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Automated high-performance liquid chromatographic determination of hydroxylysylpyridinoline and lysylpyridinoline in urine using a column-switching method☆

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ABSTRACT

An on-line urine clean-up system was developed for the simultaneous determination of free and total pyridinoline, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) by high-performance liquid chromatography (HPLC) using a column-switching technique. The method is based on a combination of gel permeation chromatography (GPC) and ion-pair reversed-phase HPLC. In the GPC column, pyridinoline is preseparated from endogenous urinary substances with 0.03 *M* heptafluorobutyric acid (HFBA) as the mobile phase. After column switching, the eluate fraction containing pyridinoline is further separated by ion-pair chromatography using an octadecylsilica (ODS) column with 0.03 *M* HFBA–acetonitrile (81:19) as the mobile phase. The detection limits were 36 and 44 pmol/ml for free and total HP, respectively, and 44 pmol/ml for both free and total LP at a signal-to-noise ratio of 3. The coefficients of variation for free and total pyridinoline were 1.5 and 3.5%, respectively. The determination of one sample including the clean-up is completed within 25 min. This system is precise and is useful for the determination of pyridinoline in large amounts of urine. The usefulness of pyridinoline as a biomedical marker for bone resorption was also examined.

INTRODUCTION

Hydroxyproline (Hyp) in urine has been widely employed as a biomedical marker for bone resorption. However, as Hyp is derived from the Clq fraction of complement and foods in addition to collagen and is also metabolized in liver, it is not a specific bone disease index [1].

In recent years, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) have been utilized as new biomedical markers for bone resorption (Fig. 1). These pyridinolines are intermolecular cross-linking amino acids of collagen and are excreted in the urine following collagen breakdown caused by bone resorption. Their urinary excretion is not influenced by foods. Thus, the specificity as biomedical indices seems to be higher than that of Hyp [1].

A number of methods using ion-pair reversed-phase high-performance liquid chromatography

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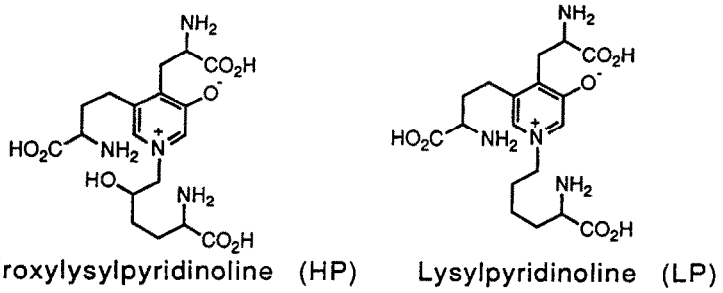

Fig. 1. Structures of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).

(HPLC) [2–4] or ion-exchange chromatography [5] with fluorescence detection have been developed for the determination of pyridinoline in urine and tissues [2–11]. Although CF1 cellulose extraction has been employed in the sample preparation [3–11], it is too tedious for the routine clean-up of large amounts of urine and/or tissue hydrolysates. Also, the reproducibility of the determination of pyridinoline in urine after the clean-up procedure is between 10 and 16% [3,6].

Recently, the column-switching technique has been widely used in HPLC for the direct injection of biological fluids [12] and for their efficient clean-up. However, there have been no reports on the use of column-switching for the determination of pyridinoline including clean-up, which prompted us to develop an on-line urine clean-up system for the simultaneous determination of free and total pyridinoline by HPLC using a column-switching technique. This paper describes the automated determination of pyridinoline in urine.

EXPERIMENTAL

Reagents and materials

Heptafluorobutyric acid (HFBA) was of amino acid analytical-reagent grade. Creatinine was determined using a Wako creatinine test kit, and the other reagents were of HPLC grade from Wako (Osaka, Japan). N-Ethyl-3-pyridinol was purchased from Aldrich (Milwaukee, WI, USA).

Preparation of pyridinoline standard

Hydroxylysylpyridinoline (HP). Bovine achilles tendon collagen (Sigma, St. Louis, MO, USA) (50 g) was heated with 6 *M* HCl (1.5 l) at 110–120°C for 16 h. After filtration, the filtrate was concentrated to 250 ml *in vacuo*. Acetic acid (250 ml) and 1-butanol (1 l) were added to the residual solution and the mixture was allowed to stand overnight at room temperature. After removal of the upper layer by decantation, the lower layer was filtered and the filtrate obtained was evaporated *in vacuo*. The residue was dissolved in 10% acetic acid (40 ml) and half of this solution was chromatographed on a Bio-Gel P2 (100–200 mesh) column (95 mm × 2.5 cm I.D.) (Bio-Rad Labs.) with 10% acetic acid as eluent at 0.5 ml/min. Each fraction (10 ml) was collected in a glass tube. The HP in each fraction was determined by HPLC [Jasco 880-PU pump, Wakosil II 5C18HG (5 μm) column (150 mm × 4.6 mm I.D.)] wih a fluorescence detector (Hitachi F1050 spectrometer) with excitation at 295 nm and emission at 395 nm. The fractions containing HP were combined and concentrated to 20 ml *in vacuo*. The residual solution was chromatographed three times on the Bio-Gel column under the same conditions.

