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Serum Galactosyl Hydroxylysine as a Biochemical Marker of Bone Resorption

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Background: **Serum-based biochemical markers of bone resorption may provide better clinical information than urinary markers because direct comparison with serum markers of bone formation is possible and because the within-subject variability of serum markers may be lower. We describe a method for the measurement of free β-1-galactosyl-*O*-hydroxylysine (Gal-Hyl) in serum.**

Methods: **The assay used preliminary ultrafiltration of serum, dansylation, and separation by reversed-phase HPLC with fluorescence detection. Healthy subjects were recruited from population-based studies of bone turnover.**

Results: **The within-run (n = 15) and between-run (n = 15) CVs were 7% and 14%, respectively, at a mean value of 48 nmol/L. In women and pubertal girls, serum free Gal-Hyl correlated with urine free Gal-Hyl (r = 0.84; P <0.001). Serum Gal-Hyl was higher during puberty and increased after menopause. The fractional renal clearance of free Gal-Hyl relative to that of creatinine was 0.90 (95% confidence interval, 0.82–0.98). Serum free Gal-Hyl decreased by 36% (SE = 4%) in 14 patients with mild Paget disease treated with an oral bisphosphonate, and this decrease was significantly (P <0.001) greater than that seen for either serum tartrate-resistant acid phosphatase (9%; SE = 4%) or serum C-terminal telopeptide of collagen I (19%; SE = 8%).**

Conclusion: **Serum free Gal-Hyl may be useful as a serum marker of bone resorption.**

Biochemical markers of bone turnover reflect either the rate of bone formation or the rate of bone resorption *(1)*. The most sensitive and specific markers of bone resorption have been based on measurement of urinary analytes, such as deoxypyridinoline (Dpd),[1] telopeptide fragments of type I collagen, and galactosyl hydroxylysine *(1)*. The principal potential clinical uses of new markers of bone turnover is to identify healthy subjects at risk of future osteoporosis and to monitor relatively small changes in bone turnover in response to antiresorptive therapy. Serum-based measurements are likely to provide better clinical utility because the within-subject variability of serum markers may be better *(2)*. In addition, serum-based measurements of bone resorption could potentially allow direct comparison with markers of bone formation, all of which are measured in serum.

Putative serum markers of bone resorption, such as tartrate-resistant acid phosphatase (TRAcP) and an assay measuring the C-terminal telopeptide of type I collagen (ICTP), probably are not bone specific and have been disappointing in clinical studies *(3–6)*. Furthermore, different markers of bone turnover provide very different results in clinical studies *(4, 5)*, and it is not possible to rely on any single marker. Although the concentration of Dpd in serum is very low, recent studies have shown that it is possible to quantify serum Dpd in healthy subjects *(7, 8)*. The concentration of free and peptide-associated pyridinolines may, however, be affected by differential renal clearance or metabolism of these analytes *(8)*. The aim of this study was to evaluate the use of free serum β-1-galactosyl-*O*-hydroxylysine (Gal-Hyl) as a marker of bone resorption.

Hydroxylysine residues in collagen are less abundant than hydroxyproline, and are not reused in collagen biosynthesis *(9–11)*. A variable proportion of hydroxylysine residues are glycosylated to form Gal-Hyl, and this particular form is abundant in bone type I collagen. Gal-Hyl can be measured using an amino acid analyzer *(12)*, but simpler methods have been developed for measurement of urinary Gal-Hyl, using HPLC after derivatization with dansyl chloride *(13–16)*. However, this assay

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[1] Nonstandard abbreviations: Dpd, deoxypyridinoline; TRAcP, tartrate-resistant acid phosphatase; ICTP, C-terminal telopeptide of type I collagen; Gal-Hyl, β-1-galactosyl-*O*-hydroxylysine; and Cr, creatinine.

is not suitable for the measurement of Gal-Hyl in serum because of the much lower concentration of Gal-Hyl in serum and the presence of interfering analytes.

The aims of this study were (*a*) to establish an assay for free Gal-Hyl in serum by modifying the method of Moro et al. *(13)*; (*b*) to examine the effect of age on serum free Gal-Hyl; (*c*) to evaluate the renal clearance of free Gal-Hyl; and (*d*) to compare the performance of serum free Gal-Hyl to that of urinary markers of bone resorption in patients with mild Paget disease treated with a bisphosphonate.

