DETERMINATION OF PAMIDRONATE IN BISPHOSPHONATE-ENRICHED BONE CEMENT BY ION-PAIR HPLC AND CAPILLARY ELECTROPHORESIS

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Abstract

The presence of pamidronate during local use of bisphosphonates (BP)-enriched bone cement was determined. The question was whether pamidronate implanted into the bone cement is eluted. The study was performed on 10 probes of BP-enriched bone cement located in 0.9% NaCl. The probes were incubated for 3 and 6 weeks. Ion-pair HPLC was used for the detection of pamidronate. Then, capillary electrophoresis was applied for quantitative analysis of pamidronate in the 3rd and 6th week after incubation. The presence of pamidronate, eluted from BP-enriched bone cement into 0.9% NaCl solution 3 and 6 weeks after incubation, was demonstrated. These results may explain the changes in the level of cytokine RANKL and bone turnover marker osteoprotegrin in rats' serum treated with BP-enriched bone cement 3 and 6 weeks after surgery. The possibility of effective local use of BP-enriched bone cement in veterinary medicine was underlined. The results, and the former conducted research, point out that the clinical applications of BP-enriched bone cement *in vivo* may have some validity in the future.

Key words: rats, bisphosphonates, bone cement, ion-pair HPLC, capillary electrophoresis.

Bisphosphonates (BPs) are stable pyrophosphate analogs with the same basic structure, so called P-C-P group. Each BP has its own chemical, physicochemical, and biological characteristics (6). Recently an increasing number of reports have been published on the important effects of bisphosphonates on bone turnover. Therefore, BPs are widely used as inhibitors of bone resorption in the treatment of many skeletal disorders such as osteoporosis, malignant hypocalcaemia, bone metastasis, osteogenesis imperfecta or Paget's disease (2, 7, 23, 28, 33). Despite the fact that BPs act specifically on osteoblasts and osteocytes, the complete mechanism of BPs' action is still unknown (17, 34). Pamidronate, with its specific binding affinities, appears to adsorb to calcium phosphate crystals in bone, thus blocking their dissolution by inhibiting osteoclast-mediated bone resorption. The main reason for a high efficacy of pamidronate is that it acts directly on bone tissue, therefore, the local use of pamidronate provides regulation of the secretion of various serum bone turnover markers, growth factors, or cytokines, such as osteoprotegrin (OPG), receptor activator of NF-κB ligand (RANKL), and tumour necrosis factor (TNF-α). In

previous *in vivo* studies, where the BP-enriched bone cement was implanted into the tibia of rats, it was revealed that local use of pamidronate led to an increase in the quality of bone density, improved bones' microstructure, and might have had an influence on some bones' turnover markers (20, 21). Therefore, such findings might have an important effect on bone implant osteointegration in the prevention of bone collapse and also in the enhancement of implant fixation (8, 25). Many recent studies, both preclinical and clinical, also focused on further potential applications of BPs in orthopaedics (4, 8, 9, 13, 36).

The present study aimed at assessing whether pamidronate implanted into the bone cement is eluted. Therefore, an attempt has been made to determine and assert the presence of pamidronate during local use of BP-enriched bone cement. It seems that BP-enriched bone cement might be very useful in treatment of trauma or bone defects in rats or other animals.

Material and Methods

Sample preparation. The study was performed on the probes of BP-enriched bone cement. Forty grams

of the cement was mixed with 60 mg of pamidronate. Rollers were formed with a diameter of 1 cm and a length of 2 cm. Ten roller-shaped probes were prepared. The probes were incubated at 37°C in 25 mL Erlenmayer flasks containing 15 mL of 0.9% NaCl. Five probes were incubated for 3 weeks and the remaining five for 6 weeks. Ion-pair HPLC was used for detection of the eluted pamidronate. Then, capillary electrophoresis was used for quantitative analysis of eluted pamidronate under optimised conditions in the 3rd and 6th week after incubation. The time of incubation was congruous with our former experiments where effects of BP-enriched bone cement implanted into tibia bone in rats were assessed (19, 20).

Reagents. Pamidronate (3-amino-1-hydroxypropylidene) bisphosphonate was obtained from Vipha S.A. (Poland). Bone cement was purchased from CMW 1, DePuy International Ltd, England. Acetonitrile and methanol HPLC grade were purchased from Merck (Germany). N-butylamine and n-amylamine were obtained from Sigma (USA) and tetrabutylammonium chloride and cetyltrimethylammonium bromide from Fluka (Switzerland). For mobile phase preparation MilliQ water was used.

