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Extraction and measurement of pamidronate from bone samples using automated pre-column derivatization, high-performance liquid chromatography and fluorescence detection

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Abstract

A method for the determination of 3-amino-1-hydroxylpropylidene-1,1-bisphosphonic acid (pamidronate) in bone samples is described. This method combines and modifies parts of previous procedures. Pamidronate is extracted from finely ground bone with dilute hydrochloric acid. Amine-containing contaminants are removed by co-precipitation of pamidronate with calcium. Excess calcium is removed with EDTA and an ion-exchange resin. Pamidronate is automatically derivatized at the primary amine and quantified by high-performance liquid chromatography with fluorescence detection. The method assay was linear in the concentration range 7.5–600 ng/mg bone (20–1000 pmol/mg). The imprecision for repeat analyses were 16.5 and 7.8%, at pamidronate levels of 7.5 and 600 ng/mg bone, respectively. The method has been used to analyze bone samples from pharmacokinetic animal studies involving both acute and chronic dosages.

Keywords: Pamidronate

1. Introduction

Bisphosphonate drugs potently inhibit bone resorption and are used in the treatment of bone diseases such as hypercalcemia of malignancy and Paget's disease. These drugs are poorly absorbed from the gut and what is absorbed is readily taken up by bone where it can be retained for years. The amount of bisphosphonate taken up by bone has been measured primarily using radiolabelled drug in short term studies with acute dosages. Although methods have been developed to measure bisphosphonates in urine and plasma samples, measurement in bone provides the most useful information. However, bone is an extremely difficult tissue to analyze. 3-Amino-1-hydroxy-propylidene-1,1-bisphosphonate (pamidronate, I) has been measured previously in dog urine following a single dose of drug by pre-column derivatization with fluorescamine, high-performance liquid chromatography (HPLC) and fluorescence detection (limit ≈13 ng/ml with a 2 ml sample [1]). The related compound 4-amino-1-hydroxyl-butanylidene-1,1-bisphosphonate (alendronate) was measured in a similar manner from spiked human urine using different derivatizing agents that allowed a detection limit of 1 ng/ml from a 5-ml sample [2,3].

Methods to detect two third generation bisphos-

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phonates, (cycloheptylamino)methylene-1,1-bisphosphonate monohydrate (YM175) and 1-hydroxy-2-(imidazol[1,2-*a*]pyridin-3-yl)ethane-1,1-bisphosphonate (YM529) in plasma, urine and bone have recently been reported [4,5]. These bisphosphonates lack the primary amine that was used to derivatize I and alendronate but do have side chains consisting of relatively non-polar rings. This characteristic permits the use of Sep-Pak cartridges to concentrate the drug and to remove much of the excess calcium present in bone extracts, which would normally interfere with detection. This is combined with HPLC and either electrochemical detection (for YM175) or intrinsic fluorescence at alkaline pH (for YM529), resulting in very sensitive methods with detection limits as low as 5 ng/g bone [4,5]. The low detection limits are necessary for these drugs because they are effective at much lower concentrations than either I or alendronate.

We were interested in measuring I, a "second generation" bisphosphonate, in bone samples obtained from long-term animal studies. Such studies are necessary to evaluate the potential of bisphosphonate treatment for chronic conditions such as osteoporosis. We have developed and validated a method to quantify I, extracted from bone samples, using pre-column derivatization, HPLC and fluorescence detection.

2. Experimental

2.1. Chemicals and reagents

Pamidronate disodium (I) and the internal standard, 1-hydroxypentanylidene-1,1-bisphosphonate (CGP 38146, I.S.), were obtained from Ciba Giegy (Basel, Switzerland). All solvents and reagents were of HPLC or analytical grade and were obtained from VWR Scientific (Toronto, Canada), Caledon (Georgetown, Canada) or Sigma (St. Louis, MO, USA). N-Acetyl-D-penicillamine (NAP) was from Fluka Biochemika (Ronkonkooma, NY, USA). 2,3-Napthalene dicarboxylaldehyde (NDA) was obtained from Aldrich (Milwaukee, WI, USA). Standard bone was reference material H-5 animal bone from the International Atomic Energy Agency, analytical quality control services. Cation-exchange resin, AG 50W-X8 (200–400 mesh, H^+-form) was obtained from Bio-Rad Laboratories (Mississauga, Canada) and was converted to the K^+-form according to the established method [5].

2.2. Standard solutions

Compound I and the I.S. were dissolved in water at a concentration of 100 μg/ml. These solutions were stored at 4°C for use as stock solutions. Standard solutions of these compounds were prepared in polypropylene tubes by diluting the stock solutions with water to suitable concentrations.

