ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 1545–1550

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

# High-performance ion-exchange chromatography with in-line complexation of bisphosphonates and their quality control in pharmaceutical preparations

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Received for review 13 April 1995

**Abstract**

An ion-exchange chromatographic method using an anion-exchange column was developed for the analysis of a number of bisphosphonates. The bisphosphonates were in-line complexed by copper(II) ions and added to the acidic eluent, to yield an UV-absorbing complex. Chromatographic parameters were studied for eight different bisphosphonates, particularly amino-1-hydroxyalkyl-1,1-bisphosphonates; special attention was paid to the relationship between retention and complex formation. The method was applied to the quality control of pamidronate injection concentrate and olpadronate tablets. The lower detection limit was 8 ng of disodium pamidronate, and the inter-assay precision was 1.0% for both pamidronate and olpadronate standard solutions and 1.8% for a 3 mg ml$^{-1}$ disodium pamidronate injection concentrate. The method was compared with a previously described ion-exchange chromatographic method with conductivity detection, without copper(II) ions in the eluent.

*Keywords:* Bisphosphonates; Ion-exchange chromatography; Pamidronate; Olpadronate; Copper(II) complex; In-line complexation

## 1. Introduction

Recently, a simple, fast and standard method for the analysis of bisphosphonates and foscarnet was developed by den Hartigh et al. [1]. In particular, the pharmaceutical quality control of pamidronate (3-amino-1-hydroxypropane-1,1-bisphosphonate, APD) dosage forms was investigated. The method was based on high-performance ion-exchange chromatography (IEC) with conductivity detection; a nitric acid solution was applied as a mobile phase on an anion-exchange column. Analogous to this method, Tsai et al. [2] presented an assay for alendronate (4-amino-1-hydroxybutane-1,1-bisphosphonate), the butane-analogue of pamidronate, also in pharmaceutical preparations. Besides the lack of sensitivity for bioanalysis, mentioned previously by den Hartigh et al. [1], a serious drawback for widespread application as a quality control method is the availability of the detector required; a conductivity detector is rarely available in a pharmaceutical quality control laboratory. In order to increase the potential of this method it was decided not to choose phosphor selective photometric detection [3] or post-column derivatization with colorimetric- [4] or UV/vis-detection [5]. A simple modification was made of the method of Tsai et al. [6], who developed a technique for alendronate based on capillary zone electrophoresis with in-line copper(II) complexation and UV-detection. In aqueous solution, copper(II) ions react with bisphosphonates to form soluble

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*SSDI* 0731-7085(95)01572-8

Table 1
Bisphosphonates: the basic structure (bisphosphonic acids) is $RC(PO_3H_2)_2R'$

| R | R' | Abbreviation | Non-proprietary name |
|---|---|---|---|
| OH | $CH_3$ | EHDP | Etidronate |
| OH | $CH_2CH_2NH_2$ | APD | Pamidronate |
| OH | $CH_2CH(C_6H_5)NH_2$ | APPD | |
| OH | $CH_2CH_2N(CH_3)_2$ | | Olpadronate |
| OH | $CH_2CH_2CH_2NH_2$ | | Alendronate |
| OH | $CH_2CH(NH_2)CH_3$ | ABD | |
| OH | $(CH_2)_5NH_2$ | | Neridronate |
| Cl | Cl | | Clodronate |

UV-absorbing complex [7,8], facilitating their detection as a copper(II) complex (1:1 stoichiometry) by UV-absorption. Tsai et al. [6] also reported that attempts to develop a HPLC method, based on the same detection principle, were not yet successful. In the present paper such a method, based on anion-exchange chomatography, is presented. The chromatographic behaviour of several bisphosphonates (Table 1), phosphate and nitrate is reported, together with the validation of the quality control of pamidronate in a parenteral solution and olpadronate (3-dimethylamino-1-hydroxypropane-1,1-bisphosphonate) in oral dosage forms.

