Rapid Determination of Bisphosphonates by Ion Chromatography with Indirect UV Detection

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Abstract

Rapid methods for etidronate, clodronate, pamidronate, and alendronate assays are presented. The methods are based on ion chromatography with indirect UV detection, which avoids the need for chemical derivatization procedures. Each compound is analyzed on an individual basis. There is no need for having separation among these analytes because the aim of the proposed methods is to analyze each compound separately either in bulk material or pharmaceuticals. Phenosphere (150 × 2.0-mm, 5 µm) and Sphereclone (250 × 2.0-mm, 5 µm) anion exchange columns were employed with sodium citrate (20mM) as the mobile phase. The methods are simple, rapid (analysis time of 5 min for etidronate and clodronate and 7 min for alendronate and pamidronate), and demonstrate precision (relative standard deviation was lower than 2.0% in all concentrations), accuracy, and specificity. Calibration curves are linear with $r^2 > 0.99$ over the concentration range of 50 to 400 µg/mL for etidronate and clodronate, and of 100 to 500 µg/mL for pamidronate and alendronate. Furthermore, they employed silica-based columns, which are cheaper than polymeric columns frequently used in previous reported methods.

Introduction

Bisphosphonates (BPs) are bone resorption inhibitor drugs and have been widely used in the treatment of several bone metabolism disorders, including Paget's disease, malignant hypercalcemia, bone metastasis, and osteoporosis (1). They have also been employed in the treatment of rheumatoid arthritis (2). Moreover, BPs are active against several parasites, such as *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii*, and *Plasmodium flaciparum* (3,4).

Determination of these drugs by high-performance liquid chromatography (HPLC) and direct UV or fluorescence detection is hindered because of the lack of chomophore groups in their structures. Therefore, different detection systems have been employed to assay BPs in different matrices by HPLC (5). Chester developed a phosphorous-selective detector based on the molecular emission of HPO (6). Several methods using refractive index detection were also described (7–9). Electrochemical detection was used in the determination of BPs with the advantage of avoiding the analyte derivatization (10–13). However, these detectors are sensitive to external variations that can compromise the reproducibility. Recently, some methods to determine BPs using mass spectrometry (MS) were developed. Quin et al. presented a method to analyze alendronate in tablets using ion chromatography coupled to an MS detector (14). Methods employing inductively-coupled plasma and evaporative light-scattering detectors have also been reported in the literature (15–17). However, these detection systems are not common in most analytical laboratories. Several determination methods for BPs using UV–vis or fluorescence detectors in the indirect mode, or after pre- or post-column derivatization, have also been described (18–26). The derivatization step is time and solvent consuming, making this process inappropriate for routine analysis. Thus, indirect detection methods are an appropriate alternative for this kind of analysis because there is no need for additional steps. Tsai et al. (27) developed a method to analyze different BPs in pharmaceutical preparations using indirect UV

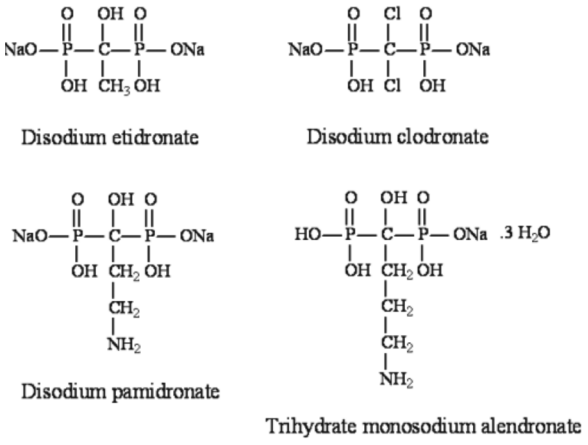

Figure 1. Chemical structures of evaluated bisphosphonates.

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detection. Quattrocchi et al. (28) proposed a method to determine olpadronate in tablets using the same detection system. In both methods, anionic exchange polymeric columns with a conventional internal diameter (4.6 mm) were used.

This study presents the development and validation of alternative methods for the routine analysis of etidronate, clodronate, pamidronate, and alendronate (Figure 1) in bulk material and pharmaceutical preparations using ion chromatography and indirect UV detection. Parameters such as precision, accuracy, specificity, limit of quantitation (LOQ) and detection (LOD), linearity, range, and robustness were evaluated according to the International Conference on Harmonization (ICH) guidelines (29,30). The developed methods are simple and rapid, making routine control quality analysis easy. These methods employ narrower internal diameter columns (2.0 mm) for reducing the amount of solvent used. Furthermore, they use silica-based columns, which were cheaper than polymeric used in previous methods described in the literature.