Materials and Methods

CHEMICALS AND REAGENTS

We used HPLC-grade acetonitrile (Rathburn Chemical), glass-distilled acetone (Rathburn), HPLC-grade isopropanol (Fluka), glacial acetic acid (BDH), anhydrous sodium acetate, 5-dimethylaminonaphthalene-1-sulfonyl chloride, dansyl chloride, sodium carbonate, and L-lysine (Sigma Chemical).

The following reagents were prepared: 0.5 mol/L sodium carbonate solution in distilled water; 20 g/L dansyl chloride in acetone; 0.05 mol/L sodium acetate; buffer A, containing 125 mL/L acetonitrile and 50 mL/L isopropanol made up to volume with sodium acetate solution, pH 6.3; and buffer B, containing 500 mL/L acetonitrile and 10 mL/L isopropanol made up to volume with sodium acetate solution, pH 6.5. All reagents were filtered through a 0.45 μm filter before use.

SAMPLE PREPARATION AND DERIVATIZATION

Partition of free from protein-bound serum components was achieved by preliminary ultrafiltration of serum (Centrifree™ MPS-1 micropartition system with YMT membranes; Amicon). This device typically retains >99.9% of serum protein but <5% of L-thyroxine (M_r 776). A 500-μL aliquot of each serum sample was placed in the sample reservoir and centrifuged at 700*g* for 90 min. The ultrafiltrate (100 μL) was pipetted into 1.7-mL polypropylene tubes, to which 50 μL of sodium carbonate solution and 50 μL of dansyl chloride solution were then added. The mixture was vortex-mixed briefly, and was incubated in a water bath at 60 °C for 30 min. After incubation, samples were cooled in a refrigerator at 4 °C for 15 min, and 100 μL was injected onto the HPLC column.

HPLC

The HPLC equipment included a Waters Model 712 autosampler (Millipore Waters), two Jasco pumps (Model 880-PU; Jasco), a fluorometric detector (Model 820-FP; Jasco), and Maxima 820 acquisition software (Millipore Waters). The excitation wavelength was 366 nm, and the emission wavelength was 490 nm. We used a C_{18} reversed-phase column (4.6 × 70 mm, 3 μm particle diameter, Ultrasphere OSD; Beckman).

The interval between each sample injection was 60 min. The flow rate was 1.0 mL/min. The ratio of mobile phase buffers A and B was varied from 9:1 (A:B) at the time of injection to 1:9 at 50 min. Quantification was by peak area, using an external calibrator consisting of didansylated L-lysine *(15)*, which has the same fluorescence yield as dansylated Gal-Hyl. Serial dilutions of 0.02–10 μmol/L didansylated L-lysine were prepared.

OTHER BIOCHEMICAL MEASUREMENTS

Urinary free Gal-Hyl was measured using the method of Moro et al. *(13)*. Total Dpd and pyridinoline in urine were measured by reversed-phase HPLC after acid hydrolysis using the method of Colwell et al. *(17)*. ICTP was estimated using a radioimmunoassay (Orion Diagnostica). TRAcP was measured using a kinetic assay with α-naphthol phosphate as the substrate (Enzyline Phosphatase acide optimisé 10 kit; BioMérieux SA). Urinary hydroxyproline was measured using a colorimetric reaction with dimethylaminobenzaldehyde after acid hydrolysis and chloramine T oxidation. Serum osteocalcin was measured using a competitive radioimmunoassay (Nichols Institute). Urinary creatinine (Cr) was measured using a kinetic Jaffé reaction.

