stearate and lactose monohydrate. Inactive ingredients of the film coating consist of hypromellose, titanium dioxide E171, hydroxypropyl cellulose, macrogol, iron oxide red (E172), colloidal anhydrous silica and iron oxide yellow (E172).

2.2. Instrumentation

Experiments were performed on an HPLC-PDA system consisting of an auto sampler model Waters 717 plus, a constant temperature oven, an isocratic pump model Waters 1515 and a photodiode array detector model Waters 996 (Milford, MA, USA). Data acquisition and analysis was attained by the use of the Empower software (Milford, MA, USA). The analytes were detected over the wavelength range of 200 to 400 nm and the chromatograms were extracted at λ = 262 nm. Chromatography was performed by using a polymeric zwitterionic ZIC®-pHILIC analytical column (150.0 × 2.1 mm i.d., 200 Å, particle size 3.5 μm) (Merck Millipore, Darmstadt, Germany). Moreover, a guard column (20 × 2.1 mm, 3.5 μm) of the same packing material was used to prolong the column lifetime. During the method development, various mobile phases consisting of mixtures of acetonitrile and ammonium formate or ammonium acetate aqueous solutions were usedand the flow rate was set at 0.25 mL min^{-1}. Aqueous solutions of ammonium acetate were prepared freshly every day. For the quantitation of risedronate, the mobile phase consisted of 38% 9 mM ammonium acetate and 1 mM sodium pyrophosphate aqueous solution pH 8.8 in acetonitrile and pumped at a flow rate of 0.15 mL min^{-1}. It was filtered through a 0.22 μm Nylon-membrane filter, Membrane Solutions (Kent, WA, USA) and degassed under vacuum prior to use. Chromatography was performed at 40 ± 2 °C, with a chromatographic run time of 6 min; a 60 μL volume was injected into a 10 μL loop.

2.3. Statistical Analysis

Regression analysis was performed using IBM SPSS Statistics ver. 22, IBM software. The ionization state of each compound was estimated using ADME boxes ver. 3.0, Pharma Algorithms software.

2.4. Stock and Working Standard Solutions

Stock standard solutions of risedronate, zoledronate and tiludronate were prepared at 500 μg mL^{-1} in acetonitrile-water mixture (60:40, v/v). Stock standard solution of risedronate was prepared in duplicate for the calibration standards and the quality control samples. These solutions were stable for several weeks when stored at −17 °C for several months. The stock standard solutions were further diluted in acetonitrile to prepare working standard solutions at two concentration levels 5 and 10 μg mL^{-1} for each analyte. These solutions were used for the method development and were stored under refrigeration at 4 °C for two months.

Calibration standard solutions of risedronate were prepared in acetonitrile over the concentration range of 1.5 to 5 μg mL^{-1}. Quality control samples of risedronate were also prepared in acetonitrile at three concentration levels (1.5, 3.5 and 5 μg mL^{-1}). Calibration standard solutions and quality control samples were prepared freshly every day and remained stable throughout the analysis.

2.5. Assay Procedure for the Pharmaceutical Samples

To calculate the tablet weight, 10 tablets containing 35 mg of risedronate sodium were weighted and then pulverized. A portion of this powder, equivalent to 35 mg of risedronate sodium, was transferred into a 100 mL volumetric flask and diluted to volume with acetonitrile/water mixture (10:90, *v*/*v*). The mixture was sonicated for 10 min and then transferred into a 2 mL Eppendorf tube for centrifugation at 4.000× *g* and 25 °C for 10 min. The supernatant was then sonicated in an ultrasonic bath for additional 10 min and filtered through a PTFE hydrophilic syringe filter. A 100 μL aliquot of the filtrate was then transferred into a 10 mL volumetric flask and diluted to volume with acetonitrile prior to HILIC-PDA analysis.

