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Urinary output of hydroxylysine glycosides and pyridinium cross-links in detecting rat bone collagen turnover rate

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Abstract Glucosylgalactosylhydroxylysine (GGHYL), galactosylhydroxylysine (GHYL), pyridinoline (PYD) and deoxypyridinoline (DPD) were measured in the urine (6 h serial specimens over 96 and 24 h urine specimens for 4 days) collected from four adult Sprague Dawley rats and in the femoral and tibial bone as well as in the dorsal skin of the same rats. No significant daily variations were found in the urine excretion of GGHYL, GHYL, PYD and DPD but significant diurnal variations. The GGHYL/GHYL ratio in rat urine (0.46 ± 0.1) reflected neither the bone collagen ratio (1.9 to 2.4) nor the skin collagen ratio (1.22 ± 1.07), a finding that may reflect GGHYL conversion into GHYL. The content of both pyridinolines was very low in the skin and high in the bone collagen and the urinary PYD/DPD ratio (1.46 ± 0.15) reflected essentially the bone collagen ratio (0.8–3.0). These results suggest the usefulness of measuring GGHYL, GHYL, PYD and DPD in 24 h urine specimen and, based on the inter-animal variations, the necessity to consider each animal as its own control when bone turnover needs to be monitored.

Key words: Rat; Bone turnover; Galactosylhydroxylysine; Glucosylgalactosylhydroxylysine; Pyridinoline; Deoxypyridinoline

1. Introduction

Hydroxylysine glycosides (galactosylhydroxylysine or GHYL, and glucosylgalactosylhydroxylysine or GGHYL) and the residues of non-borohydride reducible cross-links (hydroxylysylpyridinoline or PYD and lysylpyrydinoline or DPD) are components of collagen [1]. Since GHYL and GGHYL as well as PYD and DPD are the products of post-translational modifications in the pathway of collagen biosynthesis, they are not reutilized during collagen turnover [1,2]. Thus, the urinary excretion of hydroxylysine glycosides and pyridinium cross-links residues specifically reflects collagen breakdown [3–6]. Recently, these two classes of metabolites were proposed to represent markers of bone turnover in human bone diseases, with a specificity higher than that of hydroxyproline (HYP) [4,5,7–14], due to their selective presence in type I bone collagen. In addition their metabolism is less affected by non-specific interferences and is not influenced by renal tubular reabsorption [15,16].

Interest has also grown in the use of these markers in experimental animal models which may be relevant to human bone remodelling diseases. In particular, PYD and DPD excretion in the rat was reported to be increased by ovariectomy [17,18], experimental polyarthritis [18], dietary calcium restriction [19] and parathyroid hormone infusion [20]. Furthermore, PYD and DPD levels in ovariectomized rats rose early with a significant peak 2 weeks after surgery [18].

The bone specificity of these markers in the rat has been also investigated by Robins and Duncan [21]. Some authors [17] have found that cortical bone contains higher concentrations of cross-links than trabecular bone, as observed in humans. The bone collagen content of hydroxylysine glycosides in the rat has also been investigated by Moro et al. [22] who reported that the levels of glycosylation of type I collagen in cortical bone were not statistically different from those of trabecular bone. In particular, in these rat tissues, the GGHYL/GHYL ratio ranges from 1.6 to 2.3, a value which is considerably higher than that of 0.47 observed in man [23,24].

To date, at least two important questions, regarding the study on urinary excretion of these markers in the rat, remain open. Firstly, the biological variation of the urinary excretion of GGHYL, GHYL, PYD and DPD over time needs to be properly evaluated in order to better define which specimen (24 h vs spot urine) is most appropriate in order to measure these markers. Secondly, the glycosylation and cross-linkage of bone collagen need to be better characterised, especially in the metaphysis, epiphysis and diaphysis of the distal femur and of the proximal tibia, the two segments of bone primarily involved in the experimentally induced osteopenia in the rat. Furthermore, rat skin collagen also needs to be better characterised since, due to its turnover, it might represent a source of type I collagen catabolites.

