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Ion-exchange liquid chromatographic analysis of bisphosphonates in pharmaceutical preparations

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Abstract: A simple, fast and uniform method has been developed for the quantitative determination of bisphosphonates in the quality control of pharmaceutical preparations. The method is based on ion-exchange liquid chromatography with conductivity detection. Separation is performed on a Waters IC-PAK Anion column using 2 mM nitric acid or 25 mM succinic acid as the mobile phase. Retention of the bisphosphonates can be influenced by pH and the anion concentration of the mobile phase. Sensitivity and selectivity are sufficient for the assay of bisphosphonates in bulk drug and pharmaceutical preparations. Sample preparation comprises dissolution or dilution of the sample in the mobile phase followed, if necessary, by filtration prior to HPLC analysis. Since the method is stability indicating, it is also well suited for shelf-life studies of bisphosphonate pharmaceutical preparations. Validation of the analytical method for the assay of pamidronate injection indicated an intra-day reproducibility of 1.7% (n = 6) and an inter-day reproducibility of 2.7% (n = 6). A linear relationship between response and concentration was found in the concentration range studied from 200 ng to 10 μg pamidronate per 20 μl injected. The lower limit of detection (signal-to-noise ratio = 3) of pamidronate was about 100 ng.

Keywords: *Ion-exchange chromatography; bisphosphonates; pamidronate; APD; foscarnet.*

Introduction

Phosphonates form a group of compounds characterized by the presence of one or more O=P—$(OH)_2$ groups attached to a carbon atom. Bisphosphonates are analogues of pyrophosphate, an endogenous substance found in human urine and plasma, which plays a role in calcium homeostasis. Attempts to treat calcium metabolic disorders with exogenous pyrophosphate have been unsuccessful owing to the rapid hydrolysis of the P—O—P bond. Bisphosphonates, which possess a P—C—P rather than a P—O—P linkage, are stable towards chemical and enzymatic hydrolysis [1].

Several phosphonates have recently been introduced into clinical practice. Trisodium phosphonoformate (foscarnet), a monophosphonate, is an antiviral drug with *in vitro* activity against, for example, cytomegalovirus [2]. Bisphosphonates, and 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (pamidronate or APD) in particular, are potent inhibitors of bone resorption. They are being used successfully in those diseases where increased bone resorption is prominent such as Paget's disease [1, 3] and tumour hypercalcemia [4]. Prospective, long-term studies on the efficacy of bisphosphonates in osteoporosis are in progress [5].

Sensitive and selective analysis of phosphonates is necessary in quality control and in shelf-life studies on formulated products as well as in biological fluids especially in bioavailability and pharmacokinetic studies; analysis is complicated because of inadequacies in both the chromatographic separation and the detection of these compounds. A non-chromatographic method based upon titration with thorium diaminocyclohexanetetraacetate using a colorimetric endpoint determination has been reported for 1-hydroxyethylidene-1,1-bisphosphonate (etidronate) in faeces and urine [6].

For the analysis of the same compound in plasma and urine Bisaz *et al.* [7] developed a procedure based on coprecipitation of etidronate with calcium phosphate, elimination of inorganic phosphate as an insoluble triethylamine–phosphomolybdate complex, decomposition of the P—C—P bond with ultraviolet light and spectrophotometric determination of the inorganic phosphate released. Analysis of dichloromethylene-1,1-bisphosphonate (clodronate) by ion-exchange liquid chro-

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matography followed by on-line phosphor-selective flame-photometric detection has been reported by Chester *et al.* [8]. Ion-pair reversed-phase HPLC with UV-detection is feasible for the determination of (4-chlorophenyl)-thiomethylene bisphosphonic acid, one of the few representatives of this class of compounds with a chromophore sufficiently sensitive for direct UV-detection [9]. A method for the quantitation of APD in urine and plasma, based on derivatization with fluorescamine and fluorescence detection was described recently [10, 11]. Also recently, the determination of 4-amino-1-hydroxybutane-1,1-bisphosphonate (alendronate) in pharmaceutical dosage forms [12, 13] and biological fluids [14] was reported. All three methods utilize pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC) [12], *o*-phthalaldehyde (OPA) [13] and 2,3-naphthalene dicarboxyaldehyde-cyanide (NDA-CN) [14], respectively, followed by HPLC. Determination of phosphonoformate in biological fluids by reversed-phase HPLC with electrochemical detection has been described [15, 16]. A general method for the derivatization of α-aminophosphonic acids for HPLC analysis is described by Huber *et al.* [17]. The method involves conversion of the amino-group with FMOC followed by esterification of the phosphonic acid moiety utilizing triethyl orthoformate. Finally, the derivative is analysed by chromatography using a reversed-phase system.