2.6. Accelerated and Long-Term Stability Studies

Degradation studies were performed in risedronate under various stress conditions where degradation was stimulated by acidic or basic hydrolysis, oxidation and thermal degradation. Risedronate bulk substance was stressed under accelerated degradation conditions with 1.0 M HCl at 50 °C (± 2) for 10 days, 1.0 M potassium hydroxide (NaOH) at 50 °C (± 2) for 24 h and 3.0% *v*/*v* hydrogen peroxide (H_2O_2) at 25 °C (± 2) for 3 h. The concentration of risedronate bulk substance in the accelerated stability samples was 0.35 mg mL^{-1}. During each degradation experiment and at predetermined time intervals, appropriate aliquots were neutralized with base or acid, and analyzed according to the proposed method. The concentration of risedronate in the analyzed sample solution was 3.5 μg mL^{-1}.

Blistered tablets containing risedronate were stressed in long-term stability studies. Blistered tablets have been stored for 3 months at 50 °C (± 2) and 75% (± 2) relative humidity, and at 50 °C ± 2 °C and 15% (± 2) relative humidity. After the completion of each degradation treatment the samples were analyzed as described in the sample preparation procedure (Section 2.5).

3. Results and Discussion

3.1. Method Development

Bisphosphonates contain two phosphoric acid groups and are strongly polar and ionic compounds. The three bisphosphonates drugs studied in this work are divided into two groups: two nitrogen-containing compounds (risedronate and zoledronate) and one acidic compound (tiludronate). These compounds are poorly retained in the classical reversed phase analytical columns and their chromatographic analysis is challenging. The ZIC®-pHILIC analytical column used is a polymeric and zwitterionic sulfoalkylbetaine stationary phase. The functional group of this column consists of a sulfonic acid group (acidic), which was separated with a short alkyl spacer from a quaternary ammonium group (basic). In this zwitterionic stationary phase, the electrostatic forces of each charge were partly counterbalanced by the proximity of an ion with opposite charge. Though the accessibility to the positively charged quaternary ammonium groups was limited, the negatively charged sulfonic acid groups might be responsible for weak, but important, electrostatic interactions [25]. The studied bisphosphonates were retained adequately in this analytical column through hydrophilic interactions, even if they had the same charge with the sulfonic acid groups of the stationary phase. Electrostatic repulsions in HILIC were first described by Alpert [26] as electrostatic repulsion hydrophilic interaction chromatography (ERLIC). These kinds of interactions were of great interest and can be used to selectively antagonize the retention of analytes that normally would be best retained [27].

3.1.1. Effect of Chromatographic Parameters on the Bisphosphonates Retention

A one-variable-at-a-time approach was used to study the chromatography of bisphosphonates in the zwitterionic stationary phase. Mobile phases in HILIC typically contain high percentages of acetonitrile mixed with an aqueous salt solution. In this work, the mobile phase salts were limited to ammonium formate and ammonium acetate due to their good solubility in acetonitrile. The sulfonic acid groups of the stationary phase are responsible for weak electrostatic interactions that can be reduced by the addition of an aqueous salt solution. In preliminary experiments with a mobile phase containing 35% 10 mM ammonium formate water solution in acetonitrile, both nitrogen-containing bisphosphonates (risedronate and zoledronate) were not eluted, while tiludronate exhibited a broad asymmetrical peak. On the other hand, ammonium acetate improved the chromatography for all compounds. Consequently, ammonium acetate concentration was varied from 1 to 40 mM in mobile phases containing 35% Φ_{water}. The logarithm of retention factor (k) was used to evaluate retention of the analytes. Retention factor (k) is independent of column geometry and flow rate and was often used for reproducibility evaluation, and method validation [28].

Typical HILIC chromatograms illustrating the effect of the concentration of ammonium acetate on the retention time and the peak shape bisphosphonates are presented in Figure 1. In all of the ammonium acetate concentrations tested tiludronate exhibits good peak symmetry while tailing peaks are observed for both risedronate and zoledronate. Bisphosphonates as strong chelators are capable to interact with the metals of the liquid chromatographic (LC) system [10,29]. This binding affinity is greater in nitrogen-containing bisphosphonates where the one side chain of the molecule is a primary amino-group and allows a tridentate interaction [30]. By increasing ammonium acetate concentration, the elution of the analytes was delayed, leaving them more time to interact with the metals of the LC system; hence, peak tailing of nitrogen-containing bisphosphonates increased.