The aim of this work was to study the biological variation in the urinary output of GGHYL, GHYL, PYD and DPD in the rat and to verify their specificity as bone marker. The urinary excretion of collagen catabolites was studied in 6 h serial specimens over a 96 h period and in 24 h urines from four adult Sprague Dawley rats for 4 days. In addition, the relative content of GGHYL, GHYL, PYD and DPD was measured in the rat femoral and tibial bone as well as in the dorsal skin.

2. Materials and methods

2.1. Materials

All reagents used, purchased from BDH (UK), were of analytical grade.

2.2. Urine and tissue collection

Mature male Sprague Dawley rats (n = 4) (Charles River, Milan, Italy), aged 90 days, weighing between 350 and 380 g, were maintained at 22 ± 1°C and at 12 h light/dark cycle. The animals were

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fed with a normal calcium diet (Mucedola, Milan, Italy) containing 1% calcium, 0.85% phosphorus and 1260 IU/kg vitamin D_3, and were allowed free access to food and water. After a week of acclimatisation, urine specimens were collected every 6 h for 4 consecutive days. All the urine specimens were collected in dark, sterile containers. Aliquots of urine were frozen at −80°C pending analysis.

On the fifth day, the rats were killed with inhalation of diethyl ether. Left femurs and tibiae were collected and cartilage, tendons and adhering tissues were accurately removed. The bones were then submitted to densitometry (Hologic QDR 1000 Plus, USA) and physical measurements in order to accurately dissect out segments of their respective diaphysis, metaphysis and epiphysis. The methaphysis dissection limits were established in order to ensure inclusion of the growth plate; the bone segments were submitted to densitometry to confirm their trabecular or cortical structure. A dorsal skin specimen (approx. 1 cm^2) was obtained from each animal and immediately frozen and kept at −135°C pending analysis.

2.3. Measurement of GGHYL, GHYL, total PYD and DPD and creatinine in urine

GGHYL and GHYL were assayed according to a previously described method [25] using 500 μl of the urine specimens diluted with 9 ml of deionized water and 1 ml of 100 mM acetic acid. The detection limit was 2 μmol/l for both the analytes; total inter-assay and intra-assay coefficients of variation (CV) were 5 and 7%, respectively. Total PYD and DPD were determined according to the method of Black et al. [26] using 250 μl of the urine specimens hydrolysed with 250 μl of 12 N HCl for 16 h at 110°C. The detection limit was 20 pmol/ml for both the analytes; total inter-assay and intra-assay CV were 10 and 13%, respectively. Urinary creatinine was measured by Jaffe' method (Boehringer Mannheim, Italy) using a Hitachi 911 analyzer. In the 6 h urine specimens, the concentrations of GGHYL and GHYL were expressed as a function of the urinary creatinine (CR), in terms of nmol of GGHYL (or GHYL)/μmol of CR; analogously, the PYD and the DPD concentrations were expressed as pmol of PYD (or DPD)/μmol of CR. In the 24 h urine, the output of GGHYL, GHYL, PYD and DPD was expressed as nmol/24 h.

2.4. Measurement of GGHYL, GHYL, total PYD and DPD, and HYP in tissues

The segments of tibiae and femurs were delipidized with chloroform:methanol 2:1 for 18 h at 4°C and decalcified with changes of 0.5 M EDTA for 3 weeks. After washing with abundant distilled water, the segments were delipidized again, dried by vacuum centrifugation (Savant 290, USA) and homogenised. The homogenate was hydrolysed in 6 N HCl at 110°C for 18 h. An aliquot of the homogenate was also hydrolysed with 2.5 M sodium hydroxide at 110°C for 18 h in order to determine hydroxylysine glycosides content as well (indeed, the strong acidity of the first procedure of hydrolysis with 6 N HCl results in the hydrolysis of GGHYL and GHYL into hydroxylysine). For the measurement of GGHYL and GHYL, 60 μl of the basic hydrolysate were diluted with 30 ml of deionized water and 0.8 ml of 5 M acetic acid; the sample was then submitted to the above-described procedure. To determine PYD and DPD, 500 μl of the acid hydrolysate were subjected to the above-described procedure.