The other half of the 10% acetic acid solution was also chromatographed on a Bio-Gel column. A total amount of 250 g of collagen was treated by the same procedure mentioned above. The fractions eluted from the Bio-Gel column and containing HP were combined and concentrated to 6 ml *in vacuo*. HFBA (1 ml) was added to the residual solution and a 500-μl portion of this solution was injected into the preparative HPLC system [Hitachi 655 A11 pump, YMC ODS A324

(5 μm) column (300 mm × 10 mm I.D.)] with 0.03 *M* HFBA–acetonitrile (81:19, v/v) as the mobile phase. The eluted fractions containing HP were collected using a UV detector (Shimadzu SPD-6A) at 297 nm, and the combined fractions were concentrated to 6 ml *in vacuo*. HFBA (1 ml) was added to the residual solution and a 500-μl portion was again purified by preparative HPLC according to the procedure described above. The fractions containing HP were combined and concentrated *in vacuo*. The residue was dissolved in 1 *M* HCl (1 ml) and evaporated *in vacuo*. The same procedures were repeated three times. The residue was dissolved in water (3 ml) and a 500-μl portion was injected into the HPLC system (Hitachi 655 A11 pump) with a gel permeation column (Asahipak GS 220, 9 μm, 1000 mm × 7.6 mm I.D.) and water as the mobile phase for desalting. The fractions containing HP were collected using a UV detector at 297 nm, concentrated to 2 ml and the residual solution was lyophilized to give 12.93 mg of HP as the monochloride–trihydrochloride form.

Fast atom bombardment mass spectrometry (FAB-MS): *m/z* (M^+) 429. Analysis: calculated for $C_{18}H_{26}N_4O_8Cl \cdot 1.35\ H_2O$, C 36.12, H 5.84, N 9.36, Cl 23.69%; found, C 35.86, H 5.89, N 9.65, Cl 23.50%.

Isolation of lysylpyridinoline (LP). Chicken bone (4 kg) was decalcified according to the conventional procedure. The decalcified bone (136 g) was treated according to a procedure similar to that used for the isolation of HP. LP (0.47 mg) was obtained as the monochloride–trihydrochloride form. FAB-MS: *m/z* (M^+) 416.

Determination of molar absorptivity of HP

A Hitachi U3200-10 ultraviolet–visible spectrometer was used.

Instruments and conditions for column-switching

The HPLC system consisted of a Jasco 880-PU pump (Japan Spectroscopic, Tokyo, Japan) and an L-6000 pump (Hiachi, Ibaragi, Japan), a Jasco 851-AS autoinjector, a Jasco 870-UV detector, an RF-535 fluorescence detector (Shimadzu, Kyoto, Japan) and a Jasco 892-01 two-column-switching valve unit. Relief valves (Nupro spring kits) were used. A Jasco 807-IT integrator for quantification and a Hitachi 056 recorder for checking the eluate from column 1 (C1) were employed.

Determination of free pyridinoline

A schematic diagram of the HPLC system with the column-switching valve is shown in Fig. 2. C1 is a gel permeation (GPC) column, Asahipak GS-220M (9 μm, 100 mm × 7.5 mm I.D.) (Asahikasei, Miyazaki, Japan). L1 is 0.03 *M* HFBA solution at a flow-rate of 1.0 ml/min. DET1 is a UV detector at 297 nm. C2 is a YMC ODS A302 (5 μm) column (150 mm × 4.6 mm I.D.) (Yamamura Kagaku, Kyoto, Japan). L2 is 0.03 *M* HFBA–acetonitrile (81:19, v/v) at a flow-rate of 1.0 ml/min. DET2 is a fluorescence detector with

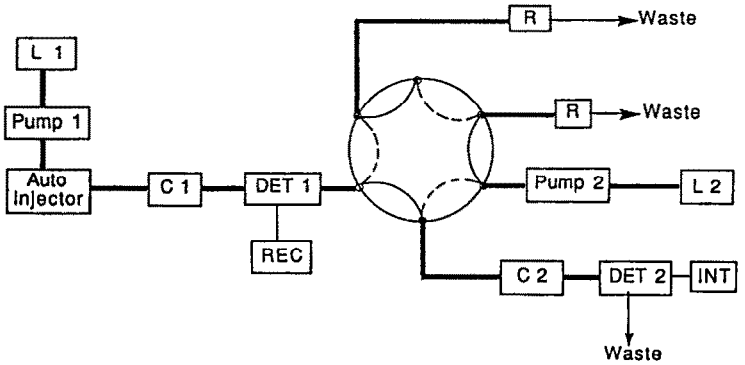

Fig. 2. Schematic diagram of the column-switching system. L1, L2 = Mobile phases 1 and 2; C1 = precolumn (GPC, Asahipak GS220M); C2 = analytical column (ODS, YMC A302 or AM303); DET1 = detector (UV, 297 nm); DET2 = detector (fluorescence, λ_{ex} 295 nm, λ_{em} 395 nm); REC = recorder; INT = integrator; R = relief valve.

excitation at 295 nm and emission at 395 nm.