Assay of polyphosphonates by ion chromatography with conductivity detection [18] or UV-spectrophotometric detection after post-column reaction with ferric nitrate [19] has been described. Hirai *et al.* [20] have reported the HPLC analysis of monophosphonates by ion-exchange chromatography in combination with flow-injection analysis as a post-column reaction detector. With minor modifications Waldhoff and Sladek [21] have applied this method to the analysis of bisphosphonates. Combination of this complex detection method with an efficient ion-chromatographic separation technique led Daley-Yates *et al.* [22] to the development of a method to analyse pamidronate and related bisphosphonates in plasma and urine. After coprecipitation of the analyte with calcium phosphate, the sample components were separated by high-performance, metal-free, anion chromatography coupled with a simplified two-stage post-column reaction, finally resulting in the formation of the phosphomolybdate chromophore; visible detection was performed at 820 nm.

Gas chromatographic analysis with flame ionization detection of etidronate and several other bisphosphonates is feasible after conversion of the free phosphonic acids into trimethylsilyl (TMS) derivatives [23, 24]. Capillary gas chromatography–mass spectrometry (GC–MS) has been reported for the analysis of clodronate in urine [25].

All these methods involve complicated sample pretreatment procedures or require instrumentation typically not available in a routine pharmaceutical analysis laboratory. Moreover, they lack the simplicity, convenience and speed preferred for an analytical assay in pharmaceutical quality control. Therefore a study was started aimed at the development of a simple uniform analytical method for the determination of bisphosphonates currently of pharmacotherapeutic interest. This paper reports such a method based on ion-exchange liquid chromatography and conductivity detection. Sample preparation simply comprises dissolution or dilution of the sample in the mobile phase, followed, if necessary, by filtration prior to HPLC analysis. Sensitivity and selectivity are sufficient for the assay of bisphosphonates in the bulk drug and in pharmaceutical preparations.

Experimental

Chemicals

Pamidronate (as the disodium salt) and its dimethylated analogue (dimethyl-APD, disodium salt) were obtained from BUFA BV (Uitgeest, The Netherlands). Foscarnet sodium was a gift from Astra (Södertälje, Sweden). Etidronate (sodium salt) was obtained from Nogepha (now Centrafarm, Etten-Leur, The Netherlands). The internal standards 3-phenyl-3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid and 6-amino-1-hydroxyhexylidene-1,1-bisphosphonic acid were generously provided by Dr C.W.G.M. Löwik (Department of Endocrinology, University Hospital Leiden, The Netherlands). ILC Regenerant A was from Millipore–Waters (Part no. JWNL00700, Etten-Leur, The Netherlands). Nitric acid 65%, Suprapur was obtained from Merck (Darmstadt, Germany).

All other chemicals were supplied by standard chemical sources and were of analytical reagent grade.

Apparatus

The chromatographic system comprised a LKB 2150 HPLC pump (LKB-Produkter AB, Bromma, Sweden), equipped with a Waters high-sensitivity noise filter and restrictor tube (Millipore–Waters, Milford, MA), a Promis autosampler (Spark, Emmen, The Netherlands), a Waters temperature control system and a Waters 430 conductivity detector (both from Millipore–Waters). The 50 × 4.6 mm i.d. column was packed with 10-μm Waters IC-PAK Anion and was used in combination with a Waters Guard-PAK precolumn module. Chromatograms were recorded on a Model SP 4270 integrator (Spectra-Physics, San José, CA).