2.3. Chromatographic conditions

The HPLC system consisted of a HP 1050 (Hewlett-Packard, Mississauga, Canada) series pump and autosampler, an LC 240 (Perkin-Elmer, Beaconsfield, UK) fluorescence detector, a PLRP-S column (15 × 0.46 cm I.D., Phenomenex, Torrance, CA, USA) and a guard column. Data acquisition and analysis was performed using HP ChemStation software (Hewlett-Packard). The mobile phase consisted of 16% acetonitrile in 0.025 *M* citrate–phosphate buffer, pH 6.5, delivered at a flow-rate of 1.0 ml/min. The column eluate was monitored fluorometrically at excitation and emission wavelengths of 436 and 508 nm, respectively.

2.4. Sample extraction

Bone samples (25 mg), consisting of finely ground and sifted (<20 μm) powder, were dissolved in 2 ml of 0.2 *M* HCl. Each sample received a 5-μg aliquot of I.S. For each set of bone samples processed, a high and a low spiked bone control was included. Spiked bone controls consisted of bone without endogenous I that was spiked with various amounts of I standards. Samples were vortexed and incubated overnight at room temperature. After centrifugation, 500-μl aliquots, equivalent to 6.25 mg of bone were processed further. The samples were diluted with 1 ml of 0.01 *M* NaOH and 50 μl of 10 *M* NaOH to co-precipitate I with calcium phosphate. The precipitate was isolated by centrifugation (1000 *g*, 10 min) and the pellet was washed with 1 ml of water to

remove any trapped amine-containing compounds. After further centrifugation, the supernatant was discarded and the calcium salt pellet was dissolved in 200 μl of 0.2 *M* phosphoric acid. To remove calcium from the dissolved sample, 250 μl of 0.2 *M* EDTA in 0.2 *M* NaOH, pH 10.3, was added to the sample followed by 200 μl of AG 50W-X8 (K^+-form). After vortexing and centrifugation, a 550-μl aliquot was filtered through a 0.2-μm membrane and alkalized with 10 μl of 10 *M* NaOH. Samples were prepared manually to this point in batches of up to twenty samples. These samples were stable for over one week at 4°C, and for over one month at −20°C.

Compound I from a 50-μl aliquot of each prepared sample was derivatized by the automated addition of 50 μl of 1 *M* carbonate buffer, pH 10.7, followed by 10 μl of 1 mg/ml NDA and 10 μl of 1 mg/ml NAP. The sample was mixed and incubated for 2 min prior to the application of either a 20- or a 50-μl aliquot onto the HPLC column. Each sample was derivatized at precisely the same time before analysis.

2.5. Preparation of calibration curves

Calibration curves were constructed by plotting the peak area versus the concentration of standard solutions injected directly onto the HPLC column. Unknown sample concentrations were calculated from the calibration curve by the HP ChemStation software.

2.6. Recovery, precision and accuracy

The precision of the method was examined by the replicate analysis ($n = 5$) of bone standards spiked with I, at five different concentrations. The analytical recovery of I was determined by comparing the peak area of the I derivative extracted from these samples to that of derivatized standards, injected directly. In addition to the spiked bone controls, two experimental dog bone samples that were available in larger quantities were included in each set of samples and were analyzed as quality controls.

2.7. Bone samples

Data from two representative sets of bone samples are presented. One set of bone samples was obtained from the ilium of dogs that had received a daily oral dose of I for one year. These data illustrate the ability of the method to measure a dose–response curve in high-dose animal studies. We also obtained bone samples from dogs that had received 150 ng/kg body weight of [^{14}C]I per day, for seven days intravenously. These data provide results for radioactive I recovery and demonstrate the potential of the method for use in acute studies.

3. Results

Typical chromatograms of I, extracted from bone samples of dogs that had received acute intravenous and chronic oral doses of I are shown in Fig. 1A and Fig. 1B. Compound I and I.S. derivatives eluted at 5.0 and 13.5 min, respectively. The specificity of the assay was confirmed by the analysis of different bone blanks (see Fig. 1A and Fig. 1B). Control bone samples from short-term experiments, involving intravenous application of I and bone standards, did not have any interfering substances (Fig. 1A). Animals that had received no I and therefore acted as zero-dose controls in long-term studies where I was delivered orally did exhibit an interfering peak with the same retention time as I (Fig. 1B). This peak could be separated from I using a mobile phase of 20% acetonitrile in phosphate buffer (data not shown). Under these conditions the retention times of I and I.S. were 7 and 21 min, respectively. Because of the long retention time under these conditions and reduced sample throughput, 16% acetonitrile was used for routine analysis.

