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Urinary Pyridinium Cross-Links as Markers of Bone Resorption in Tumor-Associated Hypercalcemia*

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ABSTRACT. Osteoclastic activity is increased in tumor-associated hypercalcemia, which, thus, constitutes an excellent opportunity to assess new markers of the bone resorption rate. We have measured the fasting urinary excretion of the pyridinium cross-links pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) in 36 hypercalcemic cancer patients (mean ± SD, 3.2 ± 0.4 mmol/L for total serum Ca and 1.66 ± 0.24 mmol/L for Ca^{2+}). Thirty-two of them were reevaluated after treatment with iv bisphosphonates. Urinary Pyr and D-Pyr levels were higher than those in healthy controls (130 ± 62 *vs.* 40 ± 19 nmol/mmol creatinine for Pyr and 20 ± 15 *vs.* 6 ± 3 nmol/mmol creatinine for D-Pyr; $P < 0.001$ for both). This represented a mean 3.3-fold increase over the normal mean compared to 5.8- and 3.4-fold increases for fasting urinary Ca and hydroxyproline, respectively. Individual values were elevated in 83% and 75% of the cases for Pyr and D-Pyr compared to 97% and 83% for urinary Ca and hydroxyproline, respectively. The levels of Pyr and D-Pyr tended to be higher in patients with head and neck tumors than in patients with breast cancer. Urinary Pyr and D-Pyr correlated with each other ($r = 0.72$; $P < 0.001$) and were highly correlated with hydroxyproline ($r = 0.68$ and 0.83, respectively; $P < 0.001$ for both), but poorly correlated with urinary Ca ($r = 0.21$; P = NS and $r = 0.42$; $P = 0.01$, respectively), suggesting that these markers reflect different events of bone resorption. Similarly, after bisphosphonate therapy, urinary Pyr and D-Pyr levels fell by 31% and 50%, respectively, compared to 38% for hydroxyproline and 76% for urinary Ca. There was a significant correlation between posttreatment D-Pyr and serum Ca levels ($r = 0.43$; $P < 0.05$). In summary, we found that the urinary excretion of Pyr and D-Pyr was markedly increased in hypercalcemic cancer patients and was adequately lowered by bisphosphonate therapy. The urinary excretion of the pyridinium cross-links, especially D-Pyr, should be helpful to specifically quantitate bone matrix resorption and monitor the inhibition of bone resorption in cancer patients receiving antiosteolytic drugs. (*J Clin Endocrinol Metab* **74:** 471–475, 1992)

TUMOR -associated hypercalcemia is characterized by a marked increase in the bone resorption rate, frequently unmatched by an increase in the bone formation rate (1–3). This stimulation of osteoclastic activity by tumor products explains why bisphosphonates have been so successfully used for the treatment of tumor-associated hypercalcemia (4, 5) and why they are now tested for the treatment and even the prevention of metastatic bone disease (6). The availability of sensitive and specific markers of bone resorption would be particularly helpful for selecting the optimal therapeutic scheme for bisphosphonates in this clinical setting (7).

Urinary hydroxyproline is a conventional parameter for measuring bone resorption, but hydroxyproline also comes from sources other than bone, and its clinical value is probably more limited in cancer patients than in osteoporotic patients, because of the frequent soft tissue invasion by the tumor cells (8–10). Fasting urinary calcium excretion is currently the most reliable and easily measured parameter to monitor the inhibition of bone resorption induced by bisphosphonates (6, 7, 10). However, tumor products such as PTH-related peptide (PTHrP), which is the most important pathogenic factor in humoral hypercalcemia of malignancy, increase the tubular reabsorption of calcium in a PTH-like manner (11, 12). This increased calcium reabsorption by the kidneys can play an important contributory role in the genesis of cancer hypercalcemia, particularly humoral hypercalcemia of malignancy, and perhaps also in hypercalcemia caused by breast cancer (13, 14).