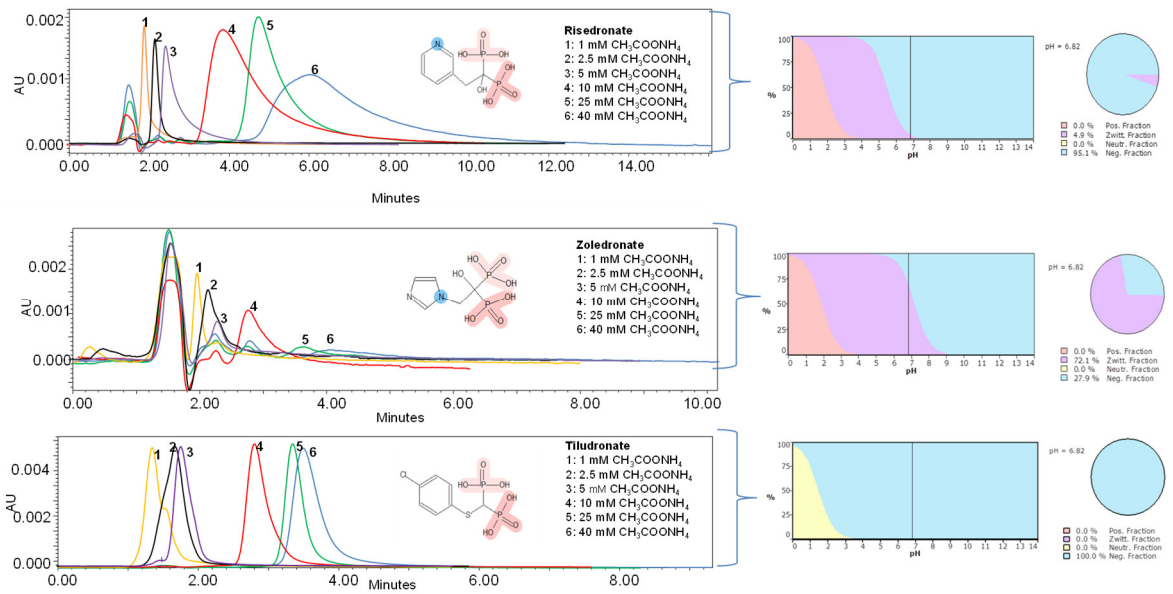

Figure 1. Typical HILIC chromatograms displaying the effect of ammonium acetate concentration on the peak shape and the retention of bisphosphonates, accompanied by diagrams of their ionization state at pH 6.8 as calculated ADME boxes ver. 3.0, Pharma Algorithms software. Chromatographic conditions: ZIC®-pHILIC analytical column, mobile phase: aqueous solution of ammonium acetate pH 6.8/acetonitrile (35:65, v/v), 0.25 mL min^{-1} flow rate and wavelength of detection at 262 nm.

As illustrated in Figure 2A, the retention of both the nitrogen-containing bisphosphonates (risedronate and zoledronate) and the negatively charged tiludronate increased by increasing ammonium acetate concentration due to the reduction of the electrostatic repulsions between the analytes and the stationary phase. From these experiments, we concluded that by using a 10 mM

ammonium acetate concentration all bisphosphonates are adequately retained and well separated from the solvent front.