Chromatographic conditions

All chromatographic analyses were performed at a column temperature of 35 ± 0.5°C. The mobile phases were prepared from nitric acid and several organic acids, usually at low millimolar concentrations. Of all acids tested, two were selected for a thorough investigation of the analytical methodology: 2 mM nitric acid and 25 mM succinic acid. The mobile phases were filtered through an 0.5-μm filter and de-aerated ultrasonically before use. The flow rate was 1.0–1.2 ml min^{-1}, depending on the mobile phase composition. The injection volume was 20 μl, unless otherwise indicated.

Procedures

Column pretreatment. Before use the Waters IC-PAK Anion column as well as the entire flowpath of the chromatographic system had to be pretreated by flushing with ILC Regenerant A, water, 100 mM nitric acid, water and again ILC Regenerant A; each solvent was flushed for 30 min at a flow rate of 1.2 ml min^{-1}. After conditioning, the column could be used with the selected mobile phase for several months.

Standard solutions. Stock standard solutions of pamidronate, dimethyl-APD, etidronate, foscarnet and the internal standards were prepared in water purified by deionization and kept at 4°C. Working standard solutions of 5–2500 μg ml^{-1} were prepared weekly by appropriate dilution of the stock solutions.

Sample preparation. The bulk drug was dissolved in an appropriate amount of purified water or mobile phase to which the internal standard had been added and was analysed as such. Tablets were assayed by suspending the whole tablet in an appropriate amount of purified water or mobile phase; after automatic shaking (15 min) followed by ultrasonification (5 min), an aliquot of the solution was filtered through an 0.22-μm disposable filter and analysed by HPLC. After addition of the internal standard infusion fluids and injection concentrates were diluted to an appropriate concentration prior to HPLC analysis.

Results and Discussion

Ion-exchange liquid chromatography of bisphosphonates is hampered by the strongly adsorptive character of these compounds, particularly through the chelation of trace metal ions such as iron leaching from the stainless-steel HPLC system or present at low concentrations in some reagents. For this reason several substances of this class are used as detergents. Similar behaviour was observed by Pettersson *et al.* [15] in the development of a chromatographic assay for foscarnet where they added pyrophosphate to the mobile phase as a tailing suppressor. Because of the adsorption problem, column selection appeared to be a critical parameter in the development of the chromatographic analysis of phosphonates. Of the many ion-exchange columns tested, silica- as well as polymer-based, only the Waters IC-PAK Anion column showed an acceptable chromatographic behaviour for the test compound (pamidronate), although this could only be achieved in combination with pretreatment of the column and chromatographic system. Flushing with ILC Regenerant A, an aqueous solution containing several polyvalent anions such as EDTA and tripolyphosphate, proved to be an efficient way to prevent excessive adsorption of the phosphonates. In the authors' experience, the column can be used for several months after conditioning. If the chromatographic separation becomes worse, column performance can be restored by repeating the pretreatment of the chromatographic system. Another way to overcome interaction of the bisphosphonates with the chromatographic set-up might be the use of a completely non-metallic system [13, 19, 22]. However, even then, selection of the column

packing material remains critical, since the use of a metal-free system prevents only the chelation of bisphosphonates with metal ions but not the aspecific interaction of bisphosphonates with the column packing material. As temperature fluctuations of the mobile phase greatly influence detector performance in conductivity measurement the column and detector flow-cell were both equilibrated at 35°C. Moreover, the stainless-steel tubing between column and detector was kept as short as possible and was enclosed in 10-mm o.d. PVC tubing in order to minimize temperature fluctuations. This shielding of the metallic tubing appeared to improve the noise level of the detector signal by about a factor of 5. Another important contribution to detector noise can be pump pulsation. Therefore, the HPLC pump was equipped with a noise filter in order to smooth out flow noise.