The recently introduced measurement of the intermolecular cross-linking compounds of collagen could overcome this relative lack of specificity of urinary calcium and hydroxyproline as markers of osteoclastic activity (8, 15). Indeed, pyridinoline (Pyr) and deoxypyridinoline (D-Pyr), also called hydroxylysylpyridinoline and lysylpyridinoline, respectively, are two mature cross-linking amino acids that form covalent cross-links be-

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tween adjacent collagen chains in extracellular matrix (16). They are present in many connective tissues, such as bone and cartilage, and to a lesser extent in other connective tissues, except skin (17). Pyr is the major mature component of these tissues, while D-Pyr is present in significant amounts only in bone tissue. They can be measured precisely in urine by fluorescence detection after high pressure liquid chromatography (18, 19). Urinary concentrations of Pyr and D-Pyr have been reported in normal children and adults (15, 20, 21), in patients with osteoarthritis or rheumatoid arthritis (22, 23), and more recently in patients with various metabolic bone diseases (15, 24, 25). Urinary Pyr was markedly increased in patients with primary hyperparathyroidism and Paget's disease of bone. Intravenous treatment of Pagetic patients with a bisphosphonate induced a rapid and dramatic decrease in Pyr and D-Pyr, demonstrating that their excretion reflects resorption and not formation (15). Finally, urinary Pyr and D-Pyr are markedly increased after the menopause and return to premenopausal levels after hormone replacement therapy (25).

In this study we have measured urinary Pyr and D-Pyr in a well characterized population of patients with tumor-associated hypercalcemia before and after treatment with bisphosphonates, and the data were compared to serum and urinary calcium and urinary hydroxyproline levels.

Materials and Methods

Patients

We have studied 36 hypercalcemic cancer patients before any specific hypocalcemic therapy besides iv saline rehydration. There were 17 males and 19 females, and the mean (±SD) age was 59 ± 11 yr. Tumor types were as follows: 14 breast cancers, 9 head and neck tumors, 4 lung neoplasms, and 9 miscellaneous tumors (3 multiple myeloma, 2 melanoma, 1 prostate, 1 uterus, 1 penis, and 1 adenocarcinoma of unknown origin). Bone metastatic involvement was defined as previously described (3); 6 patients were considered negative, 2 as doubtful, and the 28 others as positive, including 4 cases with bony destruction due to local tumor infiltration.

We reevaluated 32 of 36 patients after treatment with iv bisphosphonates as part of previously described protocols (5, 26); 27 were then normocalcemic, and 5 were still mildly hypercalcemic. Of these 32 patients, 28 had received pamidronate (Aredia, Ciba-Geigy, Basel, Switzerland) at dosages of 0.5 mg/kg over 24 h (n = 5), 1.0 mg/kg over 24 h (n = 3), 1.5 mg/kg over 24 h (n = 10), or daily 2-h infusions (0.5 mg/kg·day) over 3 days (n = 10); the 4 other patients had received tiludronate (SR41319B, Sanofi, Montpellier, France) at a dosage of 6.0 mg/kg·day for 3 days, followed by oral therapy (n = 4).

Assays

As previously described (4, 5, 10), our measurements included total serum calcium (Ca; normal values, 2.12–2.57 mmol/L) corrected for protein levels according to the formula of Parfitt (corrected Ca, Corr.Ca), ionized Ca (Ca^{2+}; normal, 1.05–1.26 mmol/L), urinary calcium excretion in 2-h morning fasting specimens (uCa/Creat; normal, <0.59 mmol/mmol), and hydroxyproline in the same specimens (normal, <40 μmol/mmol creatinine). The normal values have been prospectively defined during the study period in 76 healthy subjects who were not taking any drug or hormone known to influence bone metabolism; there were 15 males and 61 females, and their mean age was 51 ± 11 yr.

Pyr and D-Pyr were measured in the same fasting urine specimens as calcium and hydroxyproline, according to a previously described method (15). Briefly, the cross-links were extracted from the hydrolyzed urine sample by cellulose chromatography, separated by reverse phase high pressure liquid chromatography, and identified by spectrofluorimetry. The area of the fluorescent peak was quantified by comparison with calibrated Pyr and D-Pyr external standards purified from human cortical bone. The intra- and interassay variations were less than 10% for Pyr and less than 15% for D-Pyr (15). All measurements were made in a blind fashion, and the code was broken after completion of the study. Values in hypercalcemic patients were compared to the ones measured in the group of healthy subjects defined above. These control values were 40 ± 19 nmol/mmol creatinine for Pyr and 6 ± 3 nmol/mmol creatinine for D-Pyr; the respective upper limits of normal were, thus, 78 and 12 nmol/mmol creatinine.

Statistical analysis

Data are expressed as the mean ± 1 SD, except when indicated. We performed classical statistical tests, using the Statview TM512+ program (Brainpower, Inc., Calabasas, CA).

