Urinary Excretion Level of Hydroxyslypyridinoline as an Index of Bone Resorption

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Hydroxyslypyridinoline (HP), an intermolecular cross-linking amino acid, is present only in bone and cartilage collagen. We examined urinary HP levels in order to assess bone collagen metabolism using HPLC. Urinary HP excretion levels (nmol/mmol creatinine) of healthy control groups gradually increased with aging. Urinary HP excretion levels of females were higher than those of age-adjusted males. On the other hand, the urinary excretion levels of HP in osteoporotic patients were significantly higher than those of age and sex-adjusted controls. It is suggested that the determination of urinary HP excretion levels is useful in the clinical evaluation of metabolic bone diseases, especially osteoporosis.

Keywords hydroxyslypyridinoline; urinary excretion level; osteoporosis; bone resorption; biomedical marker; HPLC

It is important to have an accurate and sensitive marker to determine bone turnover, especially in the clinical evaluation of patients with osteoporosis. Bone loss always results from an imbalance between bone resorption and formation, so the clinical evaluation of osteoporosis at an early stage is necessary for preventing crush fractures. Serum alkaline phosphatase and urinary hydroxyproline are commonly used to assess bone formation and resorption, respectively, but their sensitivity and specificity are limited.

Hydroxyslypyridinoline (HP) is one of the cross-linking amino acids present only in bone and cartilage collagen, and is based on a fluorescent 3-hydroxypyridium ring derived from three residues of hydroxylysine. Another analogous compound, lysylpyridinoline (LP), appears to be present in appreciable amounts only in bone collagen. HP and LP are excreted in urine following collagen breakdown caused by bone resorption, so determination of the urinary excretion of the pyridinium cross-links of collagen has been suggested as a sensitive and specific marker of bone resorption.

In this paper, we inspected the urinary excretion levels of HP in healthy controls and in patients with osteoporosis in order to assess the usefulness of the determination of urinary HP excretion levels as an index of bone collagen metabolism in the case of osteoporosis.

MATERIALS AND METHODS

Chemicals CF-11 cellulose was obtained from Whatman Biochemicals. n-Heptafluorobutyric acid (HFBA, amino acid analyzing grade) and acetonitrile (HPLC grade) were purchased from Wako Pure Chemical Industries, Ltd. Other reagents obtained from commercial suppliers were of special-reagent grade and/or analytical grade and were used without further purification.

Urine Samples The first micturition urine after arising was collected from 20 ambulatory patients (19 females and 1 male ranging in age from 66–83 years old) with osteoporosis. Two of them (1 female and 1 male) were at their first medical examination for osteoporosis and the other patients were within 60 d from their first medical examination. They were administered a preparation of vitamin D orally, and calcitonin intra muscularly in order to improve their back pain. These patients were in good health except for osteoporosis, and had no recognizable disease or history of the use of drugs known to produce osteoporosis. As controls, urine samples were obtained from 317 healthy persons (238 females and 79 males) ranging from 40 to 89 years of age who had no back pain or lumbago. Their urine was also collected at their first micturition. After collection, urine samples were stored at −20°C and kept frozen until the time of analysis.

Hydrolysis and Fractionation of Urine Samples The elution solvent was made up of a mixture of n-butanol, acetic acid, and water (4:1:1, v/v). CF-11 slurry was prepared by making a 5% (w/v) suspension of CF-11 cellulose in an elution solvent. The pretreatment columns were prepared by adding 15 ml of CF-11 slurry to a glass column.

A urine sample of 1 ml was hydrolyzed with 1 ml of 12 M HCl for 18 h at 108°C in a screw-capped Pyrex tube with a teflon liner. The supernatant of cold hydrolyzates (250 μl) was added with 2 ml of n-butanol, 250 μl of acetic acid and 250 μl of CF-11 slurry. This mixture was applied to the top of the pretreatment column (bed size; 9 mm l.d. × 60 mm), then washed with 30 ml of the elution solvent. Next, HP was eluted from the pretreatment column with 20 ml of water. The water eluent was collected, evaporated to dryness, and then redissolved in 500 μl of HPLC solvent (A). Prior to being loaded into an HPLC apparatus, the sample was filtered through a membrane filter (pore size; 0.5 μm, poly tetrafluoroethylene, Advantec Toyo).

Preparation of Collagen-Derived HP Standard Twenty-five g of bovine Achilles tendon collagen (Sigma Chemical, U.S.A.) was hydrolyzed with 6 M HCl for 24 h at 108°C. After removal of HCl, the cooled hydrolysate was applied to a pretreatment column (40 mm l.d. × 300 mm), then washed with 300 ml of the elution solvent. The pretreatment column was then eluted with 200 ml of water and the procedure of the urine sample treatment. The water eluent was collected, evaporated to dryness, and then redissolved in 3 ml of 1 M acetic acid. This solution was then chromatographed on a Bio-Gel P-2 gel (Bio-rad Labs., fine, 45–90 μm) column (19 mm l.d. × 100 cm) with

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l m acetic acid as an elution solvent at a flow rate of 6 ml/h. Then, the fluorescence intensity of each fraction (4.0 ml) was determined with Ex: 295 nm, and Em: 395 nm. The fractions containing HP were put together and evaporated to dryness, then redissolved in 5 ml of HPLC solvent (A). This HP fraction was applied to the preparative chromatographic procedure. Briefly, a preparative ODS column (Inertsil ODS-2, 7.6 mm i.d. × 250 mm, GL Sciences Inc.) was used and the flow rate was maintained at 2.7 ml/min, and other conditions were the same as urinary HP analysis. The HP fraction was collected, evaporated to dryness, and then redissolved in 10% (w/v) acetic acid to form HP salt. After removal of excess acetic acid, a freeze-dried HP standard was employed as the external standard for urinary HP analysis by HPLC.