For the measurement of hydroxyproline, aliquots of the acid hydrolysate were dried by vacuum centrifugation and derivatised with 4-dimethylaminoazobenzene-4'-sulfonyl chloride. The derivatisate was injected (20 μl) into a Spherisorb ODS2 25 cm × 0.46 cm I.D., 5 μm C18 reverse-phase column (Phase Separation, Deeside, Clwyd, UK), and the separation of the analyte was achieved in a gradient mode as previously described [27]. The detection limit was 22 ng/ml and total inter-assay and intra-assay CV were 6 and 9%, respectively.

The tissue content of the analytes was expressed in terms of nmol of GGHYL (or GHYL) and pmol of PYD (or DPD)/nmol of collagen type I chains. The molarity of collagen type I chains was calculated on the basis of the hydroxyproline tissular content, expressed in nmol, assuming a ratio hydroxyproline/α1 (I) chain equal to 100.

2.5. Bone mineral density (BMD) measurement

BMD values were determined by using an X-ray bone densitometry (Hologic QDR 1000 Plus, USA). The absorptiometer was adapted with a specially designed 1 mm collimator and a special software to measure BMD of small animals in vitro and in vivo. The CV of the technique was 1%. The BMD values were expressed in terms of g/cm^2.

2.6. Statistical analysis

Data are presented as mean ± S.E.M. in tables and graphs. Significance of difference, between days and between rats, of the data obtained from the 24 h urine, was evaluated with analysis of multiple variance (MANOVA).

3. Results

Table 1 summarises the results of analysis of multiple variance performed on data obtained by analysing 24 h urine samples from mature male rats: no significant variation between days was noted in the excretion of hydroxylysine glycosides and pyridinolines. The same results were obtained when the data were expressed as a function of creatinine excretion.

Fig. 1a–d shows the fractional daily rhythms of the urinary excretion of GGHYL, GHYL, PYD and DPD, respectively. The excretion of GGHYL and GHYL attained a peak value approximately at 19:00 h (Fig. 1a,b) whereas the urinary output of PYD and DPD attained a peak value at 07:00 h (Fig. 1c,d).

Fig. 2 summarises the results obtained by measuring the absolute content of glycosides (a), and pyridinolines (b) in bone collagen. Epiphyses appear to be the most glycosylated bone compartments, in both femurs and tibiae, whereas the GGHYL/GHYL ratio is substantially constant in the different segments, ranging from 1.89 to 2.38. The absolute content of 3-hydroxypyridinium cross-links is comparable in all the bone segments, whereas the PYD/DPD ratio is greater in epiphyses (2.91–3.18), decreases in diaphyses (1.09–1.37) and is lowest in metaphyses (0.70–0.82), in both femurs and tibiae.

The results of the analyses performed on dorsal skin specimens are shown in Fig. 3. It is interesting to underscore that, due to the great sensitivity of the method employed, it was also possible to measure the small quantities of PYD and DPD in the dorsal skin collagen of the rat.

The differences between human and rat in terms of relative content of GGHYL, GHYL, PYD and DPD in urine, bone collagen and skin collagen are summarised in Table 2. The urinary GGHYL/GHYL ratio is lower in the rat than in the human and does not reflect the relative content of the glycosides either in bone or in skin collagen. In contrast the urinary PYD/DPD ratios in the rat, although lower than in humans, reflects the relative content of pyridinolines in bone.