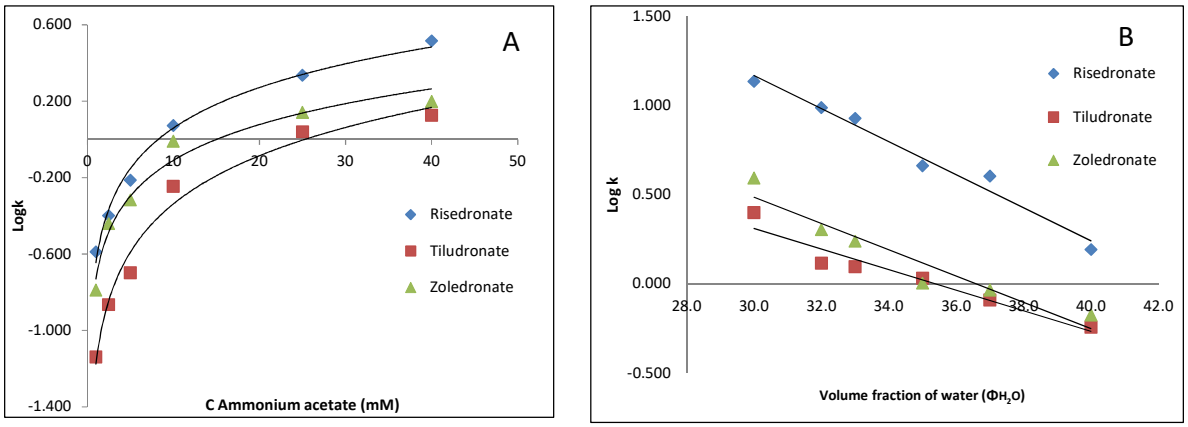

Figure 2. (**A**) Impact of the concentration of ammonium acetate (mM) on the log k. ZIC®-pHILIC column; mobile phase: acetonitrile/ammonium acetate aqueous solution pH 6.6 (65:35, *v*/*v*), (**B**) Impact of the percentage of water, Φ_{water}, on the log k. ZIC®-pHILIC column; mobile phase: acetonitrile/ammonium acetate aqueous solution pH 6.8 containing 3.5 mM ammonium acetate in whole monile phase.

In HILIC a minimum percentage of water, Φ_{water}, at 2% to 3% in the mobile phase is crucial for the creation of the water layer around the stationary phase. In mobile phases with high percentages of acetonitrile, the elution of polar compounds was increased, since the water interacts strongly with the polar stationary phase. To study the effect of Φ_{water} on the retention of bisphosphonates, the concentration of ammonium acetate in whole mobile phase stayed constant at 3.5 mM, while Φ_{water} varied from 30% to 40 %. As shown in Figure 2B the retention of all analytes decreases linearly with increasing Φ_{water}, implying partition as the dominant retention mechanism for bisphosphonates in HILIC.

The studies presented above indicate that both hydrophilic partition and secondary electrostatic interactions contribute to the retention of bisphosphonates on the ZIC®-pHILIC analytical column. Bisphosphonates are strong chelators and their interaction with the metals of the LC system causes serious peak tailing [29]. This binding affinity of bisphosphonates is greater in nitrogen-containing bisphosphonates, where the one side chain of the molecule is a primary amino-group and allows a tridentate interaction [30]. The presence of phosphate groups in the mobile phase can be critical for the analysis of bisphosphonates on a standard stainless steel LC system [31]. To overcome peak tailing for nitrogen-containing bisphosphonates, sodium pyrophosphate was added to the aqueous content of the mobile phase. As can be seen in Figure 3, risedronate and zoledronate peak tailing is seriously reduced in the presence of sodium pyrophosphate, since pyrophosphate anions interact selectively with the metals of the LC system [10]. Tiludronate retention is not seriously affected by the presence of sodium pyrophosphate in the mobile phase.