Using pamidronate as a test-compound, the chromatographic analysis was studied with nitric acid and several organic acids as the mobile phase. Of the acids tested, nitric acid and succinic acid were selected for further optimization of the chromatographic analysis of phosphonates. Table 1 shows for both acids a comparison of the experimental characteristics in the optimized chromatographic analysis of pamidronate. The corresponding chromatograms are presented in Fig. 1. The polarity of the pamidronate signal is different in these two mobile phases: in 2 mM nitric acid the pamidronate peak corresponds to a decrease in conductivity whereas in 25 mM succinic acid an increase in conductivity is observed for the pamidronate peak. This phenomenon is obviously correlated to the large difference in background conductivity for both solvents (700 μS versus 475 μS; see Table 1). With

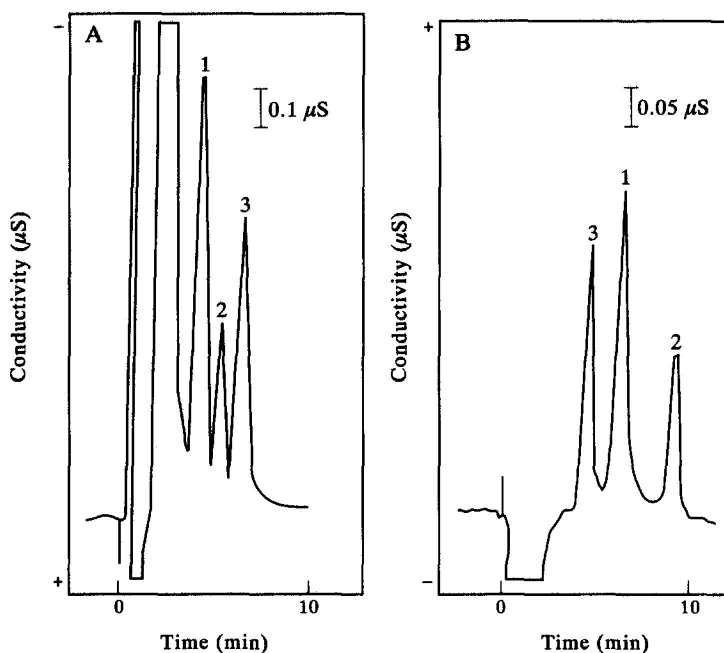

Figure 1
Representative chromatograms of an aqueous solution of (1) pamidronate (50 μg ml^{-1}), (2) phosphate and (3) internal standard. Mobile phases (A) 2 mM nitric acid; (B) 25 mM succinic acid.

Table 1
Characteristic parameters for the analysis of pamidronate using the two mobile phases studied

Mobile phase	Nitric acid	Succinic acid
Molar concentration	2 mM	25 mM
pH	2.7	3.0
Background conductivity	700 μS	475 μS
Flow rate	1.0 ml min^{-1}	1.2 ml min^{-1}
t_R pamidronate	4 min 10 s	6 min 12 s
Sensitivity	1 ng APD ≙ 1.1 nS	1 ng APD ≙ 0.4 nS
Noise	30 nS	10 nS
Detection limit pamidronate	100 ng	100 ng

2 mM nitric acid as the mobile phase, the internal standard of choice is 3-phenyl-3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid; in contrast, 6-amino-1-hydroxyhexylidene-1,1-bisphosphonic acid is preferred in the chromatographic separation with succinic acid.

Of the parameters known to influence retention time of a solute in ion-exchange liquid chromatography, column temperature and the addition of an organic modifier (acetonitrile) to the mobile phase appeared to have little influence on the retention of pamidronate. In contrast, pH and/or anion concentration of the mobile phase strongly influenced the retention behaviour of pamidronate. This influence of the molar concentration of the mobile phase using nitric acid as an example is depicted in Fig. 2. The data show that an increase in molar nitric acid concentration from, for example, 1–5 mM results in a decrease of retention for all solutes. This decrease can mainly be attributed to an increase in the concentration of nitrate ions, as is clear from comparison of Fig. 2(A) and 2(B); pH, at least in this concentration range, has only little influence on the retention of the solutes. Strong influence of pH is observed at extremely low concentrations of H^+ ions [<1 mM; see Fig. 2(C)]. Overall, control of the molar composition of the mobile phase offers good opportunities to tailor the retention of the bisphosphonate of interest. Of the two mobile phases studied extensively, nitric acid is preferred in most cases as this mobile phase proved to be more stable than succinic acid.

