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https://doi.org/10.5109/20311

出版情報：九州大学大学院農学研究院紀要. 56 (2), pp.213-216, 2011-09. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **56** (2), 213–216 (2011)

Determination of Bisphosphonates by Ion–pair HPLC

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(*Received April 4, 2011 and accepted May 9, 2011*)

The optimization of the two bisphosphonates (disodium pamidronate and zoledronic acid) separation by ion–suppression and ion–pair high–performance liquid chromatography is described. The influence of the eluent pH, composition of the mobile phase and the concentration of complexing ion–pairing agents was investigated. This developed method provides high separation and specificity to bisphosphonate analysis. In quantitative analysis in the optimized conditions (10 mM n–amylamine or n–butylamine; 14% acetonitrile in 0.1 M sodium phosphate buffer, pH=7.0), the method showed good linearity (r=0.9999–0.9998) and sufficient sensitivity (10^{-7}–10^{-8} g/ml). It can be easily and conveniently adopted for the routine quality control analysis.

INTRODUCTION

Bisphosphonates are a broad class of synthetic compounds possessing a P–C–P bridge structure and are typically used to inhibit bone resorption. They are extensively used in the management of skeletal disorder including osteoporosis, malignant hypercalcemia, bone metasis and Paget's disease (Sparidans and Hartigh, 1999). Bisphosphonates play a important role in aseptic environment of total join replacements and may contribute to the *in vivo* longevity of total joint replacements. Some data suggest that they can decrease in flexural strength and flexural modules of cured cement (Lewis and Janna, 2006). When these compounds have been administrated to the patient they have two possibilities of their delivery options *e.g*. systematic (oral ingestion or intravenous injection) (Morris and Einhorn, 2005) or local (Sabokbar *et al*., 1998). It has been shown that oral administration of bisphosphonates can be used to reduce osteoclastic reaction to wear debris and the resultant resorption in arthroplasty surgery (Antoniou *et al*., 2000). Local delivery system is that bisphosphonates are mixed with bone cement coating deposited implants (Peter *et al*., 2005). This delivery method must be carefully tested in some ways. First the clinical studies must determine the kind of specific bisphosphonate, mode of its delivery and dosage. Next the mechanism(s) of its action on cultures of osteoblast cells on human bone slices as well as the influence of this compound on the properties of the cured cement must be determined. The details of bisphosphonate leaking from the cured cement into a biosimulating solution must be also evaluated (Lewis and Janna, 2005). The bisphosphonate drugs are also used in veterinary for the suppression of bone remodeling and tumor osteolysis as a palliative treatment of animals with osteosarcoma (Tomlin *et al*., 2000; Ashlon *et al*., 2005).

The chemical nature of bisphosphonates causes some analytical difficulties. These compounds are strongly polar and ionic that causes a problem with their extraction from biological fluids into an organic solvent and they are no volatile (Sparidans and Hartigh, 1999).

The method that has been used so far for the investigation of these compounds are non–chromatographic methods based on either titration with thorium diaminocyclohexanetetra acetate (Liggett, 1973) or on detection of phosphate as a triethylamine–phosphomolybdate complex after decomposition of the bisphosphonate (Bisaz *et al*., 1975). The newest class of bisphosphonates which are used in lower doses increase the demands on the sensitivity of the analysis which can be only realized by chromatographic methods (Fleisch, 1991). For the development of analytical separation methods, several strategies have been followed mainly IEC (ion–exchange chromatography) (Daley–Yates *et al*., 1989), and reversed–phase liquid chromatography (RPLC) (Kwong *et al*., 1990) often in combination with an ion–pairing agent. Gas chromatography technique is rather limited due to difficulties in transferring the ionic bisphosphonate into an organic solvent (Auriola *et al*., 1989). Recently capillary electrophoretic (CE) separations have also been described (Huikko and Kostiainen, 2000).

In this investigation, the conditions for the separation of two bisphosphonates were examined and optimized. N–amylamine, n–butylamine or tetrabutyl–ammonium chloride was used as the complexing agent (counter ion). The influence of the eluent pH, counter–ions concentration, and the amount of methanol and acetonitrile in the mobile phase was also examined (Bidlingmeyer, 1980; Xie *et al*., 2006).

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MATERIALS AND METHODS

Chemicals

Pamidronate (3–amino–1–hydroxypropylidene) bis–phosphonate SIGMA (St.Louis, USA) (HPLC standard) and clinical preparation from Vipharm S.A. (Warszawa, Poland), zoledronic acid [1–hydroxy–2–(1H–imidazole–1–yl) ethylidene] bis–phosphonate was obtained from LKT Lab. (St.Paul, USA) (HPLC standard) and Novartis (Nürnberg, Germany). Acetonitrile and methanol HPLC grade were purchased from Merck (Darmstadt, Germany). N–butylamine, n–amyloamine were obtained from Sigma (St.Louis, USA) and tetrabutyl–ammonium chloride was obtained from Fluka (Buchs, Switzerland). For mobile phase preparation MilliQ water was used.