## 2. Experimental

### 2.1. Chemicals

The disodium salts of pamidronate and etidronate (1-hydroxyethane-1,1-bisphosphonate, EHDP) were obtained from Bufa B.V. (Uitgeest, The Netherlands); disodium olpadronate and olpadronic acid were obtained from Henkel (Düsseldorf, BRD). All other bisphosphonates and bisphosphonic acids were generously provided by Dr. C.W.G.M. Löwik (Department of Endocrinology, University Hospital Leiden, The Netherlands). The pharmaceutical preparations of disodium pamidronate, disodium olpadronate and water for HPLC were manufactured in the department's pharmacy. Nitric acid (65% w/w, Suprapur), copper(II) nitrate (analytical grade) and sodium dihydrogenphosphate (analytical grade) were obtained from Merck (Darmstadt, BRD). ILC Regenerant A was from Millipore-Waters (Etten-Leur, The Netherlands).

### 2.2. Equipment

Chromatographic analyses were performed using a Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, USA) with a Marathon autoinjector and a built-in column thermostat (Spark Holland B.V., Emmen, The Netherlands), equipped with a 7010-80 Rheodyne injection valve (Rheodyne Inc., Cotati, CA, USA) an a 20-µl sample loop. The detector was a Spectroflow 773 variable wavelength detector (Kratos Analytical Instruments, Westwood, NJ, USA) and data were recorded on a IPC Dynasty HE 486DX personal computer (IPC Corp. (PTE)LTD, Singapore), equipped with a Gynkosoft chromatographic datasystem (SOFTRON GmbH, Gräfelfing, BRD).

### 2.3. Chromatographic conditions

Flushed loop injections (20 µl) were made on a 150 × 4.6 mm i.d. IC-PAK Anion HC column packed with 10-µm particles (capacity = 30 µeq $ml^{-1}$) (Waters Corp., Division of Millipore, Milford, MA, USA). The column temperature was 30°C. The column was pretreated by eluting ILC Regenerant A and 0.1 M nitric acid, respectively, at 1 ml $min^{-1}$ for 0.5 h. At the same flow-rate 1.5 mM nitric acid and 0.5 mM copper(II) nitrate in water was used as eluent; for retention studies several other concentrations of both nitric acid and copper(II) nitrate were used. The UV-detection wavelength was 245 nm.

### 2.4. Procedures

For the validation of the assay of the two pharmaceutical preparations, aqueous standards of 688 and 758 µg $ml^{-1}$ disodium pamidronate and 1209 and 1278 µg $ml^{-1}$ disodium olpadronate were prepared and stored at 4°C. Dilutions were made daily.

From a disodium pamidronate injection concentrate (3 mg $ml^{-1}$, 5 ml), 1 ml was diluted to 100 ml; 20 µl was injected directly into the HPLC system. The amount was calculated after injection of 25-fold dilutions of the two standards.

A 5-mg disodium olpadronate tablet (62–63 mg total weight) was disintegrated in 100 ml water and ultrasonicated for 5 min. Part of the suspension was centrifuged for 4 min at $3.6 \times 10^3 g$ and 20 µl was injected into the HPLC system. The amount was calculated af-

ter injection of 25-fold dilutions of the two standards.

## 3. Results and discussion

The presence of copper(II) ions in the eluent strongly reduced the retention of bisphosphonates in this IEC system. Chromatographic retention is dependent on the form of the analyte in the eluent; several protonation and complexation equilibria with their particular dissociation constants ($K_a$) and complexation constants ($K_c$) can play a role. For example, in the analysis of pamidronate (pH eluent = 2.8), three equilibria have to be considered ($APD^{3-} = {}^{+}H_3N-CH_2-CH_2-C(PO_3^{2-})_2OH$):