Results

Evaluation before therapy

Initial mean levels of Ca, Corr.Ca, Ca^{2+}, uCa/Creat, hydroxyproline, Pyr, and D-Pyr are summarized in the *left column* of Table 1, whereas individual concentrations of Pyr and D-Pyr are depicted in Fig. 1. All four urinary markers of bone resorption were significantly increased compared to the values measured in healthy controls.

TABLE 1. Mean (±1 SD) concentrations of various parameters of calcium metabolism before and after bisphosphonate therapy in hypercalcemic cancer patients

Parameter	Before therapy (n = 36)	After therapy (n = 32)
Ca, mmol/L	3.22 ± 0.40	2.32 ± 0.27[a]
Corr. Ca, mmol/L	3.42 ± 0.42	2.50 ± 0.27[a]
Ca^{++}, mmol/L	1.66 ± 0.24	1.20 ± 0.15[a]
uCa/Creat, mmol/mmol	1.61 ± 0.74	0.39 ± 0.39[a]
Hydroxyproline, μmol/mmol creat	71 ± 46	44 ± 22[b]
Pyr, nmol/mmol creat	130 ± 62	90 ± 60[b]
D-Pyr, nmol/mmol creat	20 ± 15	10 ± 6[a]

[a] Paired t test, $P < 0.0001$.
[b] $P < 0.001$.

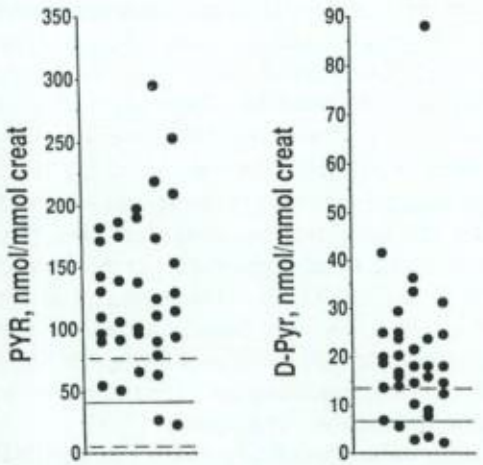

FIG. 1. Individual fasting urinary excretion of Pyr and D-Pyr measured before therapy in 2-h morning fasting urinary samples of 36 hypercalcemic cancer patients. The *horizontal lines* represent the normal range for each parameter.

There was a mean 3.3-fold increase in Pyr and D-Pyr over the normal mean, compared to 5.8- and 3.4-fold increases for fasting urinary Ca and hydroxyproline, respectively. Compared to their respective upper limits of normal, the 4 markers were, thus, increased in 35 of 36 patients (97%) for uCa/Creat, 29 of 35 (83%) for hydroxyproline, 30 of 36 (83%) for Pyr, and 27 of 36 (75%) for D-Pyr. These percentages were not significantly different from one another, and there was also no significant influence of age or sex on any of the parameters.

We analyzed the data according to the tumor type, and Table 2 compares breast cancers to head and neck tumors. The differences were not significant for any parameter, although uCa/Creat tended to be lower and Pyr or D-Pyr higher in patients with head and neck tumors than in patients with breast cancer. The data were also analyzed according to the presence of bone or soft tissue metastatic involvement. Parameters of bone resorption tended to be higher in patients with bone metastases (n = 28) than in patients without evidence of skeletal involvement (n = 6), particularly for Pyr (139 ± 64 *vs.* 87 ± 21 nmol/mmol creatinine; $P = 0.06$) and hydroxyproline (78 ± 49 *vs.* 42 ± 13 μmol/mmol creatinine; $P = 0.09$). However, we could not detect an influence of soft tissue involvement on the above-mentioned parameters.

There was no significant correlation between Ca, Corr.Ca, or Ca^{2+} and uCa/Creat, hydroxyproline, Pyr, or D-Pyr. However, the different parameters of bone resorption generally correlated with each other, although at variable degrees. Thus, uCa/Creat levels were weakly correlated with hydroxyproline ($r = 0.45$; $P < 0.01$) and D-Pyr ($r = 0.42$; $P = 0.01$), but not with Pyr ($r = 0.21$). There was a significant correlation between Pyr and hydroxyproline ($r = 0.68$; $P < 0.001$; Fig. 2A) and between D-Pyr and hydroxyproline ($r = 0.83$; $P < 0.001$; Fig. 2B), and Pyr and D-Pyr correlated with each other ($r = 0.72$; $P < 0.001$). These correlations were generally better in patients with breast cancer than in patients with head and neck tumors, *e.g.* for Pyr *vs.* uCa/Creat, $r = 0.38$ in breast *vs.* 0.20 in head and neck cancers, and for Pyr *vs.*