The influence of flow rate on the detector response was studied in the range 0.25 ml min^{-1}–1.50 ml min^{-1}, for which a decrease in the signal-to-noise ratio by a factor of almost 3 was observed. Although the data indicate a superior signal-to-noise ratio at a low flow rate, a medium flow rate of about 1 ml min^{-1} was usually preferred because of a better peak symmetry and a shorter analysis time.

Selectivity of the chromatographic system is sufficient as is demonstrated in Fig. 1 by the separation between pamidronate and phosphate, a potential impurity and decomposition product of the bisphosphonates. Figure 3 shows another example of the chromatographic selectivity: the separation of foscarnet from its decomposition products phosphate and phosphite, which are formed through decarboxylation of foscarnet in aqueous solution [26]. This example clearly demonstrates the potential of this chromatographic system as a stability-indicating assay for shelf-life studies of foscarnet and possibly other bisphosphonates.

As 2 mM nitric acid is the preferred mobile phase in most cases, the analytical method was validated for the determination of pamidronate in pharmaceutical formulations using this mobile phase. The calibration graph of the assay was found to be linear in the range 200 ng–10 µg pamidronate injected per 20 µl injection. A typical calibration line, calculated

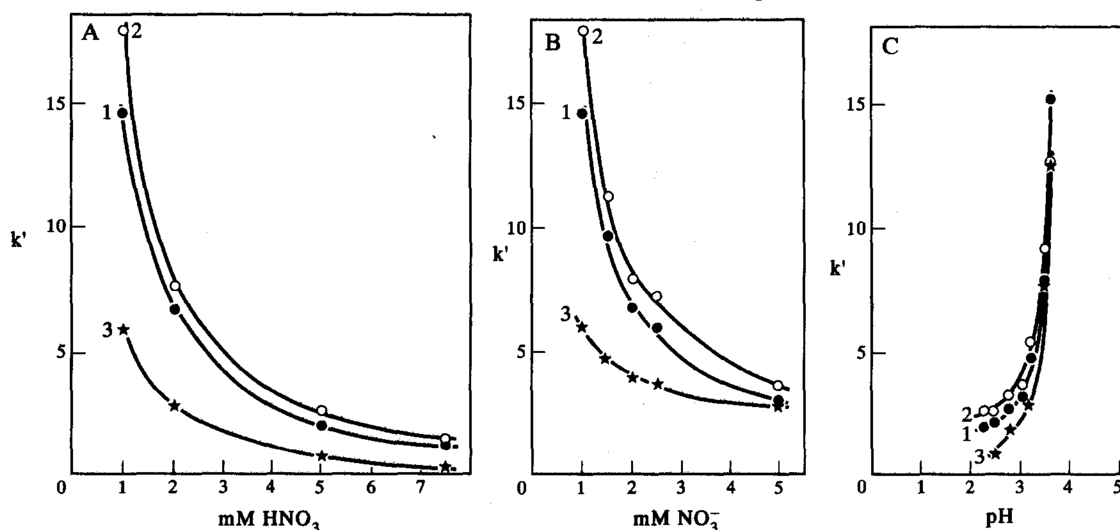

Figure 2
Influence of the molar composition of the mobile phase on the retention behaviour of (1) pamidronate, (2) phosphate and (3) the sample solvent. (A) Capacity factor (k') as a function of the molar nitric acid concentration. (B) Capacity factor (k') as a function of the molar nitrate concentration at a constant pH (1 mM H^+). (C) Capacity factor (k') as a function of pH at a constant nitrate concentration (5 mM NO_3^-).