$$H_3APD \rightleftharpoons H_2APD^- + H^+$$

$$K_{a,2}(APD) = [H_2APD^-][H^+][H_3APD]$$

$$H_2APD^- + Cu^{2+} \rightleftharpoons CuH_2APD^+$$

$$K_{c,1}(Cu-APD) = [CuH_2APD^+]/([H_2APD^-][Cu^{2+}])$$

$$CuH_2APD^+ \rightleftharpoons CuHAPD + H^+$$

$$K_{a,1}(Cu-APD) = [CuHAPD][H^+]/[CuH_2APD^+].$$

$pK_{a,2}(APD)$ was determined at 2.7 by potentiometric titration with sodium hydroxide. Reported complex formation constants for other bisphosphonates are: $K_{c,1}$(Cu–EHDP) = $6.3 \times 10^4\ M^{-1}$ for etidronate [7] and $K_{c,1}$(Cu-alendronate) = $1.7 \times 10^4\ M^{-1}$ [8]. The similarity between these two constants indicates that $K_{c,1}(Cu-APD) > 1 \times 10^4\ M^{-1}$. Thus, for an eluent copper(II) concentration of $5 \times 10^{-4}$ M, $[CuH_2APD^+]/[H_2APD^-] = K_{c,1}(Cu-APD)[Cu^{2+}] > 5$. Therefore, under the applied chromatographic conditions, $CuH_2APD^+$ will be the main form of pamidronate if, as to be expected after comparison with etidronate data [7], $pK_{a,1}(Cu-APD) > 2.8$. The positively charged pamidronate-complex can hardly be retained on an anion-exchange column and complex formation competes with the binding of the free pamidronate anion ($H_2APD^-$) to the stationary phase. Therefore, the addition of copper(II) ions to the eluent increases the total pamidronate concentration in the eluent and decreases the retention time. The behaviour of other bisphosphonates will be similar. The complexation equilibrium, which results in $CuH_2APD^+$ as the main form of pamidronate during the separation, is also responsible for the relatively small influence of pH variation (of the non-buffered eluent) on both retention and detection. For example, if $K_{c,1}(Cu-APD) = 1.6 \times 10^4\ M^{-1}$, $[CuH_2APD^+]$ is sufficiently smaller than $[Cu^{2+}]$ and $pK_{a,1}$ (Cu–APD) is sufficiently greater than the pH, an increase in pH from 2.7 to 2.8 causes $[H_3APD]$ to decrease by 19%, while $[H_2APD^-]$ (important for retention) and $[CuH_2APD^+]$ (important for detection) both increase by only 2%.

The absorption maximum of the copper(II)–pamidronate complex in the eluent was 230–235 nm, not differing from the peak at 230 nm for the copper(II)–alendronate complex, reported by Ostovic et al. [8]. However, the optimal signal-to-noise ratio in the applied eluent was obtained at 245 nm because the detector noise increased at lower wavelengths by the UV-absorption of the copper(II) ion.

When water was injected, the resulting chromatogram showed two negative peaks (Fig. 1). The first peak indicated an unretained component and was due to the absence of copper(II) and hydrogen ions in the injected sample. The second negative peak was caused by the absence of nitrate in the sample; both peaks appeared in all chromatograms of samples dissolved in pure water.

The retention times of several bisphosphonates, nitrate and phosphate are listed in Table 2 and typical chromatograms are shown in Fig. 1. The detector demonstrated an equal sensitivity for all 1-hydroxyalkyl-1,1-bisphosphonates: for the peak area divided by the molar concentration, the RSD was 5.1% ($n = 6$) without correction for the moisture content of the raw material; alendronate was not taken into account in calculating the RSD because the raw material of this bisphosphonate was strongly contaminated. Retention of the bisphosphonates varied strongly between the different types of bisphosphonates. The retention could be influenced by varying the nitric acid concentration, causing variations in eluting strength and pH, or the copper(II) nitrate concentration, causing variations in eluting strength and formation of the copper complex in the aqueous eluent. Varying the copper(II) nitrate concentration should not change the pH; between the four eluent compositions a variation of pH 0.05 was measured. The influences of both parameters on the retention of several of the compounds listed in Table 2 are shown in