Table 1
Multifactor analysis of variance between days performed on the data obtained by analysing 24 h urine of mature male rats

Parameters	1st day	2nd day	3rd day	4th day	*P* values
GGHYL (nmol/24 h)	391.00 ± 90.73	419.25 ± 56.27	407.96 ± 57.22	406.03 ± 23.61	NS
GHYL (nmol/24 h)	836.75 ± 197.20	885.25 ± 41.30	913.25 ± 66.10	907.75 ± 54.30	NS
PYD (nmol/24 h)	19.59 ± 1.88	25.81 ± 1.01	23.62 ± 2.26	25.37 ± 1.51	NS
DPD (nmol/24 h)	13.27 ± 0.98	18.78 ± 1.20	15.61 ± 0.87	17.03 ± 2.00	NS

Values (means ± S.E.M.) are expressed as absolute daily output.

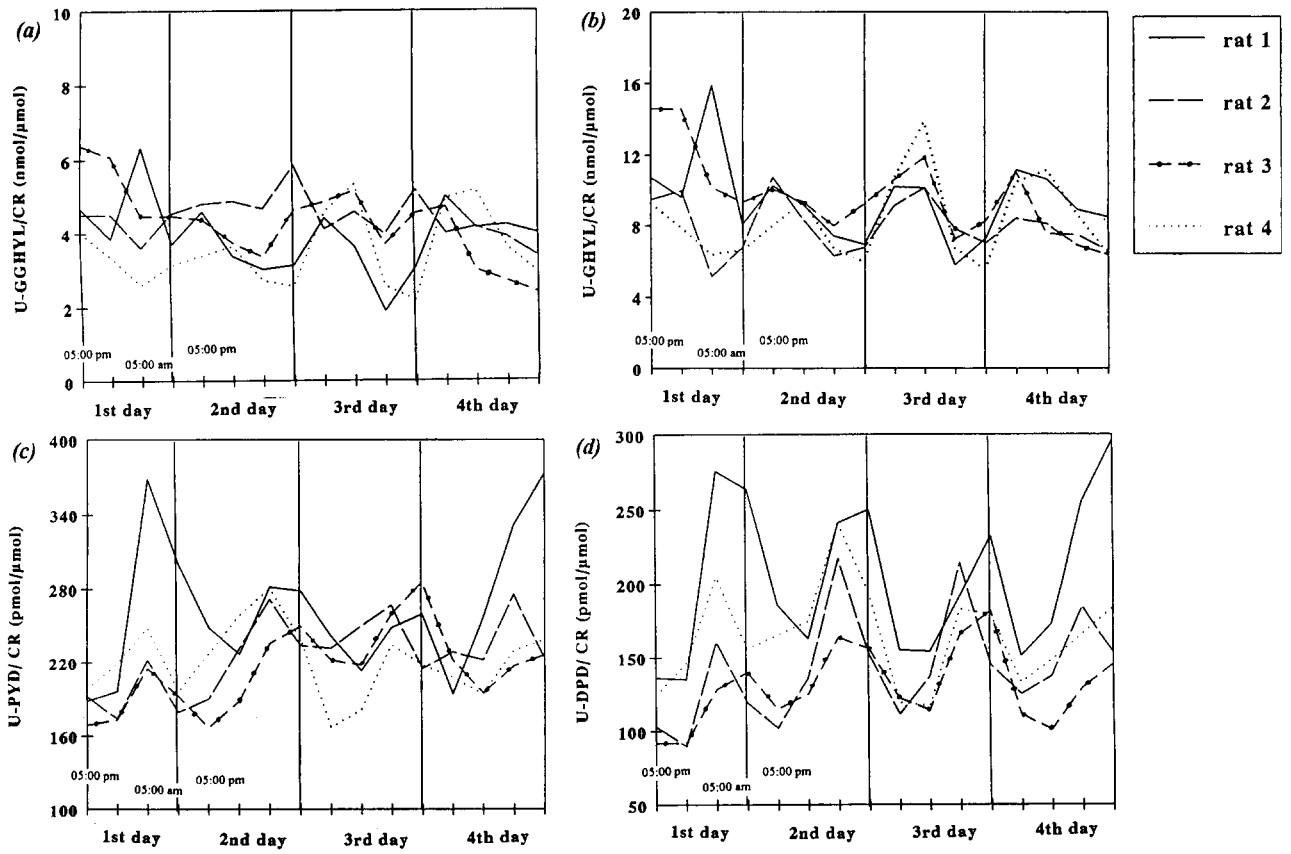

Fig. 1. Fractional daily urinary excretion of glucosylgalactosylhydroxylysine (GGHYL) (a), galactosylhydroxylysine (GHYL) (b), pyridinoline (PYD) (c) and deoxypyridinoline (DPD) (d) in mature male rats. The urine was collected, starting from 17:00 h, every 6 h for 4 consecutive days. The urinary output of GGHYL, GHYL, PYD and DPD varies significantly within day ($p < 0.001$ for GGHYL, PYD and DPD, $p < 0.01$ for GHYL).

4. Discussion

The rat is the animal most commonly used to evaluate the effects of bone turnover perturbations induced by ovariectomy, parathyroidectomy, dietary calcium restrictions and immobilisation as well as the effects of therapeutic interventions related to these pathologies [17–19]. Although it is well known that histomorphometric and densitometric techniques do not allow continuous monitoring of the bone turnover in small laboratory animals [20–22], this evaluation may be possible by measuring the urinary output of some specific bone collagen catabolites, such as urinary pyridinolines and the hydroxylysine glycosides. In order to identify the most appropriate specimen in which to measure the urinary concentration of the markers studied, the urinary output of GGHYL, GHYL, PYD and DPD was monitored over 4 consecutive days in four adult male rats (90 days old). At this age rats are characterised by a very low growth rate, and the length of the experimental period (4 consecutive days) permitted further minimisation of the effect of growth on bone turnover and, therefore, on the markers' excretion. Furthermore, male rats were chosen in order to exclude any possible interference with bone turnover caused by the ovulatory cycle.