Equipment. HPLC analysis was carried out using the reversed-phase column Phenomenex Luna C 18 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 of a LC-9A precision pump (Shimadzu, Japan), an SPD-M10A diode array detector and a Model 7125 sampling valve RHEODYNE (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 10 mM n-amylamine at a constant pH of 7.0 and acetonitrile (14%) (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 spectro-photometer (Japan) controlled by Pentium IV PC computer with PC 160 plus software (19).

The capillary electrophoresis analysis was carried out using the thermo capillary electrophoresis, Crystal 100, (Thermo Separation Products, USA). Separation was carried out using a fused silica capillary with a total length of 75 cm (50 cm to detection window) and an inner diameter of 50 µm. Applied voltage was -25 kV and the capillary temperature was maintained at 25°C. Samples were injected hydrodynamically for 0.5 s and the detection was

performed at 210 nm. Buffer solution (pH 8.6) was prepared by dissolving Na₂HPO₄ (15 mmol/L) and cetyltrimethylammonium bromide (CTAB, 0.6 mmol/L) in MilliQ water. Peak identification was performed by spiking with commercially available pamidronate.

Statistical analysis. Statistical analyses were performed using one-way analyses of variance (ANOVA). *Post-hoc* comparison of means was carried out with Tukey's test for multiple comparisons, when appropriate. In this case all data were shown as the means ±SEM. In cases of rejected hypothesis of normal distribution or homogeneity of variance nonparametric ANOVA rank Kruskal-Wallis multiple comparisons was used. The data were considered statistically significant at P<0.05. All statistical calculations were performed using 10.0 STATISTICA software (StatSoft, Poland).

Results

In the first step of the experiment, the elution of pamidronate from probes of BP-enriched bone cement, after 3 and 6 weeks of incubation, was determined by ion-pair HPLC. The ion-pair HPLC analysis was based on a previous publication in which pamidronate was quantitated on Phenomenex Luna C 18 column using 10 mM n-amylamine and a constant pH of 7.0 and acetonitrile (14%) (v/v) (16). The content of pamidronate ranged from 0.05 to 1,000 µg/mL. In the method, the correlation coefficient was higher than 0.99, demonstrating a linear correlation between the concentration and the obtained response. The linear regression equation and correlation coefficient values are shown in Table 1.

In the experiment, pamidronate was eluted from the column providing a narrow, sharp peak confirming the presence of pamidronate in the 0.9% NaCl solution after 3 and 6 weeks of incubation. The maximum concentration of pamidronate was 1.25 mg/mL after 6 weeks of incubation (Table 2) and was higher than after 3 weeks of incubation (p=0.0072).

In the next stage of the study, the results obtained by ion-pair HPLC were confirmed by capillary electrophoresis. Pamidronate was eluted from probes of BP-enriched bone cement after 3 and 6 weeks of incubation. Under the optimised CE conditions, pamidronate dissolved in a 0.9% NaCl solution gave a sharp peak at about 6.8 min migration time (Figs 1A, 1B).

Table 1
Linear regression calibration and detection limit data for pamidronate eluted from bone cement

Concentration range (µg/mL)	Regression line	Corelation coefficient (r)	Detection limit (µg/mL)
0.05–1000	y=1.2299x–6.266	0.9999	0.115

Table 2

Analysis of concentrations of pamidronate in 0.9% NaCl solution by ion-pair HPLC

Time	Concentration (mg/mL)	Found amount in standard (%)	N
3 weeks	0.35	98.68 ± 1.89	5
6 weeks	1.25	97.78 ± 1.92	5

Table 3

Analysis of concentration of pamidronate in 0.9% NaCl solution by EC

Incubation time	Migration times (min)	Peak areas	Concentration (mg/mL)	N
3 weeks	6.847	7,375.0	0.345	5
6 weeks	6.837	27,872.5	1.320	5

Fig. 1A. Electropherogram of pamidronate in 0.9% NaCl solution, after 3 weeks of incubation **Fig. 1B.** Electropherogram of pamidronate in 0.9% NaCl solution, after 6 weeks of incubation **Fig. 2.** Concentration of pamidronate standard in 0.9% NaCl solution

For injection precision, measured by integrated peak areas of five replicate injections, the RSD values of the 0.5 and 2.0 mg/mL pamidronate solution were 1.74 and 1.98%, respectively. The detector response was found to be linear ($y = 12.783x + 7430$) and showed R^2 of 0.9993 for the investigated concentration range 0.1–6.0 mg/mL using pamidronate standard solutions (Fig. 2).

The results obtained by capillary electrophoresis were similar to those obtained by ion-pair HPLC. The maximum concentration of pamidronate was 1.32 mg/mL after 6 weeks of incubation (Table 3) and was higher than after 3 weeks of incubation ($p=0.0078$).