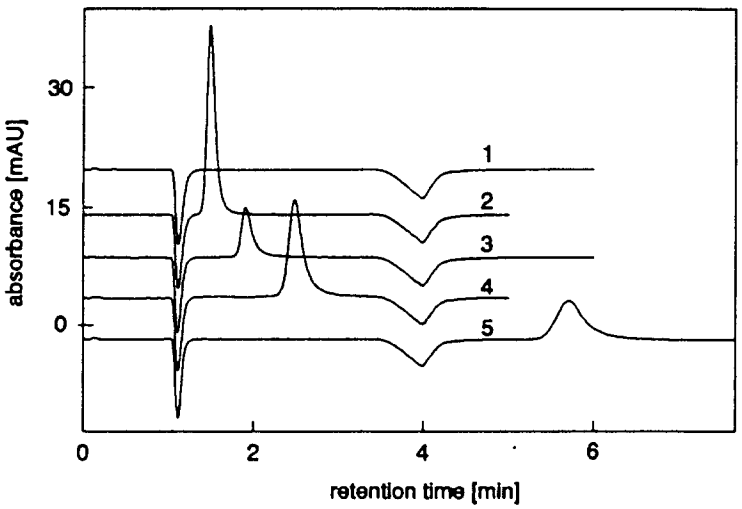


Fig. 1. Typical chromatograms of a few individual bisphosphonates. Eluent: 1 mM copper(II) nitrate and 2 mM nitric acid. (1) Blank (=water); (2) 51 μg ml$^{-1}$ neridronic acid; (3) 20 μg ml$^{-1}$ disodium pamidronate; (4) 55 μg ml$^{-1}$ APPD; (5) 37 μg ml$^{-1}$ disodium etidronate.

Fig. 2. The amino-1-hydroxyalkyl-1,1-bisphosphonates were only slightly retained on the column material; to obtain acceptable retention times and sufficient separation from nitrate for pamidronate and olpadronate, for example, a low ionic strength eluent particularly with a low copper(II) concentration was required. However, in order to facilitate detection, the last requirement is in contradiction with the desired formation of complexed bisphosphonate; sufficient copper(II) ions should be available in the eluent. For the assays of pamidronate and olpadronate, 0.5 mM copper(II) nitrate and 1.5 mM nitric acid were used as a compromise. The low copper(II) concentration appeared to cause overloading effects of the mobile phase, owing to a lack of copper(II) ions, influencing the complexation equilibrium; the relative amount of uncomplexed bisphosphonate (and the retention time) will increase at overly high bisphosphonate concentrations in the eluent. This effect will result in a concave distribution curve and was visible in the chromatograms as fronting peaks (Fig. 3). This Figure shows that the linear part of the distribution curve ended at a sample concentration of approximately 50 μg ml$^{-1}$ for both disodium pamidronate and disodium olpadronate. In other words, the formation of the copper(II) bisphosphonate complex needs an excess of copper(II) ions of at least a factor of three in order to assure complete complexation. For application as a quality control method, the limited sample capacity of the analytical method necessitated dilution of all samples prior to injection. For application in bioanalysis, the limited selectivity of IEC and possibly also the insufficient sensitivity of the UV-detection will be the limiting factors. In order to develop a bioanalytical assay for pamidronate it was decided to investigate reversed-phase HPLC in combination with derivatization and fluorescence detection. Results will be presented soon.

Table 2
Retention[a] of bisphosphonates and other compounds

| Compound | Capacity factor ($k'$) |
|---|---|
| Neridronate | 0.33 |
| Alendronate | 0.48 |
| Pamidronate | 0.71 |
| ABD | 0.80 |
| Olpadronate | 0.82 |
| APPD | 1.22 |
| Etidronate | 4.12 |
| Clodronate | 26.4 |
| Nitrate | 2.58 |
| Phosphate | 6.5 |

[a] Eluent: 1 mM copper(II) nitrate and 2 mM nitric acid. The dead time ($t_0$ = 1.11 min) was taken from the negative "proton/copper(II) peak".