SUBJECTS AND SAMPLE COLLECTION

Healthy subjects were recruited from population-based studies of bone turnover in women and teenage girls. We recruited 21 healthy pubertal girls (ages, 11–12 years), 14 healthy premenopausal women (ages, 20–40 years), and 19 healthy postmenopausal women (ages, 52–74 years). Fourteen previously untreated subjects with mild Paget disease [6 men and 8 women; ages, 59 to 87 years; mean age (± SE) 71 ± 2.1 years] were recruited from the metabolic bone clinic at the Northern General Hospital in Sheffield, UK. Clinical details relating to these patients are given elsewhere *(5)*. Patients were diagnosed on the basis of radiographic evidence and ^{99M}Tc-bisphosphonate bone scans. Patients were treated with 400 mg of oral etidronate daily for 6 months (Didronel; Proctor and Gamble). Fasting blood samples (drawn at 0900–1000) and 24-h urine samples were collected at baseline and at 2, 4, and 6 months of etidronate therapy.

Blood samples were allowed to clot for 30 min before centrifugation at 2000*g* for 10 min. Aliquots of serum were stored at −20 °C until assayed, and aliquots of urine were stored at −20 °C. The study protocol conformed with the Revised Helsinki Declaration of 1983 and was approved by the Ethics Committee at the Northern General Hospital Sheffield.

STATISTICAL ANALYSIS

All data were positively skewed and were logarithmically transformed before statistical analysis. The response of each marker following bisphosphonate therapy was expressed as the percentage of change from baseline. ANOVA was used to determine whether Gal-Hyl differed between healthy pubertal girls, premenopausal women,

postmenopausal women, and patients with Paget disease. This analysis was followed by a Scheffé test aimed at controlling the overall type I error rate at <5%. The correlation between markers of bone turnover in Paget disease before therapy was determined using the Pearson correlation analysis. Statistical analyses were performed on an IBM-compatible computer, using the Statgraphics statistical software program (STSC). All statistical tests were two-sided.

Results

ANALYTICAL PERFORMANCE

The linearity of the injector and fluorescence detector at the wavelengths chosen was evaluated by multiple dilutions of a high concentration of the calibrator. A linear response was observed between concentrations of 0.02 and 60 μmol/L. Chromatograms of dansylated Gal-Hyl and serum with added dansylated Gal-Hyl are shown in Fig. 1. The retention time of the dansylated Gal-Hyl was 36 min. Urinary Gal-Hyl was not retained by the ultrafiltration system used for sample preparation (recovery, 103%; n = 4). At a free Gal-Hyl concentration of 48 nmol/L, the within-run coefficient of variation (CV) was 7% (n = 15) and the between-run CV was 14% (n = 15).

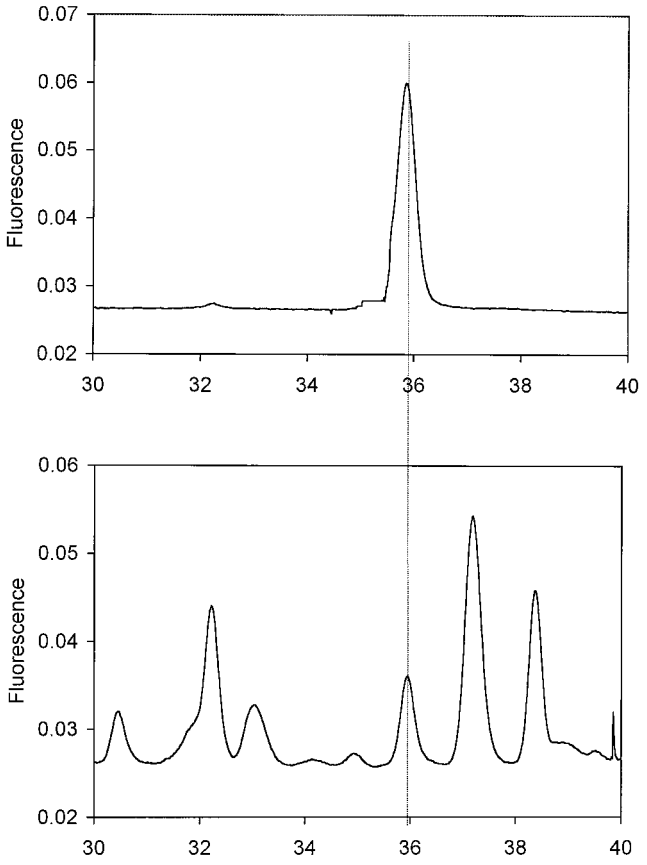

Fig. 1. Chromatograms of dansylated Gal-Hyl calibrator (*top*) and dansylated Gal-Hyl calibrator added to serum (*bottom*).