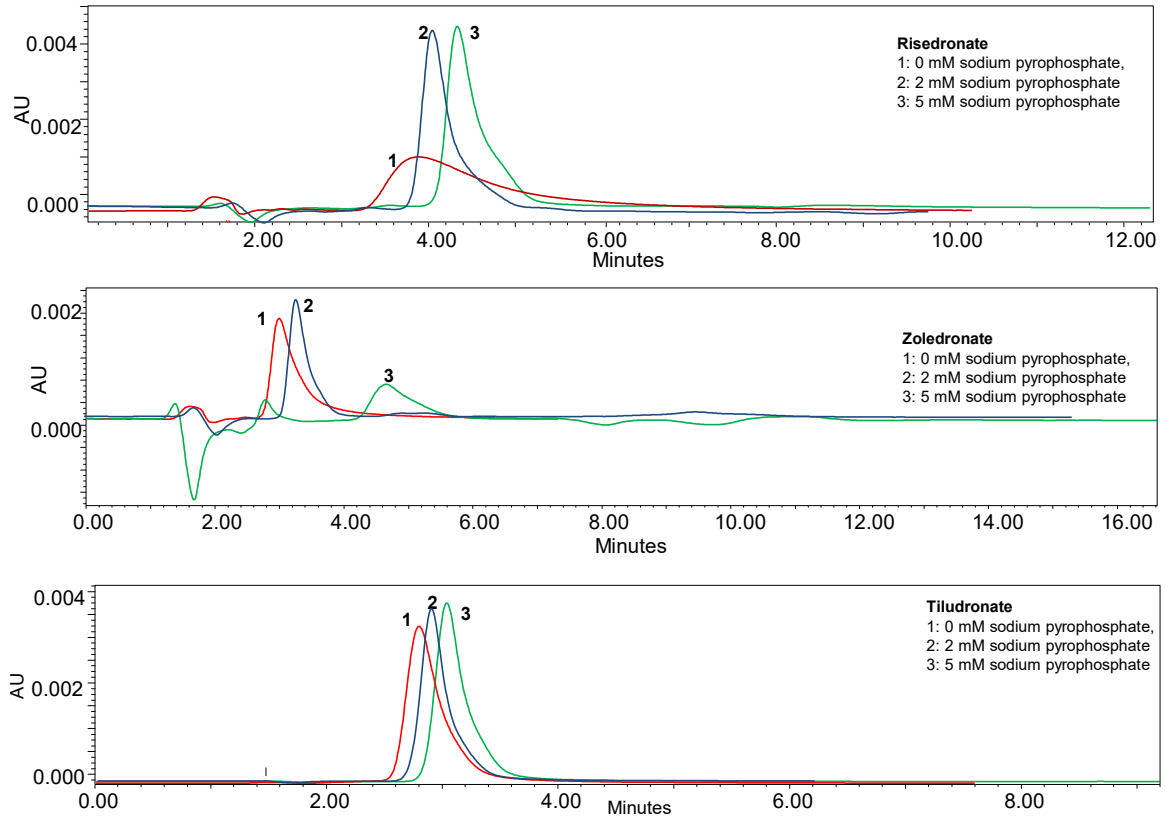

Figure 3. Typical HILIC chromatograms showing the effect of sodium pyrophosphate concentration (mM) on the on the retention time and the peak shape of bisphosphonates. Chromatographic conditions: ZIC®-pHILIC analytical column, mobile phase: acetonitrile–10 mM ammonium acetate aqueous solution pH 6.8 (65:35, v/v), flow rate of 0.25 mL min^{-1} and UV detection at 262 nm.

3.1.2. Optimization of the Chromatographic Parameters for the Quantitation of Risedronate

It was observed that the back pressure of the chromatographic system was increased by increasing sodium pyrophosphate concentration; thus, it was decided to reduce its concentration to 1 mM and to decrease the flow rate to 0.15 mL min^{-1}. Moreover, the addition of sodium pyrophosphate salt in the aqueous content of the mobile phase resulted in alkaline pH that was adjusted to 8.8 using acetic acid. By keeping sodium pyrophosphate concentration constant at 1 mM, a one-variable-at-a-time approach was used to identify the optimal mobile phase composition for the quantitation of risedronate in tablets. The parameters selected to study were the percentage of water, Φ_{water} and the concentration of ammonium acetate (mM). It was found that an increase in the percentage of water from 35% to 39% reduced the retention factor of risedronate. Moreover, it was observed that an increase in the concentration of ammonium acetate from 6 to 10 mM increased the retention of the negatively charged risedronate due to the disruption of the electrostatic repulsions between this and the negatively charged sulfonic acid groups of the stationary phase. Thus, a mobile phase consisting of 38% 9 mM ammonium acetate and 1 mM sodium pyrophosphate aqueous solution pH 8.8 in acetonitrile was finally used. At the beginning of each experiment, the column was equilibrated for 1.5 h and column temperature was set at 40 °C. Due to the isocratic separation, there was no need for time-consuming re-equilibration of the analytical column.