Our results indicate that the 24 h urine is the best specimen to measure the excretion of the considered markers. In fact, substantial variation of the urinary excretion of pyridinolines and hydroxylysine glycosides between different days was not observed, whereas a significant diurnal variation was evident. This diurnal variation represents a potentially interesting result, for at least two reasons. Firstly, the fluctuations observed seem to be periodical, in agreement with the diurnal periodicity in the metabolic activity of bone demonstrated by Mühlbauer and Fleisch [28]; this finding also confirms the high sensitivity of these markers in detecting small variations of bone turnover rate like those that physiologically occur within a day. Furthermore, the 12 h phase difference between the peaks of glycosides and pyridinolines suggests that hydroxylysine glycosides are more likely markers of both bone resorption and bone formation rather than indicating a difference in the metabolic pathways of the two classes of catabolites. In this regard, while the source of pyridinolines is undoubtedly the mature collagen, the source of GGHYL and GHYL could be also the intracellular breakdown of new synthetized procollagen demonstrated by Bienkowski et al. [29] in cultured fibroblasts.

In order to investigate the bone specificity of the markers under study, we compared their urinary concentration with their absolute and relative tissue content. Therefore, the four adult male rats were killed at the end of the experimental period, in order to analyse the tissues that, due to their turnover, could contribute to the urinary excretion of the collagen catabolites. Even if both pyridinolines are detectable in the skin, our results confirm that PYD and DPD are present, almost exclusively, in the bone tissue. Particularly, in contrast