The calibration curve derived from I standards was linear over the range of 0.5 to 200 ng/injection. The representative equation of the regression line was peak area=69.45 ng I−3.05 ($R = 0.999$, y weighted by 1/S.D.) where area is in arbitrary units. The assay was validated using spiked bone samples over a concentration range of 7.5 ng/mg to 600 ng/mg bone (Table 1). Analytical recovery ranged from 93 to 104% with a mean of 99 ± 3.9% ($n = 5$). The intra-day assay coefficient of variation (C.V.) ranged from 4.4 to 16.5%.

Inter-day assay accuracy and precision was evaluated using a high- and low-dose of I, added to three standard bone samples and two bone samples from

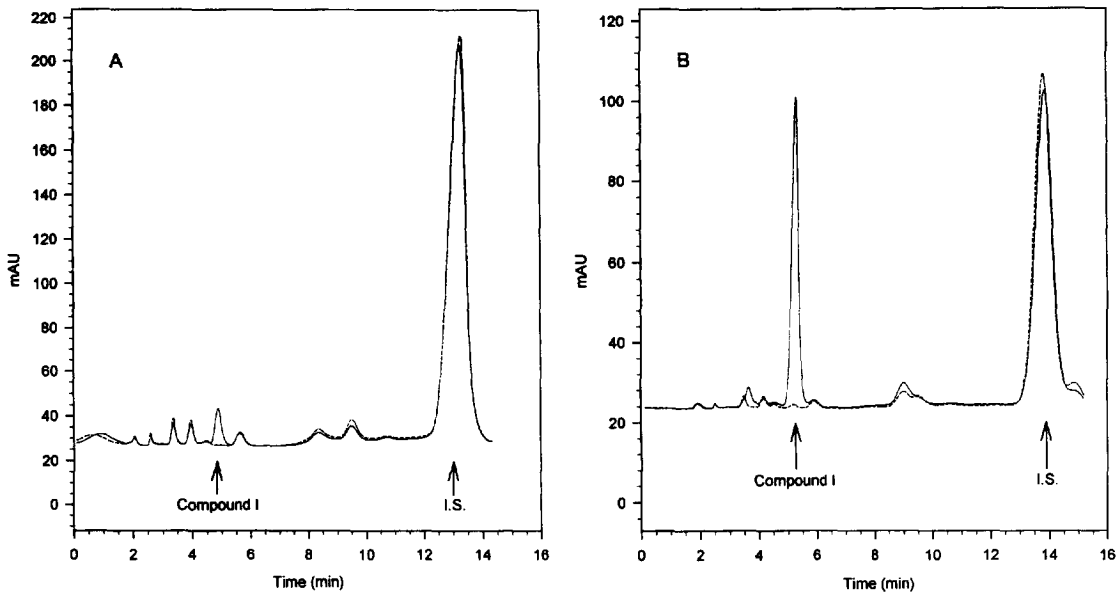

Fig. 1. Typical chromatograms of animal bone samples: (A) Bone samples from animals treated daily for one year with I; dashed line = zero-dose, solid line = 25 mg/kg body weight/day, administered orally. (B) Bone samples from animals treated daily for seven days with [^{14}C]I; dashed line = zero dose, solid line =150 ng/kg body weight/day, administered intravenously. Peaks corresponding to I and I.S. derivatives are indicated. Because the major parts of the tracings overlap, the zero-dose profiles are most evident at the retention time of the I derivative.

long-term animal studies, designated as quality controls (Table 2). Analytical recovery for the spiked bone controls ranged from 85 to 111.6% with a mean of 100.2 ± 9.7% (n = 3). The imprecision, as measured by the C.V., ranged from 5.6 to 17.5%.

This method was used for the analysis of bone

Table 1
Intra-day precision and accuracy for the analysis of spiked bone control samples (n = 5)

Sample	Amount added (ng/mg bone)	Analytical recovery (ng/mg bone)	C.V. (%)	Recovery (%)
Bone control 1	7.5	7.14 ± 1.18	16.5	95
Bone control 2	40	37.1 ± 5.6	15.1	93
Bone control 3	60	61.4 ± 2.9	4.8	102
Bone control 4	200	201.6 ± 8.9	4.4	101
Bone control 5	600	621.6 ± 48.4	7.8	104

Table 2
Inter-day precision and accuracy for the analysis of spiked I from bone control samples and quality controls

Sample	Amount added (ng/mg bone)	Analytical recovery (ng/mg bone)	C.V. (%)	Recovery (%)
Bone control 1	50	42.6 (n = 16)	10.9	85
Bone control 2	200	208.3 (n = 5)	5.6	104
Bone control 3	500	552.6 (n = 15)	17.5	111.6
Quality control 1	–	117.9 (n = 23)	9.1	–
Quality control 2	–	304.4 (n = 16)	12.0	–