3.2. Method Validation

3.2.1. Selectivity

The selectivity of the proposed HILIC-PDA method is demonstrated in Figure 4, where a representative chromatogram obtained from the analysis of standard solution containing risedronate at 3.5 μg mL^{-1} is presented overlaid with a chromatogram obtained from the analysis of risedronate commercial tablets and a blank sample (dilution solvent). No significant interfering peaks have been observed at the retention time of risedronate, which is eluted at 4.59 min.

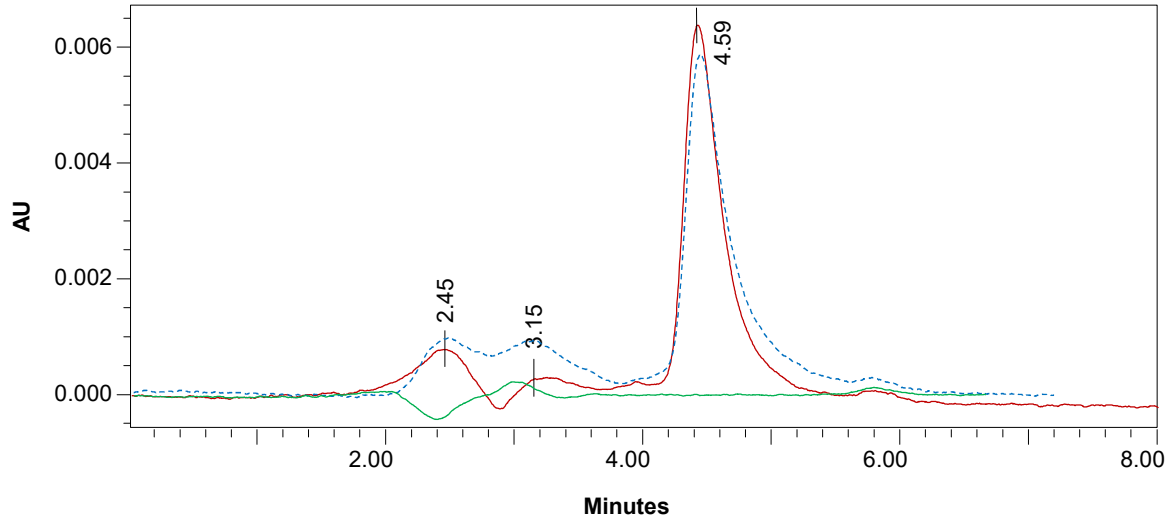

Figure 4. Typical HILIC chromatograms of a blank sample (green line) overlaid with a 3.5 μg mL^{-1} risedronate standard solution (red line) and a solution obtained from the analysis of risedronate commercial tablets (blue line). Chromatographic conditions: ZIC®-pHILIC analytical column, mobile phase: 38% 9 mM ammonium acetate and 1 mM sodium pyrophosphate aqueous solution pH 8.8 in acetonitrile, flow rate of 0.15 mL min^{-1} and UV detection at 262 nm.

3.2.2. Statistical Analysis of Data

The calibration curves of the peak area signals of risedronate versus the corresponding concentrations were linear, as shown by the results presented in Table 1. Back-calculated concentrations in the calibration curves were within 2.1% of the nominal values, which are in agreement with international guidelines.

Table 1. Statistical analysis of the calibration curves of risedronate.

Concentration Range, μg mL^{-1}	Regression Equations [a]	r [b]	Standard Deviation		S_r [c]
			Slope	Intercept	
Mean of 3 calibration curves over a period of 1 month					
1.5–5	$S_{Rsd} = 3.029 \times C_{Rsd} + 1.76$	≥0.9991	1.7×10^{-3}	3.2×10^{-2}	≤0.24

[a] Peak areas signal of risedronate, S_{Rsd} vs the corresponding concentration of risedronate, C_{Rsd}; [b] correlation coefficient; [c] standard error of the estimate.