Stock standard solutions of pamidronate and zoledronic acid (1 mM) were prepared by dissolving in water. Each solution was further diluted to obtain a series of working standarts, pipetted into 500 μl Eppendorf tubes and frozen under liquid nitrogen. Because of unstability of investigates substances all standard solutions after defrosting were used only 12 hours.

Equipment and chromatographic conditions

HPLC analysis was carried out using the reversed–phase column Phenomenex Luna C_{18}(2) column (250 mm ×4.6 mm I.D.), particle size 5 μm and was protected by a Phenomenex Security Guard column. The chromatographic system consisted LC–9A precision pump (Shimadzu, Japan), an SPD–M10A diode array detector and a Model 7125 sampling valve RHEODYNE (Berkeley, USA) with 20 μl loop and was controlled by Shimadzu Class M10A Work Station (v.1.64). The mobile phase used was a mixture of (2–36 mM n–amylamine or n–butylamine or tetrabutylamine chloride aqueous solution (adjusted to pH 5–8.5 with acetic acid) and acetonitrile (2–30%) or methanol (2–44%) (v/v). The flow–rate was 0.5 ml/min and all experiments were done at room temperature. The spectra of tested substances were determined in a Shimadzu UV–VIS 160A spectrophotometer (Shimadzu, Tokyo, Japan) controlled by Pentium IV PC computer with PC 160 plus software.

RESULTS AND DISCUSSION

The choice of the chromatograph detector in high performance liquid chromatography as well as physical properties measured substances in the column eluate influence for the detection limits. Bisphosphonates in LC can be detected by direct sensitive detection like UV adsorption, fluorescent and/or ability to oxidation (Peng *et al*., 1998; Sparidans and Hartigh, 1999). Sometimes a chromatographic separation has been preceded by one or more pretreatment steps as derivatization which enhance the detectability and/or to improve the selectivity of the separation (Fruitier *et al*., 1990; Lovdahl and Pietrzyk, 1999; Perez–Ruiz *et al*., 2009).

In the first step of the experiments we have checked the spectra of disodium pamidronate and zolendronic acid looking for the possibility of using UV–VIS detector. In the both cases the high absorption were observed in near UV region about 210 nm which was chosen as the length of wave for chromatographic detector in the next experiments. The stability of clinical used preparates was checked after 1, 3, 6, 12, 24, 48 and 72 hours by measuring their spectra profiles from 200 to 400 nm. The obtained profiles of disodium pamidronate and zoledronic acid specialy clinical grade after 24 hours showed a small unstabilities and for this reason we have used fresh solution only for 12 hours.

All the bisphosphonates are strongly polar and ionic that do not give sufficient retention on the hydrophobic stationary phase such as C_{18} or C_8 (Niemi *et al*., 1997; Xie *et al*., 2006). Very helpful for this reason is application of an ion–pairing agent which can increase the bisphosphonates retention on this kind of the sorbent. In our investigation we have used tetrabutylammonium chloride (Sparidans and Hartigh, 1999) or n–amylamine (Xie *et al*., 2006) or n–butylamine (Niemi *et al*., 1997) as ion–pair reagent. These compounds (oppositely charged ions) may combine with bisphosphonates to the form of neutral ion–pair, which can be retained on nonpolar stationary phase.

Optimization of separation conditions of the test mixture of two bisphosphonates was carried out in three stages: (a) choise of the compexing agent concentration; (b) determination of the optimal composition of the mixed mobile phase (organic compound–buffer) and (c) effect of mobile phase pH.

The effect of ion–pair agents (tetrabutylammonium chloride (TBA), n–amylamine and n–butylamine) concentration on the retention (k–retention factor) of disodium pamidronate and zolendronic acid at constant pH (7.0) and organic modifier (acetonitrile 5%) is shown in Table 1. The retention of tested bisphosphonates increased with increasing from 2 to 36mM ion–pairing agents con-

Table 1. Influence of complexing agents concentration on k of disodium pamidronate and zolendronic acid

Complexing agent and its concentration [mM]		k value	
		Disodium pamidronate	Zolendronic acid
n–amylamine	2	1.40	1.81
	10	1.67	2.46
	18	1.92	2.64
	26	2.10	2.91
	36	2.28	3.01
n–butylamine	2	1.92	2.07
	10	1.96	2.35
	18	2.00	2.47
	26	2.08	2.54
	36	2.15	2.59
tetrabutyl–ammonium chloride	2	2.94	3.43
	10	3.01	3.81
	18	3.09	4.07
	26	3.54	4.65
	36	4.07	5.29

centrations. Satisfied separations of tested compounds were obtained when concentration of n–amylamine as well n–butylamine was 10 mM. The higher concentration of these complexing agents as well using TBA caused baseline drift and increase in background noise. For this reason, in the next experiments n–amylamine at 10 mM concentration as ion–pair reagen was choosen.

The next step of this work was determination of an organic modifier (acetonitrile 2–30% and methanol 2–44%) composition in mobile phase on the retention of disodium pamidronate and zolendronic acid at constant concentration of n–amylamine (10 mM) and constant pH (7.0) of mobile phase (Fig. 1).