TABLE 2. Comparison of head and neck tumors (n = 9) with breast cancers (n = 14)

Parameter	Head and neck tumors	Breast cancers
Ca, mmol/L	3.29 ± 0.50	3.12 ± 0.30
Corr. Ca, mmol/L	3.42 ± 0.55	3.37 ± 0.37
Ca^{++}, mmol/L	1.69 ± 0.32	1.58 ± 0.19
uCa/Creat, mmol/mmol	1.52 ± 1.07	1.66 ± 0.70
Hydroxyproline, μmol/mmol creat	74 ± 71	82 ± 40
Pyr, nmol/mmol creat	144 ± 72	115 ± 64
D-Pyr, nmol/mmol creat	26 ± 24	18 ± 11

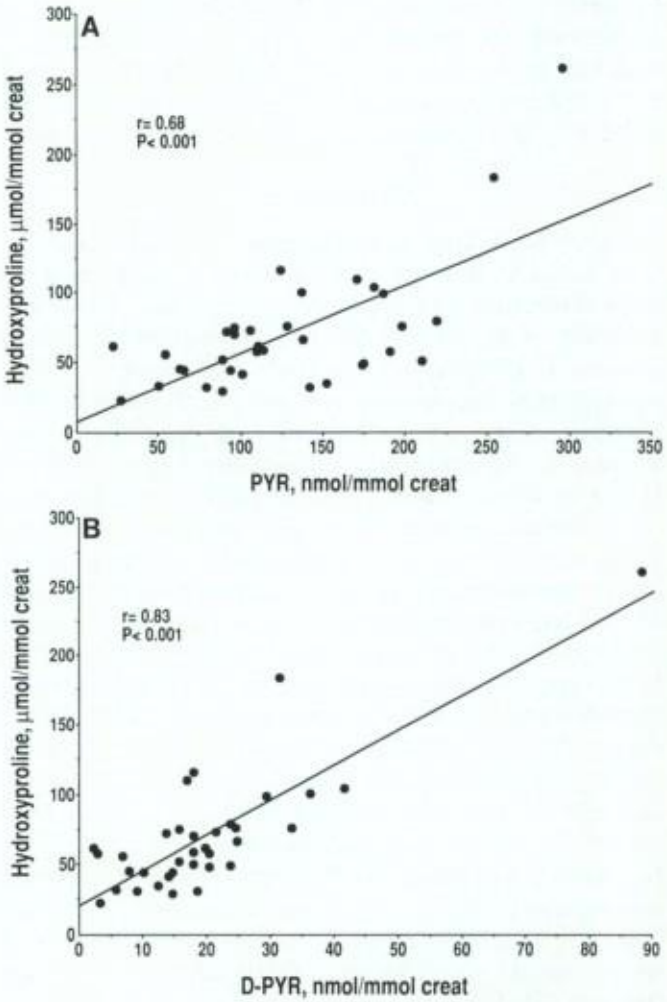

FIG. 2. Correlations between the fasting urinary excretion of Pyr and hydroxyproline (A; $y = 0.49x + 7.1$) and between that of D-Pyr and hydroxyproline (B; $y = 2.5x + 21$) measured before therapy in 2-h morning specimens of 36 hypercalcemic cancer patients.

hydroxyproline, r = 0.79 in breast *vs.* 0.30 in head and neck cancers.

Evaluation after therapy

Mean levels of Ca, Corr.Ca, Ca^{2+}, uCa/Creat, hydroxyproline, Pyr, and D-Pyr are summarized in the *right column* of Table 1. The four parameters of bone resorption fell significantly after bisphosphonate therapy, but the fall was relatively greater for uCa/Creat than for the other parameters. The mean decrease in urinary crosslink excretion after bisphosphonate therapy was 31% for Pyr and 50% for D-Pyr. There was a significant ($P < 0.05$) correlation between posttreatment D-Pyr and Ca (r = 0.43) or Corr.Ca (r = 0.36), but there was no correlation between posttreatment Ca levels and the pretreatment concentrations of the 4 parameters of bone resorption. The levels of the 4 markers remained elevated in 5 of 32 patients (16%) for uCa/Creat, 14 of 31 (45%) for hydroxyproline, 17 of 32 (53%) for Pyr, and 9 of 32 (28%) for D-Pyr, respectively (P = NS). In the 5 patients who had an elevated Ca level after therapy, Pyr or D-Pyr remained increased in 4 of them, compared to in 5 of 5 for hydroxyproline and 2 of 5 for uCa/Creat levels.