Experimental

Chemicals and reagents

Disodium salt of clodronate was obtained from Sigma-Aldrich Chemical (Steinheim, Germany). Disodium etidronate, disodium pamidronate, and trihydrate monosodium alendronate were generously provided by Bufa B.V. (Uitgeest, The Netherlands). Distilled water purified with a Milli-Q Ultra-Pure Water System (Millipore, Bedford, MA) was utilized for all sample preparations. Alendronate tablets from two different laboratories were used: a Fosamax (Merck Sharp & Dohme, Mexico City, Mexico) containing mycrocristalline cellulose, croscarmelose sodium, anhydrous lactose, and magnesium stearate as excipients (labeled a reference drug), and an Alendronato sodico (Teva, Israel) containing hydroxypropylcellulose, low-substituted hydroxypropylcellulose, hydrated silica, and sodium stearyl fumarate as excipients (labeled a generic drug) (31). Pharmaceutical preparations were purchased in a local drugstore. Citric acid was obtained from Merck (Rio de Janeiro, Brazil), and dihydrate sodium citrate was supplied by Mallinckrodt (Mexico City, Mexico).

Equipment and chromatographic conditions

The HPLC system (LC-10A) consisted of two pumps (LC-10Ai), an oven (CTO-10ASVP), a fixed wavelength UV detector (SPD-10AVP), an autoinjector (SIL-10Ai), a system controller (SCL-10AVP), a degasser (DGU14A), and an acquisition data software Class-VP (Shimadzu, Kyoto, Japan). A photodiode array detector (SPD-M10AVP) was also used. The chromatographic conditions used in the determination of etidronate and clodronate were: a Phenomenex Phenosphere SAX (150 × 2.0-mm, 5 µm) column (Torrance, CA), with a sodium citrate buffer (20mM, pH 3.6) as the mobile phase, a flow rate of 0.3 mL/min, a 50 µL injection, and a temperature of 30°C. The UV detection was set at 226 nm with inverse polarity of the detector output. To analyze the drugs alendronate and pamidronate the conditions were: a Phenomenex Sphereclone SAX (250 × 2.0-mm, 5 µm) column (Torrance), with a sodium citrate buffer (20mM, pH 4.6) as the mobile phase, a flow rate of 0.25 mL/min, a 50 µL injection, a temperature of 30°C , and a detection at 222 nm.

Preparation of standard solution

Etidronate, clodronate, pamidronate, and alendronate analytical standards were accurately weighed and transferred to a 10-mL volumetric flask, obtaining solutions of 2 mg/mL. The standard solutions were diluted with water to defined concentrations according to analytical necessities.

Preparation of alendronate tablets samples

Ten tablets of each laboratory were weighed and powdered. The equivalent of 10 mg of alendronate from the obtained powder was weighed and transferred into a 10-mL volumetric flask with water. The flask was sonicated for 10 min, filtered, and 3.0 mL was transferred into a 10-mL volumetric flask, obtaining a solution of 300 µg/mL. The samples were made in duplicate.

Validation procedure

The specificity was evaluated by comparing the UV spectrum obtained in different points of the chromatographic peak. The linearity was examined through the construction of calibration curves with five points in the range of 50 to 400 µg/mL for etidronate and clodronate and of 100 to 500 µg/mL for pamidronate and alendronate. The linear regression equation and the correlation coefficient (r^2) were calculated by the least squares method. Within- (n = 3) and between-day (n = 2) precision were examined. The LOQ was considered as the lowest concentration where the relative standard deviation (RSD) was smaller than 2.0%, and the LOD was established as the concentration where the analyte peak was three times higher than the baseline noise. Accuracy for alendronate was determined through the recovery studies using three different concentrations (n = 3). For pamidronate, clodronate, and etidronate, accuracy was inferred because of the inexistence of available pharmaceutical preparations, after the establishment of precision, linearity, and specificity according to ICH guidelines (29,30). The robustness was evaluated by checking some variations in the system, such as pH, mobile phase concentration, and column temperature.