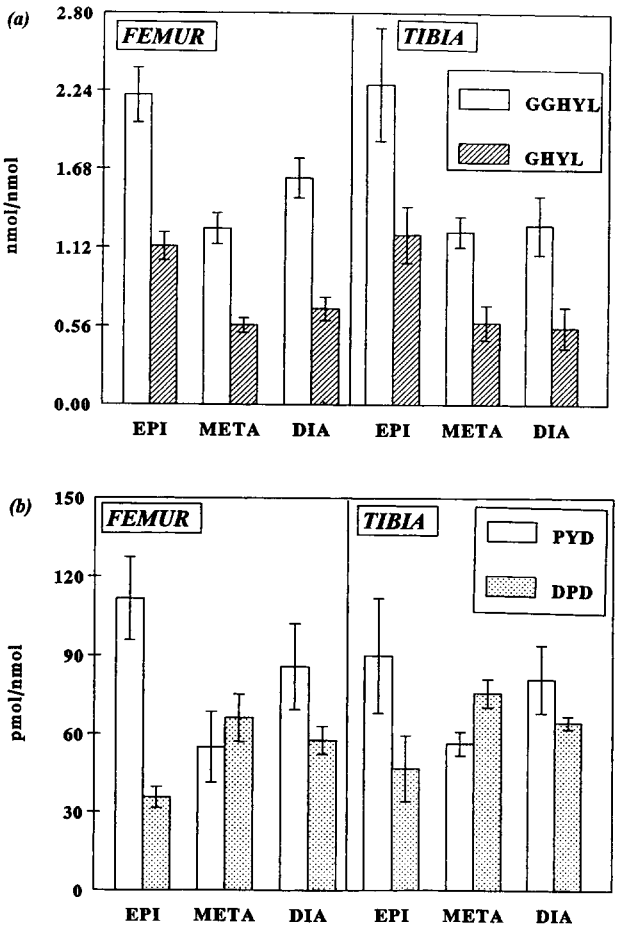

Fig. 2. Relative distribution of hydroxylysine glycosides (GGHYL and GHYL) (a) and pyridinolines (PYD and DPD) (b) in epiphysis (EPI), metaphysis (META) and diaphysis (DIA) of femur and tibia of the mature male rats.

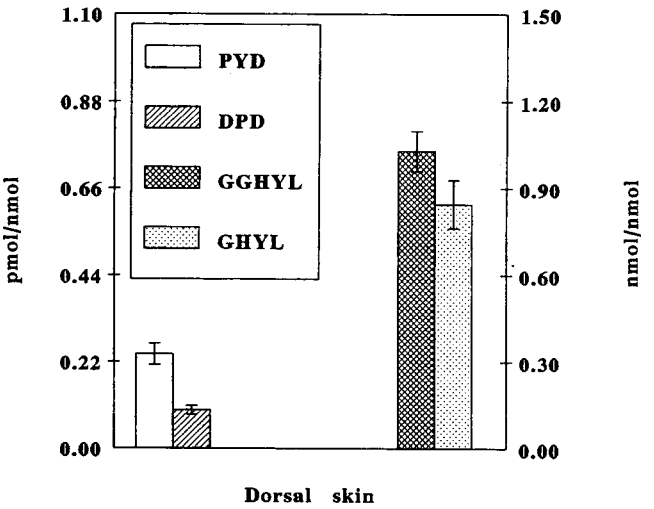

Fig. 3. Absolute and relative content of PYD, DPD, GGHYL and GHYL in dorsal skin. Values are expressed as mean ± S.E.M.

to epiphyses and diaphyses, methaphyses are richer in DPD than in PYD (with a PYD/DPD ratio ranging from 0.70 to 0.95) suggesting that hydroxylysine is relatively less available in this compartment for the formation of PYD, which embodies three hydroxylysines. As a consequence, the pathways of cross-linking in the hydroxyallysine route deviate preferentially toward the formation of DPD, which embodies two hydroxylysines and one lysine. The scarcity of hydroxylysine in methaphyses is likely due to a low lysyl hydroxylase activity, which is known to vary greatly among different tissues [30]. The glycosylation of bone and skin collagen is substantially comparable in terms of both absolute and relative content of GGHYL and GHYL, although if epiphyses appear to be more glycosylated than methaphyses and diaphyses. In rat bone collagen, we found that the GGHYL/GHYL ratio ranges from 1.9 to 2.4, confirming previous report [22]; this value is considerably higher than that observed in man [24].