Discussion

Tumor-associated hypercalcemia is an excellent human model to test the clinical value of markers of the bone resorption rate, since osteoclastic activity is dramatically increased and can be inhibited by the administration of bisphosphonates (1–5). Despite the fact that our reference values were relatively high, being mostly obtained from untreated peri- or postmenopausal women, the results clearly indicate that urinary Pyr and D-Pyr levels are indeed markedly increased in hypercalcemic cancer patients. Compared to values in healthy young adults (25), the urinary excretion of Pyr and D-Pyr in hypercalcemic patients was increased in 94% and 89% of the cases, respectively. It is interesting to note that Pyr and D-Pyr correlated much better with urinary hydroxyproline than with urinary calcium excretion, probably because of the tumoral secretion of PTHrP or similar factors, which are well known to increase the tubular reabsorption of calcium and, thus, can falsely decrease the quantification by uCa/Creat of the bone resorption rate in many hypercalcemic patients. Along the same line, Pyr and D-Pyr were relatively more increased compared to uCa/Creat in patients with head and neck tumors than in patients with breast cancer, in whom ectopic secretion of PTHrP appears to be less frequent (11–13).

Our data show that urinary Pyr and D-Pyr were adequately lowered by bisphosphonate therapy, reflecting the decrease in bone resorption. Such a decrease has been previously reported after iv bisphosphonate treatment of Paget's disease of bone (15), another human model of increased bone resorption. Noteworthy, there was a significant correlation between D-Pyr and serum Ca after therapy. The persistent elevation of Pyr and D-Pyr in four of five patients who remained mildly hypercalcemic indicates that bone resorption was insufficiently inhibited in these patients and that administration of higher doses of bisphosphonates or prolonged therapy could be beneficial (4, 5). Measurement of Pyr and D-Pyr could, thus, be very helpful to assess the optimal therapeutic scheme in trials evaluating the antiosteolytic properties of bisphosphonates in normocalcemic patients with bone metastatic involvement (6, 7).

The exact relationship of urinary Pyr and D-Pyr measurement in cancer patients to fasting urinary calcium and hydroxyproline levels remains to be determined more precisely, but these markers of bone resorption are likely to provide complementary information. As mentioned above, levels of urinary calcium also reflect the effects of tumor-secreted products on the renal tubules; despite this important limitation, baseline uCa/Creat levels were relatively more increased than the other markers, correlated poorly with Pyr or D-Pyr, and decreased relatively more after therapy than the markers of bone matrix resorption. This could suggest a preferential removal of bone mineral compared to bone matrix during the process of malignant osteolysis and a similar preferential inhibitory activity of bisphosphonates, but these intriguing possibilities need further evaluation. On the other hand, urinary hydroxyproline is derived from bone resorption, but also from the catabolism of connective tissues, some of which may be involved in the metastatic process. Conversely, urinary Pyr and D-Pyr should be specific indexes of bone matrix resorption under the direct or indirect influence of tumor cells and its modification by antiosteoclastic therapies. If the clinical development of Pyr and D-Pyr measurement is presently limited by the assay, which remains cumbersome and expensive, this limitation should be overcome in the near future by the development of immunoassays.

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References

1. Stewart AF, Vignery A, Silverglate A, et al. Quantitative bone histomorphometry in humoral hypercalcemia of malignancy: uncoupling of bone cell activity. J Clin Endocrinol Metab. 1982;55:219–27.
2. Delmas PD, Demiaux B, Malaval L, Chapuy MC, Edouard C, Meunier PJ. Serum bone gamma carboxyglutamic acid-containing protein in primary hyperparathyroidism and in malignant hyper-