Vertical lines indicate elution times of calibrator and serum Gal-Hyl. The section of the chromatogram from 30 to 40 min is shown.

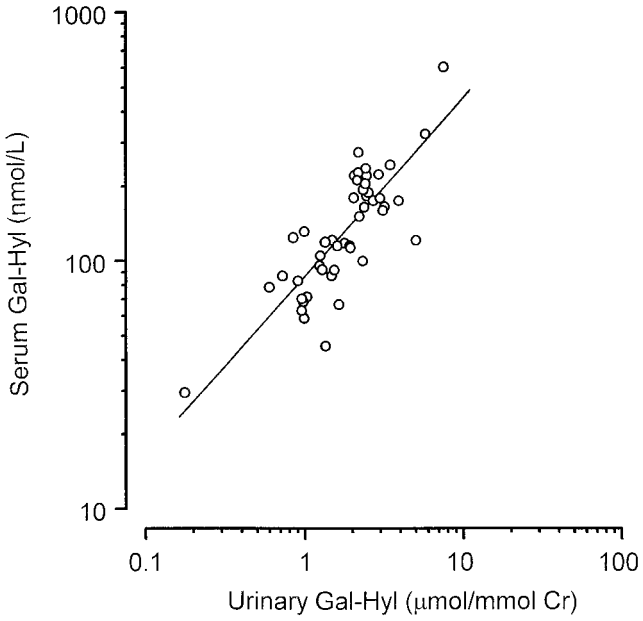

Fig. 2. Relationship between serum and urinary Gal-Hyl in healthy pubertal and adult subjects ($r = 0.84$; $P < 0.001$).

The data were log transformed before statistical analysis.

Upon serial dilution of pooled nondiseased serum, no apparent interference from baseline variability was seen at concentrations >25 nmol/L.

COMPARISON WITH OTHER MARKERS OF BONE RESORPTION

Comparisons of serum and urinary free Gal-Hyl were performed in the healthy pubertal and adult subjects (ages, 10–79 years). Both assays were significantly correlated (after logarithmic transformation, $r = 0.84$; $P < 0.001$; Fig. 2). Serum free Gal-Hyl was also correlated with the other biochemical markers of bone resorption and formation markers in the 14 patients with untreated Paget disease of bone (Table 1).

EFFECT OF MENOPAUSAL STATUS, PUBERTY, AND PAGET DISEASE

The concentrations of free Gal-Hyl in serum and urine in the four groups are shown in Fig. 3. The concentration of free Gal-Hyl in serum was 61.7 ± 8.3 nmol/L (mean ± SE)

Table 1. Correlation between serum Gal-Hyl and other markers of bone turnover in 14 patients with Paget disease.

Marker	*r*
ICTP	0.70[a]
TRAcP	0.42
Dpd	0.68[a]
Pyridinoline	0.78[a]
Hydroxyproline	0.87[a]
Osteocalcin	0.59[b]

[a] $P < 0.01$.
[b] $P < 0.05$.

