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Direct stability-indicating method development and validation for analysis of etidronate disodium using a mixed-mode column and charged aerosol detector

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Abstract

This paper describes the development and validation of a rapid, direct, and stability-indicating method for analysis of etidronate, a bisphosphonate compound without a UV chromophore. A mixed-mode column was used to separate etidronate from its impurities in an 8-min gradient method and a charged aerosol detector (CAD) was used for detection. The developed HPLC method was validated with respect to specificity, linearity, accuracy, precision, sensitivity, and stability. The method can be used for release and stability testing of etidronate and has applicability to other similar bisphosphonate compounds.

Keywords: Etidronate; Charged aerosol detector; HPLC; Bisphosphonate

1. Introduction

Bisphosphonates are potent inhibitors of bone resorption. They have been commonly used for treatment of cancer hypercalcemia and metastatic bone diseases such as osteoporosis and Paget's disease. Recent results indicate their potential application for prevention and treatment of cancer-induced bone loss and bone pain [1–4].

In spite of the wide application of bisphosphonates, the analysis of such compounds has been very challenging since they are very polar and are strong chelators. They can form multiple ionized species and have very weak or no UV chromophore, which makes it difficult for separation and UV detection. Traditional analysis of bisphosphonates involves indirect UV [5–7] or pre-column derivatization to obtain better sensitivity and selectivity [8–15]. However, this procedure is very time-consuming, and sometimes the resulting derivatives are not stable enough for complete analysis. As a result, different methods are needed for analysis of assay and degradation products, since there are potential differences in derivatization of the various degradation products.

Recent publications reported the application of ion-pair RP HPLC to the analysis of these compounds, using volatile or non-volatile ion-pair reagents [16–18] and a capillary electrophoresis (CE) method [19–20]. Recent communications summarized an ion-pair RP-LC method for analysis of zoledronic acid and etidronate and their corresponding degradation products [21–22].

This paper describes the development and validation of a direct stability-indicating method for analysis of etidronate disodium using recent analytical technology. Etidronate [(1-hydroxyethylidene) bisphosphonate] (Fig. 1), the very first compound used in clinical practice in the bisphosphonate family [1,2], is used as a worst case approach since it has no chromophore.

Because of the highly ionic characters of the etidronate disodium and impurities, the separation of those compounds is very difficult using conventional HPLC columns. In addition, the etidronate disodium and impurities do not possess UV chromophores, hence determination by ordinary spectrophotometric methods is not possible. The current United States Pharmacopoeia (USP) monograph for etidronate employs two different

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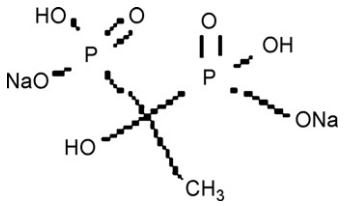

Fig. 1. Chemical structure of etidronate disodium.

methods for assay of etidronate and limit test for phosphite [23]. The etidronate assay method is based on anion-exchange chromatography with a refractive index detector. The limit test for phosphite (not more than 1.0%) is performed using a column packed with a hydroxide-selective strong anion-exchange resin and a conductivity detector.

Charged aerosol detector (CAD), a detection method based on aerosol charging, provides a new mechanism for detection of compounds with weak or no chromophores. CAD is a detector reportedly to be universal for all non-volatile analytes with response magnitude independent of the analyte chemical properties [24].

By applying recent analytical technology, such as a mixed-mode column and charged aerosol detector, an 8-min stability-indicating HPLC method was developed for analysis of etidronate and its related impurities/degradation products. The separation is performed on a Primesep SB column (3.2 mm × 50 mm, 5 μm particles), using 5% acetonitrile in water with trifluoroacetic acid (TFA) gradient (0.03–0.2% in 5 min). This method has been successfully validated for specificity, linearity, accuracy, precision, sensitivity and stability. Comparing to other published methods for etidronate [14,16,22], this method demonstrated sufficient sensitivity without time-consuming derivatization or using ion-paring reagents. It may be used for analysis of dissolution samples as well as assay/degradation products of etidronate for both release and stability testing purposes.