calcemia. J Clin Invest. 1986;77:985–91.
3. Body JJ, Cleeren A, Pot M, Borkowski A. Serum osteocalcin (BGP) in tumor-associated hypercalcemia. J Bone Mineral Res. 1986;1:523–7.
4. Body JJ, Borkowski A, Cleeren A, Bijvoet OLM. Treatment of malignancy-associated hypercalcemia with intravenous aminohydroxy-propylidene diphosphonate. J Clin Oncol. 1986;4:1177–83.
5. Body JJ, Magritte A, Seraj F, Sculier JP, Borkowski A. Aminohydroxy-propylidene bisphosphonate (APD) treatment for tumor-associated hypercalcemia: a randomized comparison between a 3-day treatment and single 24-hour infusions. J Bone Mineral Res. 1986;4:923–8.
6. Coleman RE, Woll PJ, Miles M, Scrivener W, Rubens RD. Treatment of bone metastases from breast cancer with (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD). Br J Cancer. 1988;58:621–5.
7. Dodwell DJ, Howell A, Ford J. Reduction in calcium excretion in women with breast cancer and bone metastases using the oral bisphosphonate pamidronate. Br J Cancer. 1990;61:123–5.
8. Delmas P. Biochemical markers of bone turnover for the clinical assessment of metabolic bone disease. Endocrinol Metab Clin North Am. 1990;19:1–18.
9. Niell HB, Neely CL, Palmieri GM, McDonald MW. The postabsorptive hydroxyproline in the long-term evaluation of patients with breast cancer. Cancer. 1983;52:1442–7.
10. Body JJ, Pot M, Borkowski A, Sculier JP, Klastersky J. Dose/response study of aminohydroxypropylidene bisphosphonate in mor-associated hypercalcemia. Am J Med. 1987;82:957–63.
11. Martin TJ. Humoral hypercalcemia of malignancy. Bone Mineral. 1988;4:83–9.
12. Broadus AE, Mangin M, Ikeda K, et al. Humoral hypercalcemia of cancer. Identification of a novel parathyroid hormone-like peptide. N Engl J Med. 1988;319:556–63.
13. Budayr AA, Nissenson RA, Klein RF, et al. Increased serum levels of a parathyroid hormone-like protein in malignancy-associated hypercalcemia. Ann Intern Med. 1989;111:807–12.
14. Percival RC, Yates AJP, Gray RES, et al. Mechanisms of malignant hypercalcaemia in carcinoma of the breast. Br Med J. 1985;291:776–9.
15. Uebelhart D, Gineyts E, Chapuy MC, Delmas PD. Urinary excretion of pyridinium crosslinks: a new marker of bone resorption in metabolic bone disease. Bone Mineral. 1990;8:87–96.
16. Eyre DR. Crosslink maturation in bone collagen. In: Veis A, ed. The chemistry and biology of mineralized connective tissues. Amsterdam: Elsevier; 1981;51–5.
17. Eyre DR, Paz MA, Galop PM. Cross linking in collagen and elastin. Annu Rev Biochem. 1984;53:717–48.
18. Eyre DR. Collagen cross-linking amino-acids. In: Cunningham LW, ed. Methods in enzymology. New York: Academic Press; 1987;144:115–39.
19. Black D, Duncan A, Robins SP. Quantitative analysis of the pyridinium crosslinks of collagen in urine using ion-paired reversed-phase high-performance liquid chromatography. Anal Biochem. 1988;169:197–203.
20. Fujimoto D, Suzuki M, Uchiyama A, Miyamoto S, Inoue T. Analysis of pyridinoline, a cross-linking compound of collagen fibers, in human urine. J Biochem. 1983;94:1133–6.
21. Beardsworth LJ, Eyre DR, Dickson IR. Changes with age in the urinary excretion of lysyl and hydroxylysylpyridinoline, two new markers of bone collagen turnover. J Bone Mineral Res. 1990;5:671–6.
22. Robins SP, Stewart P, Astbury C, Bird HA. Measurement of the crosslinking compound pyridinoline in urine as an index of collagen degradation in joint disease. Ann Rheum Dis. 1986;45:969–73.
23. Black D, Marabani M, Sturrock RD, Robins SP. Urinary excretion of the hydroxypyridinium crosslinks of collagen in patients with rheumatoid arthritis. Ann Rheum Dis 1989;48:641–4.
24. Delmas PD, Schlemmer A, Gineyts E, Riis B, Christiansen C. Urinary excretion of pyridinoline crosslinks correlate with bone turnover measured on iliac crest bone biopsy in patients with vertebral osteoporosis. J Bone Mineral Res. 1991;6:639–44.
25. Uebelhart D, Schlemmer A, Johansen JS, Gineyts E, Christiansen C, Delmas PD. Effect of menopause and hormone replacement therapy on the urinary excretion of pyridinium cross-links. J Clin Endocrinol Metab. 1991;72:367–73.
26. Dumon JC, Magritte A, Body JJ. Efficacy and safety of the bisphosphonate tiludronate for the treatment of tumor-associated hypercalcemia. Bone Mineral. 1997;75:257–66.