Results and Discussion

Optimization of chromatographic conditions

During the optimization, the influence of sodium citrate buffer concentration and pH, utilized as the mobile phase, in the retention time and detector response for the analytes was evaluated. Sodium citrate buffer concentrations of 20, 30, 40, and 60mM were evaluated. For each concentration, pH was varied from 3.0 to 7.0. Both buffer concentration and pH affect analyte response (area value), retention time, and resolution among analyte peak, water peak, and system peak. For clodronate and etidronate, sodium citrate buffer in the concentration of 20mM and pH 3.6 proved to be adequate for rapid analysis. The same buffer concentration was maintained for analysis of pamidronate

and alendronate, by varying the pH to 4.6. Under the optimized conditions, alendronate, pamidronate, clodronate, and etidronate were eluted as ionic species, predominantly as a divalent (–2) charged species.

This concentration of sodium citrate buffer allowed to obtain a good sensitivity because of the high difference between the mobile phase and the analyte absorbance. Moreover, this concentration allowed to obtain a rapid analysis with appropriate resolution among the analyte, water, and system peak. The system peak observed was characteristic in ion chromatography, resulting from an eluent-deficient zone formed by the sample injection; it may depend on the pH of the injected sample (32).

The wavelength used in the analyses was also varied from 220 to 230 nm in order to achieve the best response for each analyte. The indirect UV detection monitored the decrease in UV absorbance of the citrate buffer eluent, which exhibited a UV absorption maximum between 200 and 210 nm because of the replacement of the citrate anions by bisphosphonates eluted into the detector cell. The chromatograms shown in Figure 2 essentially reflect the negative (decreasing) signal with an inverse polarity of the detector output. The usable wavelength range was 220–230 nm. Wavelengths shorter than this range produced a high noise that hindered the detection of the analytes. The wavelength that produced the best response for etidronate and clodronate was 226 nm, and the best wavelength for alendronate and pamidronate was 222 nm. The former pair of analytes had a similar structure as well as the latter pair. Figure 3 shows the similarity of UV spectra between etidronate and clodronate and between pamidronate and alendronate, which explains the different response in different wavelengths.

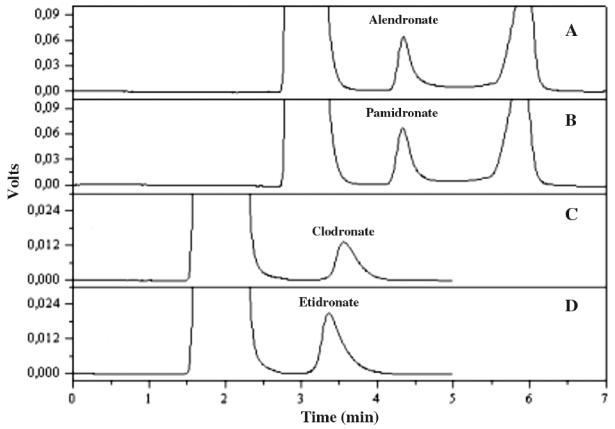

Figure 2. Typical chromatograms of standard solutions containing alendronate (A), pamidronate (B), clodronate (C), etidronate (D). A Phenomenex Phenosphere SAX (150 × 2.0-mm, 5 µm) column was used, with a sodium citrate buffer (20mM, pH 3.6) as the mobile phase, a flow rate of 0.3 mL/min, a 50 µL injection, a temperature of 30°C, and a UV detection at 226 nm for etidronate and clodronate. A Phenomenex Sphereclone SAX (250 × 2.0-mm, 5 µm) column was used, with a sodium citrate buffer (20mM, pH 4.6) as the mobile phase, a flow rate of 0.25 mL/min, a 50 µL injection, a temperature of 30°C, and a detection at 222 nm for alendronate and pamidronate.

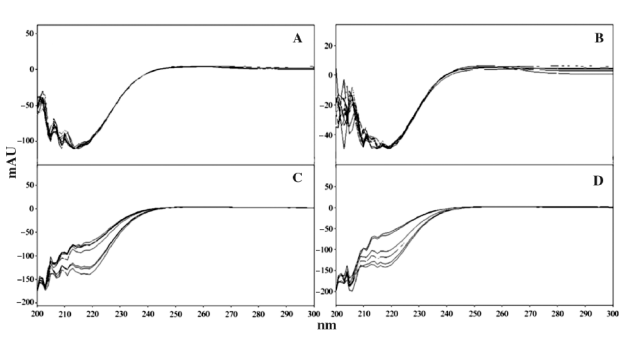

Figure 3. Etidronate (A), clodronate (B), pamidronate (C), and alendronate (D) UV spectra in the range of 200 to 300 nm.

An injection volume of 50 µL was used in order to increase the method sensitivity. Lower injection volumes, such as 10 and 20 µL, were used during the optimization, and no difference in peak broadening was observed. Furthermore, an overloading effect was not detected when 50 µL was employed.