2. Experimental

2.1. Chemicals and reagents

200 mg etidronate tablets were purchased from Genpharm L.P. (Hauppauge, NY, US). Etidronate disodium (API) and its related impurity, sodium phosphite dibasic pentahydrate (phosphite), were obtained from United States Pharmacopoeia (Rockville, MD, US). Sodium phosphate dibasic (phosphate) was purchased from J.T. Baker (Phillipsburg, NJ, US). Trifluoroacetic acid, 99.0%, HPLC grade, was obtained from Sigma–Aldrich (St. Louis, MD, US). Acetonitrile (ACN) >99.9%, HPLC grade, was purchased from Burdick & Jackson (Morristown, NJ, US). Water, HPLC grade, was prepared in-house by using Millipore Milli Q plus purification system (Billerica, MA, US). 0.45 μm Nylon membrane filters, used for mobile phase filtration, were purchased from Millipore. 0.45 μm PTFE syringe filters for sample solutions were purchased from Pall Science (East Hill, NY, US). Primesep SB HPLC column (3.2 mm × 50 mm, 5 μm, 100 Å) was obtained from SIELC Technology (Prospect Heights, IL, US). Columns were rinsed thoroughly using mobile phase A before and after each run and were stored in 50/50, ammonium formate (pH 3.5)/ACN (v/v) for long term storage.

2.2. Instrumentation

The HPLC system consisted of an Agilent 1100 Series LC (Chicago, IL, US) with a charged aerosol detector from ESA Magellan Bioscience (Chelmsford, MA, US). Dionex Chromeleon Chromatographic Data Software (Sunnyvale, CA, US) was used for HPLC system control and all data calculation.

2.3. Chromatographic conditions

Chromatographic analysis was carried out at 40 °C. The mobile phases were 5% acetonitrile in water with 0.03% TFA as mobile phase A and 5% acetonitrile in water with 0.2% TFA as mobile phase B. The gradient increased linearly from 0% B to 100% B in 5 min with a hold time of 3 min at the end. The mobile phases were filtered under vacuum through 0.45 μm Nylon filters prior to use. The flow rate was 0.5 mL/min. The injection volume was 5 μL. The CAD parameters were: gas 35 psi (house nitrogen), range 100 pA.

2.4. Preparation of standard and linearity sample solutions

A stock solution of etidronate disodium (10 mg/mL) was prepared by dissolving the appropriate amount of the compound in the diluent (5% ACN in water). Appropriate dilutions were made to approximately final concentrations of 1.25, 2.5, 4.0, 5.0, 6.0 and 7.5 mg/mL of etidronate for the calibration curve. The 5.0 mg/mL solution (100% etidronate target assay concentration level) was also used as the quantification standard.

A stock solution containing phosphate and phosphite at 0.5 mg/mL of each component was prepared in diluent. Appropriate dilutions were made to approximate 0.025, 0.030, 0.040, 0.050, 0.063 and 0.075 mg/mL of phosphate and phosphite for the impurity calibration curve, which corresponds to approximately 0.5–1.5% of the target assay concentration of etidronate. Each impurity linearity sample contained approximate 5.0 mg/mL etidronate (100% etidronate target assay concentration level). Samples used for sensitivity evaluation were further diluted.

2.5. Preparation of tablet samples

A 200 mg etidronate tablet was placed in each of the 20 mL volumetric flasks. Approximately 15 mL of diluent (5% ACN in water) were added and the suspension was sonicated for approximately 20 min. After it is cooled to room temperature, the solution was diluted to volume. A second dilution was performed to obtain a sample concentration of 5 mg/mL. The solution was filtered using 0.45 μm PTFE syringe filter before analysis.

2.6. Method development and optimization

All the bisphosphonates are strongly polar and ionic; it is difficult to retain bisphosphonates on a hydrophobic stationary phase such as a C18 column. Various columns with strong ion-exchange characteristics have been explored and the mixed-mode columns provided satisfactory separation and retention for all peaks of interest. Within the mixed-mode columns, the Primesep D column was first used. This column is a strong anion-exchange reverse phase column and provides good interaction with the negatively charged etidronate. However, etidronate exhibits four pK_a values (1.35, 2.87, 7.03 and 11.3) that span the entire pH range, which results in multiply charged ions in solution, and causes poor peak shape. The peak shapes were significantly improved when a new mixed-mode anion-exchange reverse phase column, Primesep SB, was used. This might be attributed to the fact that the Primesep SB column has a bulky basic group with a pK_a value around 13, and there is more space between ligands, which improves the peak shape for multiply charged analytes. After several optimization tests, a gradient method was successfully developed to separate the two impurities from etidronate in eight minutes. Column to column variability was assessed using a total of three columns with two columns used for method development and another one with different lot of stationary phase introduced during method validation. Satisfactory repeatability was observed and the results are summarized in the accuracy/precision tables.