Replecement of methanol with acetonitrile led to lower k values and even to shorter analysis time. In the case when methanol was used the chromatographic peaks were shown much more deformation. Sometimes asymmetry factors value were higher than 1.2 (data not shown) what exceed the acceptable value (Suprynowicz *et al.*, 1984). For these reason the acetonitrile in the concentration 14% was choosen for further studies.

Bisphosphonates belonging to quadribasic acids having several pK_a values that capable of forming multiply charged ions in solution. Therefore, the pH level of mobile phase is very important parameter in the ion–pair HPLC for analysis of bisphosphonates because of the significant effect on the retention behaviour and peak shape. The retention factor of test mixture compounds were measured for different eluent pH (5–8.5) at the constant concentration of ion–pairing modifying agents (10 mM) and 14% acetonitrile Fig. 2. The retention of tested bisphosohonates clearly increased parallely to the increasing eluent pH. At pH 7.0 the differences in k value of disodium pamidronate and zolendronic acid was so big as in higher values and we have obtained not only good separation but also short time of analysis. The similar results in the optimization procedure of four different bisphosphonates were obtained by (Xie *et al.*, 2006).

The detection limits of pamidronate and zolendronic acid obtained in the optimized condition are compared in Table 2. As we can see in the case of zolendronic acid and disodium pamidronate detection limit was about 10^{-7}–10^{-8} g/ml *e.g.* 10 to 100 times lower to compare with Xie *et al.* (2006) method where the ELSD detector was used.

Results from assay of disodium pamidronate and zolendronic acid in bulk material are shown in Table 3. The precision of the data and the agreement between the label claim and the found amount were very high. Ingradients of commercially available samples did not

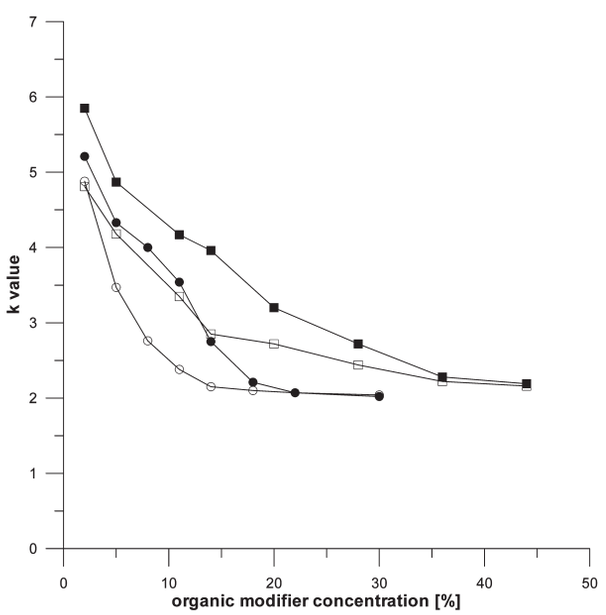

Fig. 1. The dependence of pamidronate (○, ●) and zolendronic acid (□, ■) retention factors (k) on the amount of acetonitrile (○, □) or methanol (●, ■) in mobile phase.

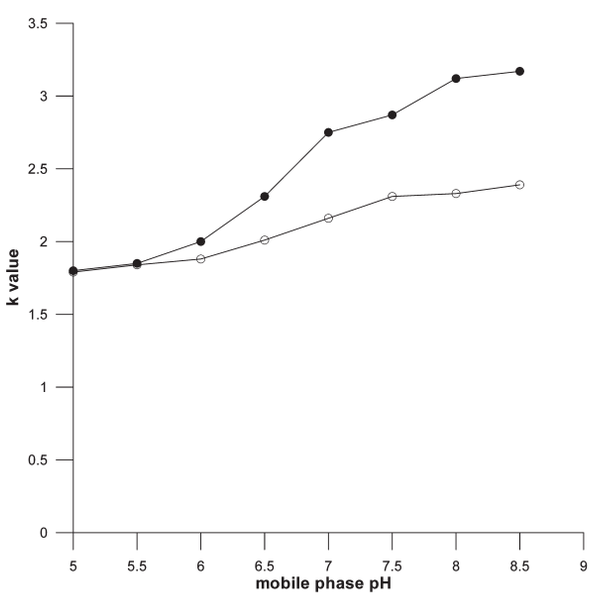

Fig. 2. Influence of mobile phase pH on retention factors (k) of pamidronate (○) and zolendronic acid (□).

Table 2. Linear regression calibration and detection limit data for bisphosphonates

Compound	Concentration range [μg/ml]	Regression line	Corelation coefficient [r]	Detection limit [μg/ml]
Disodium pamidronate	0.05–1000	y=1.2299x – 6.266	0.9999	0.115
Zolendronic acid	0.05–1000	y=1.1346x + 2.087	0.9998	0.064

Table 3. Analysis of bisphosphonates samples

Product	N	Claim amound [%]	Found amount in standart [%]
Disodium pamidronate	5	100.00	98.78±1.91
Zolendronic acid	5	100.00	97.57±2.27

interfere with analytes. Under the chromatographic analytical procedure we can easily determined these components with so high resolution in standard solution as well as in clinical samples.

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