Figure 2 presents typical chromatograms of analytical standard solutions of etidronate (A), clodronate (B), pamidronate (C), and alendronate (D), with retention times of 3.3, 3.5, 4.3, and 4.3 min, respectively. Each compound was analyzed on an individual basis. Therefore, there is no need for having a separation among these analytes because the aim of the proposed methods is to analyze each compound separately either in bulk material or pharmaceuticals. Two different methods were proposed because there was a necessity for different conditions in order to achieve an adequate separation among the analyte, water, and system peak. Furthermore, a better analyte response (higher area values) and shorter time analysis was achieved in these described conditions.

Methods validation

Linearity and range

Range was evaluated from 50 to 400 µg/mL for etidronate and clodronate and from 100 to 500 µg/mL for alendronate and pamidronate. In all methods, the correlation coefficient was higher than 0.99, demonstrating a linear correlation between the concentration and the obtained response. The linear regression equation and correlation coefficient values are shown in Table I.

Specificity

A comparison between the UV spectra obtained in different points of the chromatographic peaks (in the beginning, in the middle, and at the end of the peak) using a photodiode array detector showed a similarity higher than 98.0% for all investigated analytes. More than 10 spectra were obtained for each analyte. Each compound had a specific UV spectrum, and it was also

Table I. Linear Regression Equation and Correlation Coefficient Values for Determination Methods of BP's

Analyte	Linear regression equation	Correlation coefficient
Etidronate	$Y = -21248.6 + 1971.4x$	0.9989
Clodronate	$Y = -6868.8 + 1322.9x$	0.9993
Pamidronate	$Y = -65694.0 + 2932.0x$	0.9989
Alendronate	$Y = -8004.3 + 227.0x$	0.9985

valid for negative absorbance. Therefore, if any impurity was present in the peaks, then a different UV spectrum would be detected; however, it was not observed. This demonstrated the inexistence of impurities eluting in the same retention time of the analyte under investigation. The individual spectra, in the range from 200 to 300 nm, are shown in Figure 3 for the studied analytes.

Table II. Concentration and RSD Values Obtained to Evaluate Within- (n = 3) and Between-Day (n = 2) Precision

Concentration (µg/mL)	RSD (%)							
	Etidronate		Clodronate		Pamidronate		Alendronate	
	Within	Between	Within	Between	Within	Between	Within	Between
50	0.89	1.65	1.65	1.13	n.e.*	n.e.	n.e.	n.e.
100	1.70	n.e.	0.93	n.e.	1.06	0.76	0.85	0.60
200	1.72	0.20	0.56	0.47	1.72	n.e.	1.98	n.e.
300	1.18	n.e.	0.56	n.e.	1.87	1.62	1.42	1.96
400	0.61	1.30	1.70	0.53	1.47	n.e.	1.93	n.e.
500	n.e.	n.e.	n.e.	n.e.	1.29	0.14	1.04	1.98

*n.e. = Not evaluated.

Precision

The RSDs obtained in the evaluation of within- and between-day precision were smaller than 2.0% in all concentrations. This result demonstrates that these methods have an adequate precision (Table II).

LOQ and LOD

LOQ was established at a level of 50 µg/mL for etidronate and clodronate and 100 µg/mL for pamidronate and alendronate. The LOQ was considered as the lowest concentration where the RSD was smaller than 2.0% (29,30).

LOD values for etidronate, clodronate, pamidronate, and alendronate were 5.3, 5.1, 10.5, and 10.0 µg/mL, respectively. LOD was established as the concentration where the analyte peak was three times higher than the baseline noise (29,30).

Table III. Peak Height and Recovery Percentage Values Obtained for Alendronate

Concentration (µg/mL)	Height	Response factor	Mean response factor (n = 3) obtained in linearity	Recovery (%)
109.3	18097.3	165.57		100.67
108.6	17975.7	165.52	164.46	100.64
108.4	18348.5	169.27		102.92
327.9	64136.0	195.60		98.60
325.8	62767.7	192.66	198.37	97.12
325.2	63473.7	195.18		98.39
546.5	118393.7	216.64		101.60
543.0	118010.0	217.33	213.22	101.93
542.0	117809.0	217.36		101.94

Accuracy

Accuracy for pamidronate, clodronate, and etidronate was inferred after the precision, linearity, and specificity were established according to ICH guidelines.

Table III shows the height and the recovery percentage values for alendronate. The height values obtained in low, medium, and high concentrations in the linear range were compared with those obtained in the determination of the linearity (n = 3). The percentage recovery values were in the range from 97.12% to 102.92%, close to 100.0%, demonstrating the method accuracy.