3. Method validation

3.1. Specificity

The specificity of the method was evaluated using the 200 mg etidronate tablet, excipient blend of the etidronate tablet, degraded etidronate sample solutions and etidronate solution spiked with the two potential impurities: phosphate (a potential degradation product) and phosphite (a synthetic impurity).

Comparison of the chromatograms of the 200 mg etidronate tablet with that of the corresponding excipient blend indicates that there is no significant interference from the excipients with the detection of the etidronate or related impurities (Fig. 2). When phosphite and phosphate, the specified impurities of etidronate, were spiked into the etidronate solution, satisfactory separation of the etidronate peak from these peaks was observed (Fig. 3).

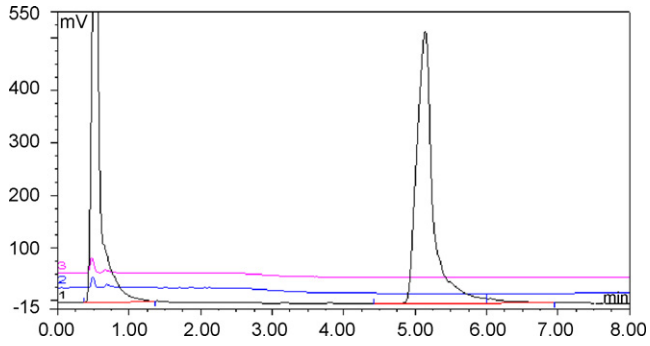

Fig. 2. Chromatogram of etidronate tablet compared to the excipient mixture and blank solution. (1) Etidronate tablet sample; (2) Sample diluent; (3) Excipient mixture.

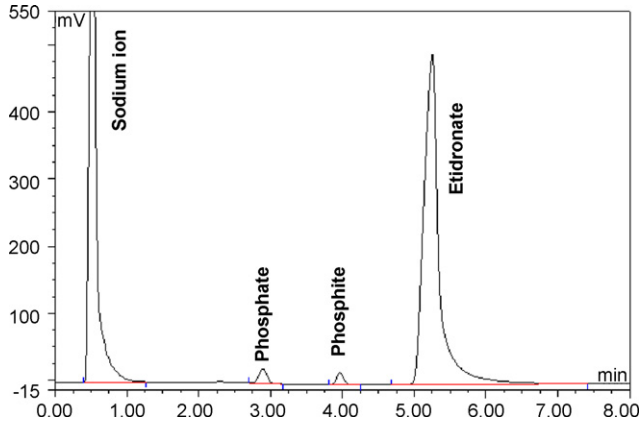

Fig. 3. Chromatogram of etidronate spiked with impurities.

Forced degradation studies were performed on the bulk API under the conditions of heat (15 days at 60 °C), acid hydrolysis (0.5N HCl, 48 h at room temperature), base hydrolysis (0.1N NaOH 48 h at room temperature), and oxidation (10% H_2O_2, 48 h at room temperature or 60 °C). No significant degradation products were observed from the heat, acid and base degradations. Forced degradation in 10% H_2O_2, 48 h at room temperature and at 60 °C generated the phosphate degradation product (Fig. 4). However, due to the nature of the CAD, the peak purity of the etidronate peak is not able to be verified. Attempts to verify the peak purity using LCMS were not successful, either.

3.2. Linearity

Linearity of the method was evaluated for etidronate at the assay levels and for both phosphate and phosphite at the impurity levels. Each sample was injected 3 times except for the samples at accuracy/precision levels, which were injected 6 times. All individual data were used for linearity evaluation.

Linearity of the etidronate response was evaluated at six concentration levels ranged from 1.25 to 7.5 mg/mL, corresponding to 25–150% of the assay concentration level of 5.0 mg/mL.