**Determination of Urinary HP** HP was determined by HPLC according to the method of Eyre et al., with some modifications. The HPLC apparatus was equipped with a gradient system incorporating a Hitachi model L-6200 pump, a model F-1050 spectrophotometer (Ex: 295 nm, Em: 395 nm) and a model D-2500 recording integrator. A 20 µl sample loop was used for application to an ODS column (Capcell Pak C-18, AG-120, 5 µm, 4.6 mm i.d. × 250 mm, Shiseido Co., Ltd.) which was run at a flow rate of 1.0 ml/min. The two solvent systems used were (A) 10 mM HFBA in 5% acetonitrile, and (B) 10 mM HFBA in acetonitrile.

Urinary HP excretion levels were expressed as nmol/mmol creatinine, and the value of each patient was the means of the two values of two successive days. Urinary creatinine was measured by the colorimetric procedure based on the Jaffe method using a clinical test kit (Creatinine-test Wako; Wako Pure Chemical Industries, Ltd., Japan).

**Statistical Methods** All results were expressed as the mean ± standard deviation (S.D.). Comparisons for significance were made using the Student's t-test, and all p values were <0.05.

**RESULTS AND DISCUSSION**

**Preparation of HP Standard** HP standard (12.2 mg) was isolated from 25 g of bovine achilles tendon collagen using cellulose chromatography, Bio-Gel P-2 gel-filtration chromatography and preparative reversed-phase HPLC. The isolated HP showed a molecular ion ([M+H]⁺) at m/z 429.6 in electrospray ionization mass spectrometry (Fig. 1), and there were no recognizable peaks except for the molecular ion.

**Chromatography of HP Standard and Urinary HP** The typical chromatograms of standard HP, urinary HP and the gradient program are shown in Fig. 2. The effluent containing HP showed fluorescence spectra with Exmax 295 nm and Emmax 395 nm, respectively, and subsequent experiments were carried out at these wavelengths. The standard curve for HP in the range of 0—100 pmol/assay was in good liner relationship between the peak area and the concentration (r=0.999). The coefficient of variation for HP was 4.5% (n=8) at 10 pmol/assay. Urinary HP was clearly separated from other components (Fig. 2B). In order to assess the precision of this method, the HP

![Fig. 1. Electrospray Ionization Mass Spectra of HP Standard](image1)

HP obtained by the proposed method was dissolved in 50% methanol. Experiments were carried out on a TSQ 700 mass spectrometer (Finnigan Mat, San Jose, California, U.S.A.). The needle voltage and chamber temperature were maintained at -40 kV and 100°C, respectively. The sample solution was infused by a syringe pump at a flow rate of 1.0 µl/min.

![Fig. 2. Reversed-Phase Chromatography of the HP Standard (A) and Urinary HP (B)](image2)

The C-18 column (Capcell Pak C-18, AG-120, 5 µm, 4.6 mm i.d. × 250 mm, Shiseido Co., Ltd.) was equilibrated with acetonitrile in water containing 10 mM HFBA as an ion-pairing reagent. The peaks were fluorometrically monitored at Ex: 295 nm and Em: 395 nm.

![Fig. 3. Urinary Excretion Levels of HP in Different Age Groups of Healthy Females ( ) and Males ( )](image3)

Each bar represents the mean ± S.D. Brackets indicate the number of samples determined.
<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Male (nmol/mmol cr.)</th>
<th>Female (nmol/mmol cr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40–49</td>
<td>15.29 ± 6.26 (6)</td>
<td>14.88 ± 5.50 (2)</td>
</tr>
<tr>
<td>50–59</td>
<td>17.29 ± 5.50 (6)</td>
<td>26.88 ± 5.29 (7)</td>
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<tr>
<td>60–69</td>
<td>21.80 ± 6.94 (78)</td>
<td>29.49 ± 6.81 (32)</td>
</tr>
<tr>
<td>70–79</td>
<td>22.82 ± 7.94 (132)</td>
<td>30.86 ± 6.27 (36)</td>
</tr>
<tr>
<td>80–89</td>
<td>22.85 ± 6.92 (16)</td>
<td>40.81 ± 13.44 (2)</td>
</tr>
</tbody>
</table>

a) Values are mean ± S.D.  b) Number of samples determined. c) The difference compared to the control (unpaired t test, p < 0.05).

**Table I. Urinary Excretion Levels of HP in Different Age Groups of Healthy Control and Osteoporotic Patients**

Thus far our experimental group has found higher urinary excretion levels of hydroxylysine glycosides, the constituent amino acids of the α-chain of the bone collagen, in osteoporotic patients compared to those in the age and sex-adjusted controls. We have also found that osteoporotic patients showed significantly higher excretion levels of HP compared to those in the age-adjusted controls. These results suggest that the determination of urinary HP, not an α-chain constituent but an intermolecular cross-linker, could be of clinical value in the screening of women for the prevention of osteoporosis.

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**REFERENCES**