In the range from 0 to 60 μg ml$^{-1}$, the detector response was linear and the calibration lines for both pamidronate and olpadronate were not significantly different. Based on the molar concentration, the combination data of the two bisphosphonates in this concentration range were used to construct the calibration curve. Calculated by least-squares regression, the calibration line was: $y$ [mAU min] =

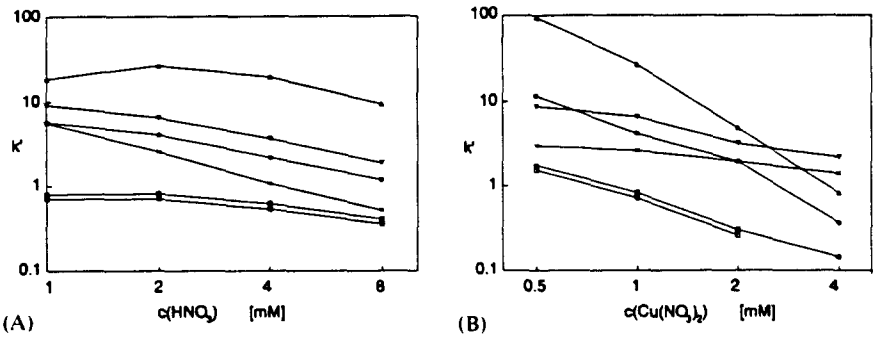


Fig. 2. Influence of the eluent composition on the retention of several bisphosphonates and other compounds. (□) Pamidronate; (■) olpadronate; (×) nitrate; (○) etidronate; (▽) phosphate; (△) clodronate. (A) Influence of the nitric acid concentration (the copper(II) nitrate concentration is 1 mM). (B) Influence of the copper(II) nitrate concentration (the nitric acid concentration is 2 mM).

$-0.018(\pm 0.032) + 14.96(\pm 0.11)x$ [mM] ($r^2 = 0.9992$; $n = 16$). The lower limit of detection (LLD; signal-to-noise ratio = 3) was about 0.4 μg ml$^{-1}$ disodium pamidronate (8 ng; 20 μl injection). The lower limits of quantification (LLQ) were calculated from calibration lines in the lowest concentration range (0–1.4 μg ml$^{-1}$). For disodium pamidronate the calibration line obtained was: $y$ [mAU min] = $0.0014(\pm 0.0023) + 0.0452(\pm 0.0016)x$ [μg ml$^{-1}$] ($r^2 = 0.992$; $n = 8$) and the corresponding LLQ, where the mean standard deviation is 10% of $y$, was 0.5 μg ml$^{-1}$. For disodium olpadronate: $y$ [mAU min] = $0.0013(\pm 0.0035) + 0.0373(\pm 0.0030)x$[μg ml$^{-1}$] ($r^2 = 0.96$; $n = 8$), resulting in 0.9 μg ml$^{-1}$ as the LLQ.

The inter-assay precision (on four subsequent days) was excellent. The RSD values were: 0.5% ($n = 4$) and 1.5% ($n = 4$) for both disodium pamidronate standard solutions (diluted 25-fold daily); 1.1% ($n = 4$) and 1.0% ($n = 4$) for both disodium olpadronate standard solutions (diluted 25-fold); and 1.1% ($n = 15$) for the 3 mg ml$^{-1}$ disodium pamidronate injection concentrate (100-fold diluted). The accuracy, calculated as a percentage of the declared amount, and precision of the complete analytical method was also determined on four subsequent days. The intra-assay ($n = 6$) accuracy (97.7%) and precision (0.7%), and the inter-assay ($n = 11$) accuracy (99.8%) and precision (1.8%) of the pamidronate injection concentrate were measured. Also, for olpadronate tablets, the intra-assay ($n = 6$) accuracy (105.5%) and precision (5.2%), and the inter-assay (four days; $n = 12$) accuracy (104.2%) and precision (5.0%) were measured. The larger variation in analysis of the olpadronate tablets was probably due to the variance in the tablet potency. To investigate this more homogeneous tablet samples were analyzed; samples were prepared from 10 tablets mixed together and disintegrated in 1 l of water. Then, the intra-assay ($n = 5$) precision was 3.0%, supporting the explanation given for the relatively poor precision of the single tablet assay.