We compared the relative urinary concentration of the markers with their relative tissue content. In rat, as well as in human, the urinary PYD/DPD ratio reflects essentially the bone collagen ratio, confirming that the bone is the main source of pyridinolines, whilst the urinary GGHYL/GHYL ratio can be related neither to the bone collagen ratio nor to the skin collagen ratio, indicating that urinary GHYL does not appear so bone-specific as in human. However, the rat urinary GGHYL/GHYL ratio could be likely explained by the conversion of GGHYL into GHYL: in these regards, the presence of a specific α-glucosidase in rat kidney has been reported [31] and, therefore, we cannot exclude the existence of such a metabolic step in one or more compartments of the rat. These considerations suggest to use total urinary excretion of hydroxylysine glycosides (GGHYL+GHYL), a parameter that is not affected by the likely transformation of GGHYL into GHYL.

Table 2
Relative content of pyridinolines and hydroxylysine glycosides in urine, bone and skin collagen from human and rat

Specimen	Human		Rat			
	PYD/DPD	GGHYL/GHYL		PYD/DPD		GGHYL/GHYL
Urine	5.17 ± 1.1[a]	1.33 ± 0.17 (3)				
	3.0 ± 0.4 (2)	1.5 ± 0.28 (7)		1.46 ± 0.15[a]		0.46 ± 0.1[a]
		1.75 ± 0.54 (33)				
Bone collagen		0.15 (1)	femoral EPI	3.182 ± 0.780	femoral EPI	2.015 ± 0.164
		0.47 (7)	femoral META	0.821 ± 0.126	femoral META	2.226 ± 0.279
	4.0 (1)		femoral DIA	1.370 ± 0.312	femoral DIA	2.361 ± 0.167
			tibial EPI	2.910 ± 2.371	tibial EPI	1.892 ± 0.237
			tibial META	0.701 ± 0.040	tibial META	2.377 ± 0.360
			tibial DIA	1.079 ± 0.283	tibial DIA	1.220 ± 0.066
Skin collagen	–	1.61 (3)		2.508 ± 0.44		1.220 ± 0.07

EPI, epiphysis; DIA, diaphysis; META, metaphysis.
[a]Our observations.

In conclusion, urinary GGHYL, GHYL, PYD and DPD seem to be very specific and sensitive markers of bone turnover rate in the rat and to represent a powerful tool for a continuous, rapid and economical monitor of bone remodelling in this animal. Our results suggest the usefulness of measuring all these markers in the 24 h urine specimen and of including the hydroxylysine glycosides in order to avoid bias due to unknown catabolic pathways in their metabolism. In addition, we suggest to express the results as mol excreted per day since the urinary excretion of glycosides and pyridinolines, and consequently their correlation with creatinine, has not yet been fully elucidated. As a consequence, the bias due to possible variations within and between methods in the measurement of urinary creatinine is avoided. Finally, to avoid a possible significant variation between subjects, it is likely necessary to consider each rat as its own control and to express the results as MK_t/MK_o (were MK_t = marker output at time t; MK_o = basal marker output) in order to normalized the variations of the bone turnover rate respect to an initial point.