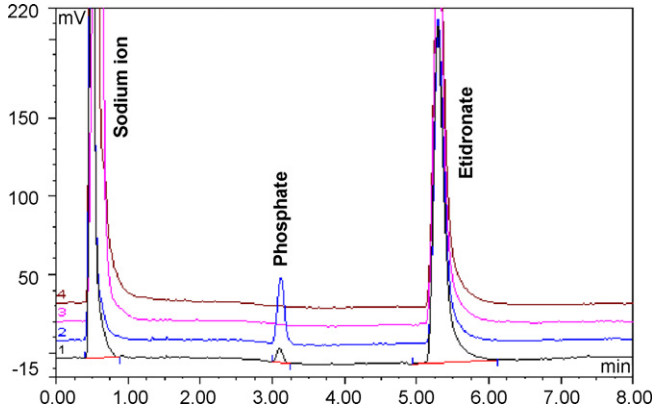

Fig. 4. Chromatograms of forced degraded etidronate samples. (1) H_2O_2 at RT; (2) H_2O_2 at 60 °C; (3) 0.5N HCl, 48 h at RT; (4) 0.1N NaOH, 48 h at RT.

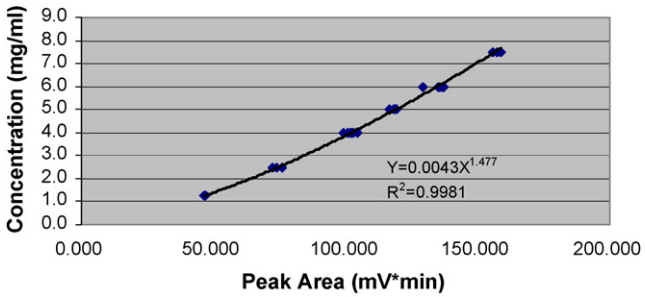

Fig. 5. Calibration curve of etidronate.

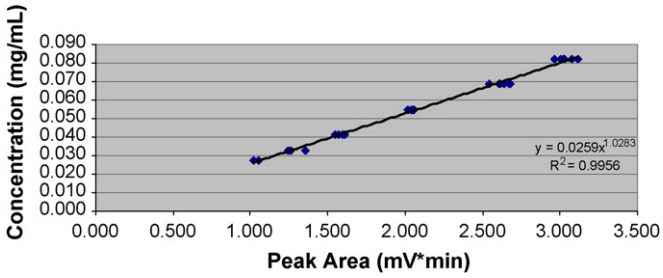

Fig. 7. Calibration curve of phosphite.

Linearity samples were prepared from the 10 mg/mL stock etidronate solution. The correlation of the peak area vs. concentration of etidronate dose not appear linear (Fig. 5). The equation for etidronate calibration curve for this range is $y = 0.0043x^{1.477}$ with a correlation coefficient (R^2) of 0.9981, where y is the sample amount and x is the peak area response from CAD. The individual recoveries of etidronate at each level were in the range of 95.9–102.6%.

Linearity of the phosphate and phosphite peaks was assessed at six concentration levels from 0.025 to 0.075 mg/mL, corresponding to 0.5–1.5% of the etidronate target assay concentration of 5.0 mg/mL. All impurity linearity samples were prepared in the presence of 100% etidronate (5.0 mg/mL). The calibration curve of phosphate is $y = 0.0214x^{0.8494}$ with a correlation coefficient (R^2) of 0.9953, where y is the sample amount and x is peak area response from CAD (Fig. 6). The individual recoveries of phosphate were in the range of 94.5–103.6%. The calibration curve of phosphite is $y = 0.0259x^{1.028}$ with a correlation coefficient (R^2) of 0.9956 (Fig. 7). The individual recoveries of phosphite were in the range of 96.5–107.9%.

3.3. Accuracy

The accuracy of the method was evaluated on the spiked samples using a 1-analyst/2-day/2-run design. Two columns with different lots of stationary phase were used. Results were summarized in Tables 1–3. The %recoveries were calculated from the corresponding calibration curves obtained from the linearity experiments.

The accuracy of the recovery for etidronate was evaluated at 80, 100 and 120% of the assay concentration levels, which correspond to 4.0, 5.0 and 6.0 mg/mL of etidronate, respectively. The mean recoveries for the 80, 100 and 120% spiked samples from each run were in the range of 99.0–102.5% with no specific trend. The overall mean recoveries from the two runs (calculated from individual values) were 99.2, 100.9 and 100.1% for the 80, 100 and 120% spiked samples, respectively.

The accuracy of the recoveries for phosphate and phosphite was evaluated by spiking each impurity at approximately 0.025, 0.05 and 0.075 mg/mL, which corresponds to 0.5, 1.0 and 1.5%, respectively, of the etidronate target concentration. The recovery samples were prepared in the presence of 5 mg/mL of etidronate. The %recoveries were calculated from the corresponding calibration curves from the linearity experiments.