## 4. Conclusions

Applying in-line complexation in HPLC can be a simple and subtle method to improve a chromatographic separation and/or detection. For bisphosphonates, this IEC method showed the following five advantages compared to the method without in-line complexation:

First, a more common detection method could be applied (UV-absorption versus conductivity). Second, detection was more sensitive (for pamidronate, improvement by a factor 12 of the LLD was obtained). Third, the precision was improved because of a more stable detection method (the variation of repeated analysis of pamidronate and olpadronate decreased by a factor of approximately 2). Combined with the more sensitive detection the LLQ of pamidronate was improved by a factor of 50. Fourth, with copper(II) ions in the eluent there was an extra tool for achieving retention of the analyte, particularly in order to separate a bisphosphonate from components of a different nature, e.g. phosphate ions. However, pamidronate and olpadronate still could not be separated from each other, only discriminated. Fifth, the method was more specific for bisphosphonates because the sensitive detection is restricted to analytes which complex with copper(II) ions. This will be advantageous when a

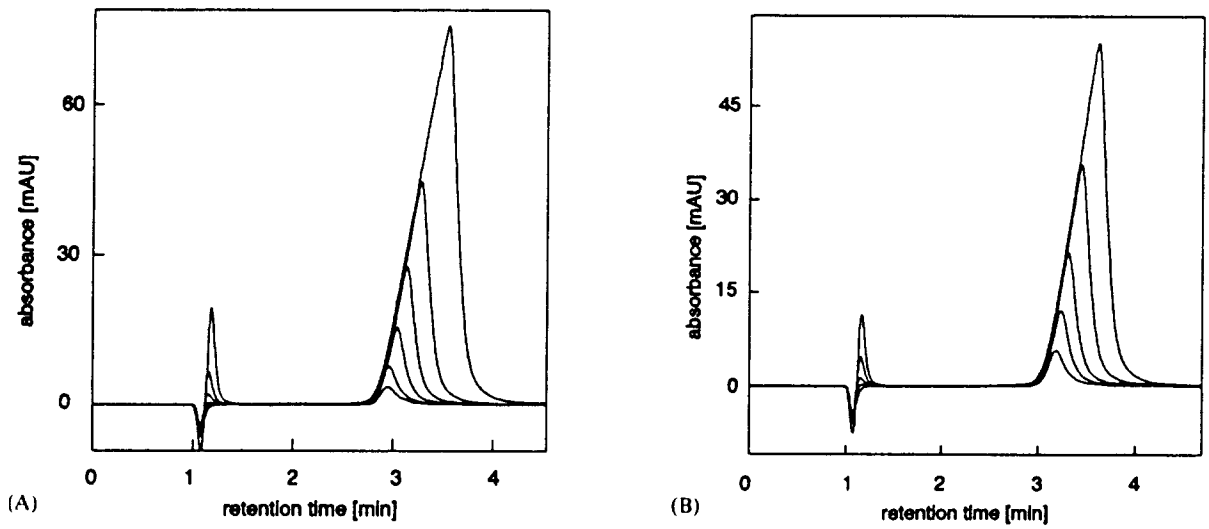


Fig. 3. Chromatograms of pamidronate and olpadronate at different concentrations, showing an overloading effect of the mobile phase. Eluent: 0.5 mM copper(II) nitrate and 1.5 mM nitric acid. (A) Disodium pamidronate concentrations are 17, 34, 69, 138, 276 and 688 μg ml$^{-1}$. (B) Disodium olpadronate concentrations are 30, 60, 121, 242 and 484 μg ml$^{-1}$.

bisphosphonate sample with an excess of other types of ions has to be analyzed.

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