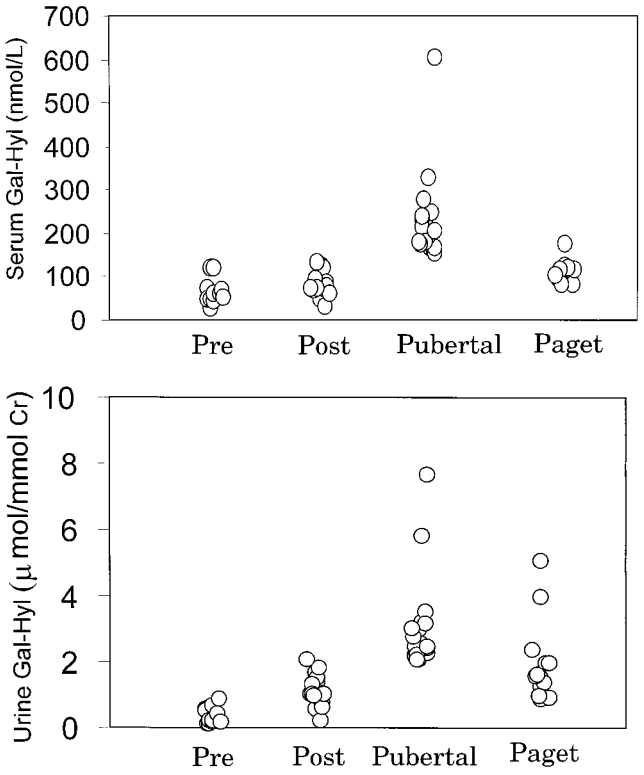

Fig. 3. Serum (*top*) and urine (*bottom*) Gal-Hyl concentrations in premenopausal women (*Pre*), postmenopausal women (*Post*), pubertal girls (*Pubertal*), and patients with untreated Paget disease of bone (*Paget*).

Overall significance of differences between groups by ANOVA, $P < 0.001$. Each group of subjects differed significantly from every other group ($P < 0.05$, Scheffé test) for both serum and urine. Data were log transformed before statistical analysis.

in premenopausal women, 80.4 ± 6.4 nmol/L in postmenopausal women, 225.7 ± 19.9 nmol/L in pubertal girls, and 110.1 ± 6.7 nmol/L in patients with Paget disease.

Response of serum free Gal-Hyl during treatment of Paget disease of bone

Free Gal-Hyl in both serum and urine decreased significantly in response to 6 months of etidronate therapy in patients with Paget disease (Fig. 4). Serum Gal-Hyl decreased by 36% (SE = 3.8%) in 14 patients with mild Paget disease treated with an oral bisphosphonate. This response was significantly ($P < 0.001$ by ANOVA; $P < 0.05$ by Scheffé test) greater than that seen for either serum TRAcP (9% decrease; SE = 4%) or serum ICTP (19% decrease; SE = 8%), but similar to that shown for Dpd and pyridinoline in urine [see Ref. *(5)* for further details].

Fractional excretion of free Gal-Hyl

The fractional excretion of free Gal-Hyl was calculated from urinary and serum Gal-Hyl and Cr in 21 girls, ages 10–12 years, using the equation:

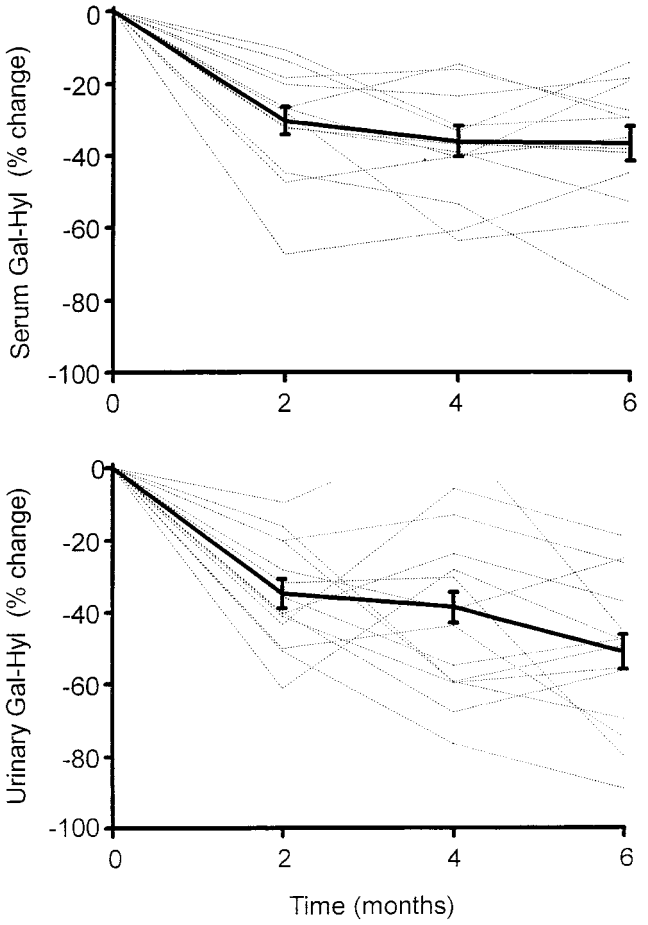

Fig. 4. Changes in Gal-Hyl following therapy with etidronate in patients with Paget.