The mean recoveries for phosphate at 0.5, 1.0 and 1.5% levels from each run were in the range of 96.5–101.6%. The overall mean recoveries for phosphate from the two runs were 98.9, 100.5 and 98.1% for the 0.5, 1.0 and 1.5% spiked samples, respectively. The mean recoveries for phosphite at 0.5, 1.0 and 1.5% levels from each run were in the range of 95.4–102.8%. The overall mean recoveries from the two runs for phosphite at 0.5, 1.0 and 1.5% levels were 97.0, 98.8 and 100.1%, respectively.

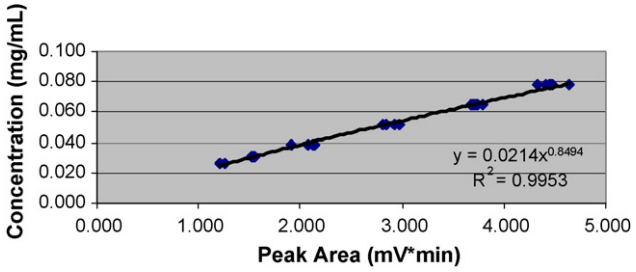

Fig. 6. Calibration curve of phosphate.

3.4. Precision

The precision of the method, expressed as the relative standard deviation (R.S.D.), was assessed using the spiked samples with a 1-analyst/2-day/2-run design. Two columns with different lots of stationary phase were used. The system precision of each run was calculated using 6 injections of the same assay solution. The intermediate precision of the method was calculated using all individual results from both runs. In addition, the precision of the method was evaluated using the 200 mg etidronate tablets.

As indicated in Tables 1–3, the precisions for the 80, 100 and 120% of etidronate spiked samples from each run were in the range of 1.1–2.9%. The intermediate precisions for etidronate from the spiked samples were 2.1, 2.3 and 2.0%, corresponding to 80, 100 and 120% of the spiked samples, respectively. The %R.S.D. from triplicate injections of the 200 mg etidronate tablet samples (total of six sample preparations) ranged from 0.1 to 1.4%.

The precisions for the 0.5, 1.0 and 1.5% phosphate spiked samples from each run were in the range of 0.6–2.2%. The intermediate precisions for phosphate were 1.6, 1.5 and 2.8%, corresponding to 0.5, 1.0 and 1.5% of the phosphate spiked samples, respectively.

The precisions for the 0.5, 1.0 and 1.5% phosphite spiked samples from each run were in the range of 0.8–2.5%. The intermediate precisions for phosphite were 2.5, 1.9 and 2.6%,

Table 1
Accuracy/precision of etidronate

Concentration (mg/ml)	Level (%)	Day 1		Day 2		Mean recovery (%)	Intermediate precision (%)
		%Recovery ($n=6$)	%R.S.D. ($n=6$)	%Recovery ($n=6$)	%R.S.D. ($n=6$)		
4.0	80	99.5	2.6	99.0	1.8	99.2	2.1
5.0	100	99.3	1.1	102.5	2.0	100.9	2.3
6.0	120	100.5	2.9	100.2	1.1	100.1	2.0

Table 2
Accuracy/precision of phosphate

Concentration (mg/mL)	Level (%)	Day 1		Day 2		Mean recovery (%)	Intermediate precision (%)
		%Recovery ($n=6$)	%R.S.D. ($n=6$)	%Recovery ($n=6$)	%R.S.D. ($n=6$)		
0.025	0.5	98.4	2.2	98.1	0.7	98.9	1.6
0.05	1.0	101.3	2.1	101.6	1.7	100.5	1.5
0.075	1.5	98.2	1.9	96.5	0.6	98.1	2.8

corresponding to 0.5, 1.0 and 1.5% of the target concentration level, respectively.

3.5. Limit of quantitation (LOQ) and limit of detection (LOD)

The LOQ and LOD of this method was determined using signal-to-noise ratio by injecting a series of diluted solutions with known concentration. The relative standard deviations were also evaluated at LOQ (S/N = 10:1) and LOD (S/N = 3:1) levels during method validation.

The LOQ and LOD for etidronate were 144 and 50 ng, with %R.S.D. of 3.1 and 14.7, respectively.

The LOQ for phosphate and phosphite was 75 ng with R.S.D. of 2.0%. When the injection volume is 5 μL, the LOQ level for phosphate and phosphite was equivalent to 0.015 mg/mL, or 0.3% of the etidronate assay concentration. The LOD for phosphate and phosphite is 50 ng, with R.S.D. of 4.4% for phosphate and 10.7% for phosphite.