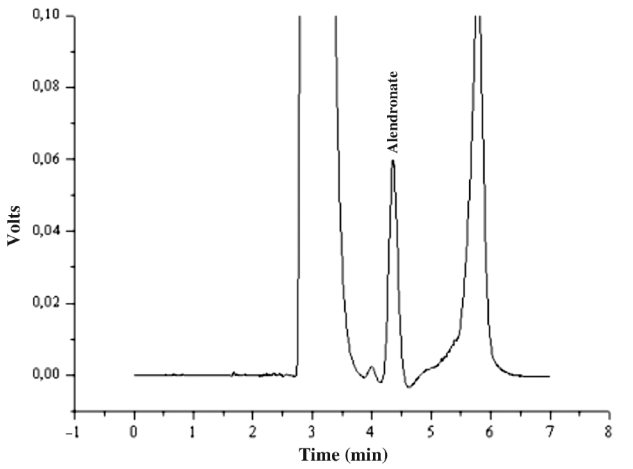

Figure 4. Chromatogram of a tablet containing the alendronate reference drug in the concentration of 300.37 µg/mL. A Phenomenex Sphereclone SAX (250 x 2.0-mm, 5 µm) column was used, with a sodium citrate buffer (20mM, pH 4.6) as the mobile phase, a flow rate of 0.25 mL/min, a 50 µL injection, a temperature of 30°C, and a detection at 222 nm.

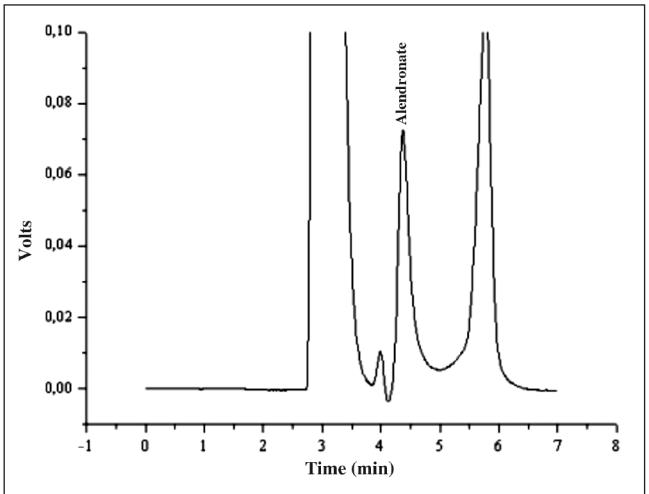

Figure 5. Chromatogram of a tablet containing the alendronate generic drug in the concentration of 300.21 µg/mL. A Phenomenex Sphereclone SAX (250 x 2.0-mm, 5 µm) column was used, with a sodium citrate buffer (20mM, pH 4.6) as the mobile phase, a flow rate of 0.25 mL/min, a 50 µL injection, a temperature of 30°C, and a detection at 222 nm.

Robustness

Both mobile phase composition and pH modify the retention time and detector response for the analytes, as well as the resolution between the peaks, which is a characteristic of ion chromatography. Therefore, these parameters should be well controlled in order to guarantee method reproducibility. Temperature has not shown to have an important influence in the system. An evaluation of temperature effects was performed between 30°C and 50°C. There was no difference in retention time, resolution, or area value in this range.

Alendronate assay in tablets

The amount of alendronate found in the assay was 102.7% for the reference drug and was 96.7% for the generic drug. These values were within the range from 90.0% to 110.0%, demonstrating the quality of the products relating to the amount of drug. Furthermore, the results show that the methods are appropriate to analyze alendronate in tablets. Chromatograms obtained in the assay of reference and generic drugs are presented in Figures 4 and 5.

Conclusion

The methods described in this work for the determination of etidronate, clodronate, pamidronate, and alendronate in bulk material and pharmaceuticals are simple, and they demonstrate precision, accuracy, and specificity. The methods are rapid, with an analysis time of 5 min for etidronate and clodronate and 7 min for pamidronate and alendronate. Moreover, these methods utilize UV detection commonly found in analytical laboratories. Narrower internal diameter columns (2.0 mm) were used, reducing solvent consumption by approximately 80% when compared with conventional columns (4.6 mm). Silica-based columns, which were cheaper than polymeric columns employed in most previously described methods, were used. Furthermore, a sodium citrate buffer was used as the mobile phase in this work because it was less harmful to the chromatographic system than the nitric acid used in some previously reported papers.

Acknowledgments

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