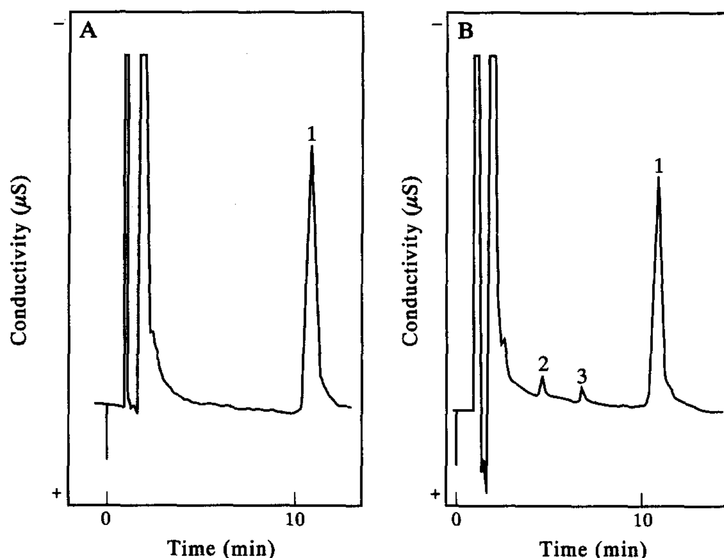

Figure 3
Chromatograms of (A) an aqueous reference solution of foscarnet (approximately 0.3 mg ml^{-1}) and (B) a partly decomposed aqueous solution of foscarnet. 1, Foscarnet; 2, phosphate; 3, phosphite.

by least squares regression analysis, was $y = -3.2\ (\pm 3.6) + 39.9\ (\pm 0.8)\ x\ (r = 0.9992;\ n = 6)$. The limit of detection (signal-to-noise ratio = 3) was about 100 ng pamidronate per 20 μl (5 ppm).

The precision of the analytical system, calculated as the RSD of six repeated injections of a pamidronate standard solution, was tested at two concentration levels (0.5 μg and 5 μg pamidronate per 20 μl injected) and found to be acceptable (2.2–8.2% and 1.3%, respectively; $n = 6$). The precision found for repeated injections of 0.5 μg pamidronate appeared to vary significantly from day to day, as indicated above. The accuracy and precision of the complete analytical method was demonstrated by determination of the intra-day and the inter-day recovery and reproducibility of the analysis of a pamidronate injection concentrate (1 mg ml^{-1}; 5 ml). The intra-day recovery and reproducibility are excellent, as shown by a six-fold analysis of the pamidronate injection (recovery = 100.3%; RSD = 1.7%). The inter-day recovery and reproducibility are good, as indicated by analysis of the pamidronate injection on six subsequent days (recovery = 102.4%; RSD = 2.7%). Typical data for the accuracy and precision of the analysis of pamidronate tablets (150 mg) are a recovery of 102.7% and an intra-day reproducibility (RSD) of 2.1% ($n = 8$).

The lower limit of quantitation of the assay (LLQ; defined as the lowest absolute amount of pamidronate that can be determined at a 95% confidence level) was calculated from calibration graphs, using standard statistical methods. For pamidronate, dissolved in water, an LLQ of 500 ng 20 μl^{-1} injection volume (25 ppm) was established. Attainment of a lower LLQ is hindered by interference of the large solvent peak, which overwhelms the pamidronate peak and prevents the use of an increased injection volume. Using the mobile phase as the injection solvent, the LLQ for pamidronate can be improved to approximately 500 ng 100 μl^{-1} injection volume (5 ppm).

The analytical method has been applied in the authors' laboratory for the quality control of pharmaceutical preparations of pamidronate and dimethyl-APD for several years. The method has proved to be simple and fast, demanding only minimal sample preparation. In all these aspects the method is superior to other methods using mostly pre-column or post-column derivatization of the bisphosphonate. Although selectivity of the method is sufficient for quality control purposes, separation of pamidronate and dimethyl-APD with this particular chromatographic system is not possible, either with nitric acid or with succinic acid as the mobile phase. Studies are being conducted to overcome this problem. The analytical technique has only moderate sensitivity, which makes the proposed assay less suitable for bio-analysis, at least for the

bisphosphonates pamidronate and dimethyl-APD; however, bio-analysis of foscarnet by this method should cause few problems as this drug is administered in much higher dosages [27]. For the bio-analysis of dimethylpamidronate, on-line sample enrichment and column-switching techniques are being applied to improve the method.

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