Data are expressed as the percentage of change from baseline for serum Gal-Hyl (*top*) and excretion of Gal-Hyl in urine over 24 h (*bottom*). The *solid lines* represent the means, and the *error bars* represent the SE. Results for individual patients are shown as *dotted lines*. Serum Gal-Hyl decreased by 36% and urinary Gal-Hyl by 41% following 6 months of therapy.

$$FE_{Gal\text{-}Hyl} = \frac{U_{Gal\text{-}Hyl} \cdot S_{Cr}}{U_{Cr} \cdot S_{Gal\text{-}Hyl}}$$

where *FE* is the fractional excretion, *U* is the urinary concentration, and *S* is the serum concentration.

The mean fractional excretion of free Gal-Hyl was 0.90 (95% confidence interval, 0.82–0.98).

Discussion

The method developed by Moro et al. *(13)* for the measurement of Gal-Hyl in urine is not suitable for measurement of the concentration of Gal-Hyl in serum because of the much lower serum concentration and protein interference. Sample preparation was modified by preliminary ultrafiltration of samples, an increase in the volume of sample used in the dansylation procedure, and an increase in the concentration of dansyl chloride to avoid the production of mono-dansylated Gal-Hyl.

The concentration of free Gal-Hyl in both serum and urine discriminated between premenopausal women,

postmenopausal women, pubertal girls, and patients with untreated Paget disease, as would be expected. The fourfold increase of serum free Gal-Hyl concentrations in girls compared with premenopausal women reflects the high bone turnover we and others reported previously for urinary Gal-Hyl *(4, 18–20)*. The increase in serum free Gal-Hyl in postmenopausal compared with premenopausal women is in agreement with urinary Gal-Hyl results that have been published *(13, 14, 19)*.

There are several potential problems associated with the use of Gal-Hyl as an indicator of bone resorption. Differential renal handling and metabolism may significantly influence excretion of biochemical markers of bone resorption, and this has been demonstrated for free pyridinolines *(8)*. β-galactosidase or α-glucosidase enzymes may be present in the renal cortex in animals *(21)*, raising the possibility of a similar problem for Gal-Hyl. However, Moro et al. *(16)* reported that α-glucosidase and β-galactosidase are not detectable in the human renal cortex. It is also possible that Gal-Hyl may be metabolized or reused in other tissues, although the extent of this is uncertain. Robins *(22)* showed that after injection of tritium-labeled glycosides of hydroxylysine in rabbits, 100% of the compounds were recovered in the urine in 48 h. In this study, the fractional excretion of Gal-Hyl by the kidney was close to one, suggesting minimal additional metabolism in humans. This is in contrast to our findings with free Dpd *(8)*.

It is also possible that Gal-Hyl could be absorbed from the diet. This has only been explored in one study *(11)*, which involved a single experimental subject. In a preliminary study *(23)*, we evaluated the effect of oral administration of 10 g of gelatin (containing 38 μmol of Gal-Hyl) on urinary excretion of Gal-Hyl in healthy adults and were unable to demonstrate increased Gal-Hyl excretion.

Serum Gal-Hyl was increased in patients with Paget disease of bone, and decreased rapidly in response to bisphosphonate therapy. The relative decrease in serum Gal-Hyl was similar to that seen for urinary Gal-Hyl, but less than that seen for cross-linked telopeptide fragments of type I collagen in the same patients *(5)*. This greater response of urinary telopeptide fragments of type I collagen may imply greater sensitivity of these analytes, but could be a result of the differential metabolism of free and peptide-associated cross-linked telopeptides, as has been suggested *(8)*. In contrast, the other two putative serum markers of bone resorption (ICTP and TRAcP) showed a minimal response to antiresorptive therapy, as has been found in previous studies *(5, 6, 24–26)*, probably because of the nonspecificity of these analytes for bone resorption.

In conclusion, measurement of free Gal-Hyl in serum by HPLC is possible. The assay developed in our laboratory is relatively simple and has good reproducibility, and its clinical performance is likely to be better than that of serum TRAcP and ICTP.

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