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References

[1] Kucharz, E.J. (1992) Collagen: Biochemistry and Pathophysiology, Springer, Berlin, pp. 31–53.
[2] Eyre, D.R. (1987) Methods Enzymol. 144, 139–155.
[3] Askenasi, R. (1974) J. Lab. Clin. Med. 83, 673–79.
[4] Beardsworth, L.J., Eyre, D.R. and Dickson, I.R. (1990) J. Bone Miner. Res. 5, 671–76.
[5] Moro, L., Modricky, C., Rovis, L. and De Bernard, B. (1988) Bone Miner. 3, 271–76.
[6] Seibel, M.J., Robins, S.P. and Bilezikian, J.P. (1992) Trends Endocrinol. Metab. 3, 263–70.
[7] Uebelhart, D., Schlemmer, A., Johansen, J.S., Gineyts, E., Christiansen, C. and Delmas, D. (1991) J. Clin. Endocrinol. Metab. 72, 367–373.
[8] Seibel, M.J., Gartenberg, F., Silverberg, S.J., Ratcliffe, A., Robins, S.P. and Bilezikian, J.P. (1992) J. Clin. Endocrinol. Metab. 74, 481–485.
[9] Hamdy, N.A.T., Papapoulos, S.E., Colwell, A., Eastell, R. and Russel, R.G.G. (1993) Bone Miner. 22, 1–8.
[10] Body, J.J. and Delmas, P.D. (1992) J. Clin. Endocrinol. Metab. 74, 471–475.
[11] Moro, L., Modricky, C., Rovis, L. and De Bernard, B. (1988) Bone Miner. 3, 271–276.
[12] Bettica, P., Moro, L., Robins, S., Taylor, A.K., Talbot, J., Singer, F. and De Bernard, B. (1992) Bone Miner. 3, 271–276.
[13] Moro, L., Gazzarini, C., Modricky, c., Rovis, L., de Bernard, B., Galligioni, E., Crivellari, D., Morassut, S. and Monfardini, S. (1990) Clin. Chem. 36, 772–774.
[14] Schonau, E., Kruse, K., De Bernard, B. and Moro, L. (1992) Clin. Chem. 81, 633–634.
[15] Askenasi, R. (1976) in : Methodology of Connective Tissue Research (Hall, D.A. ed.) pp. 263–273, Johnson-Bruver, Oxford.
[16] Tohme, J.F., Seibel, M.J., Silverberg, S.J., Robins, S.P. and Bilezikian, J.P. (1991) Z. Rheumatol. 50, 133–141.
[17] Black, D., Farquharson, C. and Robins, S.P. (1989) Calcif Tissue Int. 44, 343–347.
[18] Tordiman, C., Lhumeau, A., Pastoureau, P., Meunier, F., Serkiz, B., Volland, J.P. and Bonnet (1994) J. Bone Miner. 26, 155–167.
[19] Egger, C.D., Muhlbauer, R.C., Felix, R., Fleisch, H. and Delmas, P.D. (1992) Bone Miner. 17, 137–140.
[20] Jerome, C.P., Colwell, A., Eastell, R., Russel, R.G.G. and Trechsel, U. (1992) Bone Miner. 19, 117–125.
[21] Robins, S.R. and Duncan, A. (1987) Biochim. Biophys. Acta 914, 233–239.
[22] Michalsky, M., Norris-Suarez, Karem., Bettica, P., Pecile, A. and Moro, L. (1993) Biochem. Biophys. Res. Commun. 192, 1281–1288.
[23] Kakimoto, Y. and Akazawa, S. (1970) J. Biol. Chem. 245, 5751–5758.
[24] Krane, S.M., Kantrowitz, F.G., Byrne, M. and Pinnel, F.R. (1977) J. Clin. Invest. 59, 819–827.
[25] Alfano, M., Casari, E., Stenico, A., Murone, M. and Grazioli, V. (1994) Clin. Chem. 40, 2113–2114.
[26] Grazioli, V., Casari, E., Murone, M. and Bonini, P.A. (1993) J. Chromatogr. 615, 59–66.
[27] Casari, E., Ferrero, C.A. and Grazioli, V. (1992) Clin. Chem. 38, 2337–2338.
[28] Mühlbauer, R.C. and Fleisch, H. (1990) Am. J. Physiol. 259, 679–689.
[29] Bienkowski, R.S., Baum, B.J. and Crystal, R.G. (1978) Nature 276, 413–416.
[30] Tanzer, M.L. (1973) Science 180, 561–566.
[31] Sternberg, M. and Spiro, R.G. (1979) J. Biol. Chem. 254, 10329–10336.