The limit of detection (LOD) and the limit of quantitation (LOQ) were estimated experimentally by analyzing risedronate samples spiked at low concentrations. These limits were defined according to a signal-to-noise ratio (S/N) corresponding to 3:1 for the LOD and to at least 10:1 until a %CV of less than 2.5% was obtained for the LOQ. The LOD was found to be at 0.3 μg mL^{-1}, and the LOQ at 1.5 μg mL^{-1}.

Risedronate can be determined by appropriate precision and accuracy as is indicated by the intra- and inter-assay precision data that are presented in Table 2. Precision was evaluated by one-way

analysis of variance. Intra-assay relative standard deviation values, %RSD, were between 0.3% and 0.6%, while inter-assay %RSD was no more than 0.6%. The overall accuracy of the method was expressed by the relative percentage error, Er%, that ranged from 2.3% to 1.8%.

Table 2. Accuracy and precision data.

Risedronate	**Concentration (μg mL^{-1})**		
Added concentration	1.5	3.5	5
Run 1 (mean ± SD)	1.4669 ± 0.0051	3.556 ± 0.022	5.013 ± 0.046
Run 2 (mean ± SD)	1.4589 ± 0.0033	3.567 ± 0.012	5.022 ± 0.088
Run 3 (mean ± SD)	1.4715 ± 0.0044	3.5628 ± 0.0091	4.973 ± 0.012
Overall mean	1.4658	3.5618	5.0031
Intra-day CV(%) [a]	0.3	0.5	0.6
Inter-day CV(%) [a]	0.5	0.05	0.6
Overall accuracy Er% [b]	−2.3	1.8	0.1

[a] (n = 3 runs; 5 replicates per run).

During the method development, it was observed that both the volume fraction of water (Φ_{H_2O}) and the concentration of ammonium acetate affected the chromatography of risedronate. To assess method robustness, small deliberate variations were performed in the aforementioned parameters and in the wavelength of detection. Each parameter was changed at two levels (0 and 1) using a univariate approach and robustness was estimated by measuring the peak area signal. Ammonium acetate concentration was altered by 1.0 mM (range 8 to 9 mM), volume fraction of water Φ_{water} was altered by 2% (range 38% to 36%) and the wavelength of detection was altered by of 2 nm (range 262 to 264). A standard solution of risedronate at 3.5 μg mL^{-1} was injected in three replicates under changes of the parameters mentioned above. The proposed method could be considered as robust, since %RSD values of the peak area signal of risedronate does not exceed 3.2% (acceptance criteria < 10%) in all of the tested conditions.

3.2.3. Accelerated and Long-Term Stability Studies

The results of the accelerated and the long-term stability studies are presented in Table 3. In the acid stressed samples and after 10 days, a 21.9% of risedronate was degraded using 1.0 M HCl at 50 °C, while no degradation products have been detected. In the base stressed samples and after 1 day, a 21.2% of risedronate was degraded using 1.0 M NaOH at 50 °C, while no degradation products were detected. In the acid stressed samples and after 3 h, a 17.3% of risedronate was degraded using 3.0% (v/v) H_2O_2 at 25 °C while the degradation products could not be detected. After the degradation of risedronate blistered tablets under low (15%) and high (75%) humidity conditions for three months, the percentage recovery of the analyte was 95.9% and 78.5%, respectively.

Table 3. Stability data for risedronate by HILIC-PDA.