3.6. Stability

The stability evaluation, conducted on the 100% spiked samples, indicated that the solutions are stable for at least 10 days at room temperature. The recovery was 99.3% with an R.S.D. of 1.1% at time zero and 99.4% with 1.4% R.S.D. at day 10. No degradation products above the LOD were observed at the end of the study.

3.7. System suitability

As summarized in Table 4, the method demonstrated satisfactory system suitability with respect to peak area, retention time, resolution and peak tailing.

4. Discussion

4.1. Quantification using calibration curve vs. response factor of a corresponding working standard

A previous publication [24] as well as the results from this study indicated that, unlike the UV signals, the signals from CAD are not linear at the parent assay levels. The calibration curve for the etidronate response in the range from 1.25 to 7.5 mg/mL, corresponding to 25–150% of the assay concentration level of 5 mg/mL, can be expressed by $y = ax^b$ where x is the peak response (area), y the sample amount, and b is the exponential response factor, which reflects sensitivity. For the etidronate assay, $a = 0.0043$ and $b = 1.4769$. However, when the level is very low or when the range is small, the calibration curve is close to a linear curve [25].

Calculation results using a calibration curve vs. response factor of a corresponding working standard were summarized in Table 5. The etidronate, phosphate and phosphite peaks were quantified using 5 mg/mL etidronate, 0.05 mg/mL phosphate or 0.05 mg/mL phosphite working standard, respectively. The results indicated that it is feasible to use a work-

Table 3
Accuracy/precision of phosphite

Concentration (mg/mL)	Level (%)	Day 1		Day 2		Mean recovery (%)	Intermediate precision (%)
		%Recovery ($n=6$)	%R.S.D. ($n=6$)	%Recovery ($n=6$)	%R.S.D. ($n=6$)		
0.025	0.5	98.6	1.9	95.4	1.9	97.0	2.5
0.05	1.0	98.4	0.8	99.1	2.5	98.8	1.9
0.075	1.5	98.8	1.8	102.8	1.5	100.1	2.6

Table 4
Summary of system suitability results

Compound	Run 1				Run 2			
	%R.S.D. of peak area	RRT	Resolution (USP)	Tailing (USP)	%R.S.D. of peak area	RRT	Resolution (USP)	Tailing (USP)
Etidronate	0.8	1.0	–	1.17	2.0	1.0	–	1.5
Phosphate	2.1	0.47	5.53	1.09	1.7	0.47	5.85	1.2
Phosphite	0.8	0.71	4.58	1.22	2.5	0.70	4.77	1.4

Table 5
Quantification using calibration curve vs. response factor of a working standard

	Concentration (mg/mL)	Using calibration curve of corresponding working standard		Using response factor of corresponding working standard	
		%Recovery	%R.S.D.	%Recovery	%R.S.D.
Phosphate	0.05	101.3	2.1	101.4	2.4
Phosphite	0.05	98.4	0.8	98.0	2.4
Etidronate	5	99.3	1.1	100.9	0.8

Table 6
Recovery of phosphate and phosphite (0.05 mg/mL) using etidronate standard at impurity levels

	Using response factor %recovery	Using calibration curve %recovery
Phosphate	189.5	159.8
Phosphite	105.5	109.7

ing standard at a similar concentration level for quantitation purposes.

4.2. Quantification of phosphate and phosphite using etidronate standard

The response factor (peak area/concentration) of phosphate, phosphite and etidronate is 50.3, 37.5, and 24.2, respectively, using 0.05 mg/mL phosphate or phosphite and 5 mg/mL of etidronate for determination.

When etidronate standard was used for quantification, more accurate recoveries were consistently observed for phosphite, compared to those of the phosphate, as can be seen in Table 6. This observation might be attributable to the fact that, even though CAD is reportedly a universal detector with response magnitude independent of the analyte chemical properties [24], the CAD response itself is not linear and also is directly proportional to mobile phase composition. Thus the use of other standards for quantification needs well-characterized, especially when a gradient method is used.

5. Conclusions

By applying the charged aerosol detector and a mixed-mode column, a fast, direct, and stability-indicating method was developed and validated for the analysis of etidronate and its related substances. Similar methodologies may be applied for pharmaceutical analysis of other bisphosphonates, which may significantly improve the efficiency and accuracy of the analysis of these pharmaceutical compounds.

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