Discussion

Bisphosphonates play an important role as antiresorptive drugs for the prevention and treatment of osteoporosis. They increase bone mass and produce a reduction in fracture rates to approximately half of control rates in the spine, hip, and other sites in postmenopausal women. In addition to the inhibition of osteoclasts, the ability of bisphosphonates to reduce the activation frequency and birth rates of new bone remodelling units, and possibly to enhance osteon mineralisation, may also contribute to the reduction in fractures (28, 29). The compounds have been registered for various clinical applications. They are usually given parenterally, or orally. However, there are some reports concerning side effects, especially during oral and long-term treatment (18, 21, 26, 33).

Arthroplasty patients with an orthopaedic implant device are at high risk for osteopenia or osteoporosis. Total joint replacement can be complicated by subsequent aseptic loosening of the implant. The pathogenesis of implant-associated osteolysis includes inflammatory and osteolytic processes (1, 11, 26). Recent studies have indicated that the use of BPs is connected with the improvement of the implant survival after total arthroplasty of the knee or hip. It may be very helpful in prevention of periprosthetic bone loss and failure of joint prosthesis (10, 26).

A modular prosthesis, which is based on many of the technical advances found in human prostheses, is now available for use in veterinary medicine. Currently, total hip and elbow replacements are commercially available options viable for use in dogs. Aseptic loosening that develops osteoarthritis is likely to be developed in the near future. The bisphosphonate drugs are also used in veterinary medicine for the suppression of bone remodelling and tumour osteolysis as a palliative treatment of animals with osteosarcoma (32).

The details of bisphosphonate leaking from the cured bone cement into a biosimulating solution are described by Lewis *et al.* (16). BPs are strongly polar and ionic and are non volatile (31). Nowadays, bisphosphonates are used in lower doses, which increases the demands for sensitive analyses, therefore, it can be only detected by chromatographic methods (6). For the development of analytical separation methods, several strategies have been applied, mainly IEC (ion-

exchange chromatography) and reversed-phase liquid chromatography (RPLC), often in combination with an ion-pairing agent (5, 15). In 2006, Jia *et al.* (14) described the development, optimisation, and validation of ion-pair HPLC with UV detection procedure for the analysis of risedronate in rat plasma. They reported that this method is simple and the most economic of the extraction procedures. They proposed that ion-pair HPLC method is a robust and reliable method for determination of risedronate in plasma and can be used to detect low concentrations of other bisphosphonates usually found in such samples (14). In this study, ion-pair HPLC method, which was described in an earlier report (19), had detection limit of pamidronate about 10^{-7} – 10^{-8} g/mL, *i.e.* 10 to 100 times lower than method of Xie *et al.* (35) where the ELSD detector was used. The precision of the data and the agreement between the label claim and the amounts found were very high.

Capillary electrophoresis was the next method used in the study. Many authors claim that this method is very useful (12, 30, 37). Perjesi *et al.* (24) reported that CE is a simple and easy to perform method for quantification of clodronate in liposomal formulation. This reproducible method may be routinely applied as quality control during the preparation of liposomal formulation of bisphosphonate. A capillary zone electrophoretic (CZE) method has been developed for the determination of impurities (phosphite and phosphate) in technical-grade ibandronate, which is a potent nitrogen-containing bisphosphonate (3, 27). The potential of multidimensional capillary electrophoretic methodological approaches, which eliminate sample preparation have been evaluated with regard to the analysis of bisphosphonates in aqueous and urinary matrices. Capillary isotachopheresis (cITP) was employed as a pre-separation and on-line sample concentration step prior to analytical determination using either cITP or CZE with conductivity detection (3).

The results obtained by ion-pair HPLC and CE methods showed that pamidronate is eluted from the BP-enriched bone cement after both 3 and 6 weeks of incubation in 0.9% NaCl solution. Wherein, after 6 weeks of incubation, the amount of pamidronate released from a sample was more than 4-fold higher than after 3 weeks of incubation. The release of pamidronate with BP-enriched bone cement also explains enhancement of the microstructure of bones in rats treated with BP-enriched bone cement in our former experiments (20). All these factors improved bone turnover toward reducing bone resorption and significantly improved the bone quality. Such methods of administering a BP-enriched bone cement, *e.g.*, pamidronate, to a patient's periprosthetic bone area may prevent bone resorption and aseptic loosening at the site of the medical implant devices. The slowing of the rate of bone resorption, is hypothesised to be associated with an improvement in the local bone turnover in the periprosthetic bone, which may provide a greater integrity to the overall bone and prosthesis structure. It is also suggested that BP-enriched bone cement can be successfully used in animals for filling bone defects that

arise as a result of a variety of diseases or injuries and in joint replacements as well. Thus, it is thought that the use of BP-enriched bone cement may be very beneficial in veterinary medicine. The results obtained with BP-enriched bone cement in rat models encouraged to prepare further clinical *in vivo* studies.

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