Degradation Conditions/Time	**Time**	**Concentration (μg mL^{-1}) (Mean ± S.D., n = 3)**	**% Recovery (Mean ± S.D., n = 3)**	**Degradation Products Retention Time (min)**
1.0 M HCl, 50 °C	1 day	3.467 ± 0.038	99.0 ± 1.1	-
	2 days	3.469 ± 0.041	99.1 ± 1.2	-
	8 days	3.036 ± 0.040	86.7 ± 1.1	<3
	10 days	2.736 ± 0.032	78.1 ± 0.9	<3
1.0 M NaOH, 25 °C	1 day	2.757 ± 0.073	78.8 ± 2.1	<3
3 % v/v H_2O_2, 25 °C	1 h	3.211 ± 0.042	91.7 ± 1.2	-
	2 h	3.094 ± 0.041	88.4 ± 1.1	<3
	3 h	2.895 ± 0.047	82.7 ± 1.4	<3

Table 3. *Cont.*

Long-Term Stability Studies	Time	Amount (mg) Per Tablet (Mean ± S.D., n = 3)	% Recovery (Mean ± S.D., n = 3)	Degradation Products Retention Time (min)
50 ± 2 °C 15% humidity	1 month 3 months	32.79 ± 0.51 32.41 ± 0.46	100.9 ± 1.5 99.7 ± 1.3	- -
50 ± 2 °C 75% humidity	1 month 3 months	32.38 ± 0.44 25.22 ± 0.63	99.6 ± 1.2 77.6 ± 1.8	- <3

Based on the results presented in Table 3, the proposed HILIC-PDA method is stability-indicating, since it is able to quantitate risedronate in commercial formulations without any interference from degradation peaks.

3.3. Analysis of Commercial Tablets

The applicability of the proposed method was evaluated through the analysis of commercially available tablets containing 35 mg of risedronate sodium (equivalent to 32.5 mg risedronic acid). The analysis was performed on accurately weighted amount of the pulverized tablets. Percentage recovery was found to be 99.3 ± 0.7% of the label claim, or 32.3 ± 0.2 mg of risedronic acid per tablet (n = 10, RSD = 0.6%). Additionally, this method was used for the content-uniformity testing, in which many assays on the individual tablets are required. Percentage recovery was found to be 100.2 ± 1.2% of the label claim, or 32.6 ± 0.4 mg of risedronic acid per tablet (n = 10, RSD = 1.1%).

Table 4 presents data obtained from the analysis of real samples and indicate that the proposed HILIC-PDA method is applicable to the accurately quantitation of risedronate in commercially available tablets.

Table 4. Risedronate quantitation in a commercial formulation.

Test	Amount (mg) Per Tablet (Mean ± SD, n = 10)	% Recovery (Mean ± SD, n = 10)
Quality control	32.3 ± 0.2	99.3 ± 0.7
Content uniformity	32.6 ± 0.4	100.2 ± 1.2

4. Conclusions

HILIC is a popular chromatographic method for the analysis of hydrophilic compounds. The separation mechanism in HILIC is more complicated in regards to reversed phase HPLC due to the various kinds of interactions that rule the retention. Despite the increased number of publications in the last years, the separation mechanism in HILIC is still under investigation and many analysts are puzzled over the use of this chromatographic method [32,33]. Thus, understanding the influence of operational parameters in HILIC is crucial for an effective method development. The chemical nature of bisphosphonates, both polar and ionic, makes them perfect candidates for HILIC. In this work the chromatographic behavior of two nitrogen-containing bisphosponates, namely risedronate and zoledronate, and one non-nitrogen-containing bisphosphonate, namely tiludronate, has been thoroughly investigated under zwitterionic HILIC conditions. The results indicate that apart from partition, which is the dominant separation mechanism in HILIC, electrostatic repulsions play an important role to the retention of bisphosphonates. Peak tailing of the two nitrogen-containing bisphosphonates was improved by the addition of sodium pyrophosphate to the mobile phase. A zwitterionic hydrophilic interaction liquid chromatography method coupled to diode-array detection was developed, validated and applied to the quantitation of risedronate in pharmaceutical formulations. The proposed method is stability-indicating since it allows accurate and precise quantitation of risedronate in tablets without any interference from excipients or degradation products, and it is

applicable to routine quality control of risedronate in tablets. A run time of less than 6 min ensures rapid quantitation.

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