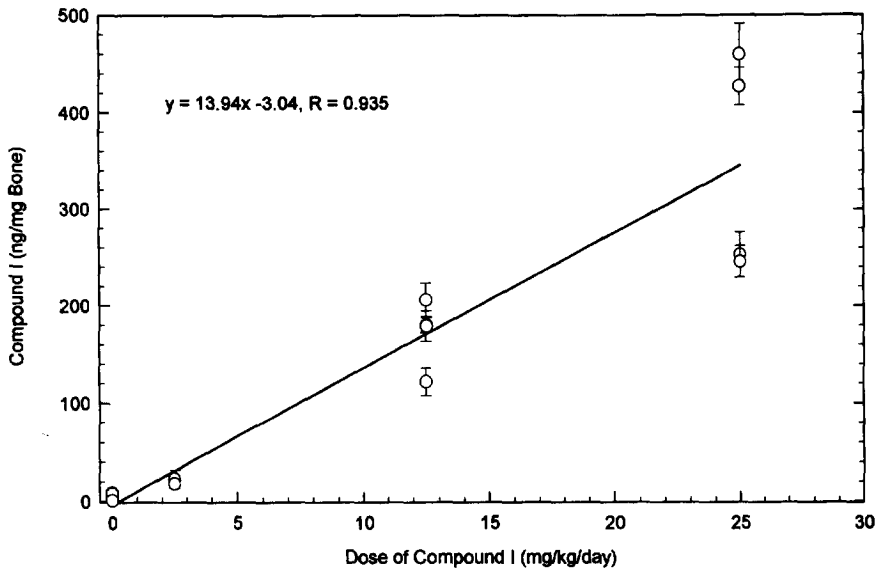

Fig. 2. The content of I (ng/mg bone) versus dose (ng/kg body weight/day) for dog bone (ilium). Animals received either 0, 2.5, 12.5 or 25 mg of I per kg of body weight, administered orally, each day for one year. Each point represents the mean ± the standard deviation of three different measurements by HPLC after extraction. The solid line is the regression line for all the data (n = 20, 4/dose).

sample from dosage studies. Bone samples from animals that received acute doses of [^{14}C]I for 7 days were found to contain 8.2 ± 2.3 ng/mg bone (n = 4). This is very similar to the amount of I predicted from liquid scintillation counting of [^{14}C]I from HCl extracts of the same bone samples and the specific activity of the administered [^{14}C]I (6.2 ± 1.5 ng/mg, n = 8).

Representative concentration data for bone samples taken from the ilium of dogs that had received daily oral doses of I for one year are presented in Fig. 2. A linear relationship was observed between the dose of I and its content in bone (Fig. 2). The equation for the regression line through all the data was I (ng/mg) = 13.94 × dose + 3.04 (R = 0.935).

4. Discussion

Derivatization of the amino group on I using NAP and NDA was based on a procedure developed for alendronate [3]. Minor changes were required for optimum derivatization and HPLC. The fluorescent derivative of I was unstable and timed pre-column derivatization was required. An incubation period of 2 min was needed for adequate conversion, rather than 1 min. Slight changes in mobile phase pH significantly affected the retention time of the derivative of I, and a pH of 6.5 rather than 6.3 was used to reduce run times. No late interfering peaks were observed which obviated the gradient elution used by Kline and Matuszewski [3].

Extraction of I from bone was based on approaches used for other bisphosphonates [1–5]. Both the ion-exchange resin and the EDTA were required for removal of calcium. Omission of one or the other of these, or the use of lower amounts of either, reduced or eliminated the recovery of I. Efficient derivatization of I requires a pH of 10.7. Calcium phosphate and calcium pamidronate precipitate at this pH, therefore removal of excess calcium before the sample is alkalized is critical for the detection of I. As a result, concentration of I from large amounts of bone was not feasible. This method is sensitive to the amount of bone processed (and hence the amount of calcium) and is most useful for bone samples of less than 25 mg.

Only two other bisphosphonates have been measured in bone samples using non-radioactive methods. The bisphosphonates YM175 and YM529 have been measured in bone using methods that take advantage of these drugs' inherent fluorescence or

electrochemical nature [4,5]. Neither of these approaches was suitable for I. The method described here was able to detect at least 7 ng/mg of I in bone samples. This detection limit is more than adequate to quantify I in bone samples from chronic oral studies, and following acute moderate intravenous dosage, where approximately 50% of the applied drug is taken up by bone. Most importantly, our method makes possible the measurement of I in its target tissue, rather than in plasma or urine.

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