The retention time of pyridinoline in C1 is checked each day before analysis to determine the time programme for the column-switching. At time zero, a urine sample (25 μl) is injected into the clean-up column (C1) via an autoinjector. C1 is eluted with L1 while the analytical column (C2) is being conditioned with L2 (dotted-line route in the six-port valve). Just before pyridinoline is eluted from C1, the six-port valve is switched to the full-line route. The fraction containing pyridinoline is introduced into C2 from C1. Just after pyridinoline is eluted from C1, the six-port valve is switched back to the dotted-line route again. The pyridinoline fraction is then separated into HP and LP by C2 with L2 and they are detected with a fluorescence detector (DET2), while C1 is being conditioned with L1 for the next injection. The valve operation is all carried out automatically by the Jasco 807-IT integrator according to the predetermined time programme.

Determination of total pyridinoline

The system is similar to that mentioned above, but the flow-rate of L1 is 0.5 ml/min and C2 is a YMC ODS AM303 (5 μm) column (250 mm × 4.6 mm I.D.).

Preparation of standard pyridinoline solutions

Both HP and LP were dissolved in non-fluorescence water to prepare aqueous standard solutions in the range 0.2–10 μ*M*.

Urine samples

Ten-week-old female SD rats were divided at random into two groups of ten. Bilateral ovariectomies were carried out on the first group and sham operations were performed on the second group. Both groups were maintained under identical conditions for four weeks and were allowed free access to food and water. The 24-h urine collections were done for each animal under fasting conditions but with free access to water to avoid contamination of the urine by food. Urine was also collected from normal rats.

Urine was stored at −70°C until the time of analysis. Defrosted urine was filtered using a Sartorius Ministart SRP15 (pore size 0.45 μm) and the filtrate was injected into the HPLC system via the autoinjector to determine free pyridinoline.

For the determination of total pyridinoline, the defrosted urine (250 μl) was heated with concentrated HCl (250 μl) in a screw-capped glass tube at 108°C for 18 h. After cooling, the hydrolysed urine was filtered and the filtrate was injected into HPLC system via the autoinjector.

Recovery from rat urine

Pyridinoline standard solution (40 μl) was added to a normal rat urine (160 μl) and filtered. The filtrate (25 μl) was injected into the HPLC system via the autoinjector. According to the procedure mentioned above, the pyridinoline standard solution (40 μl) was added to hydrolysed rat urine (160 μl). The filtrate (25 μl) was injected into the HPLC system.

Determination of creatinine in urine

The creatinine concentration in urine samples was determined according to the Jaffe method using the creatinine test kit.

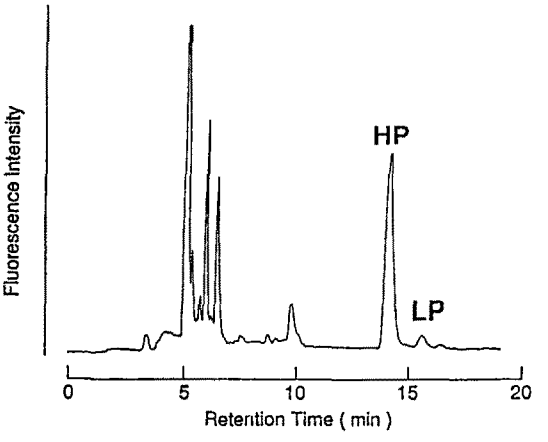

Fig. 3. Typical chromatogram of free HP and LP in rat urine using the column-switching system. Analytical conditions: precolumn (C1), Asahipak GS-220M (100 mm × 7.5 mm I.D.); mobile phase (L1), 0.03 *M* HFBA; flow-rate, 1.0 ml/min; connection time of C1 with C2, 2.0–3.2 min after injection; analytical column (C2), YMC A302 ODS (150 mm × 4.6 mm I.D.); mobile phase (L2), 0.03 *M* HFBA–acetonitrile (81:19); flow-rate, 1.0 ml/min; detection, fluorescence (detector 2), λ_{ex} 295 nm, λ_{em} 395 nm; concentration, HP 0.72 nmol/ml, LP 0.07 nmol/ml.

RESULTS AND DISCUSSION

Isolation of pyridinoline standard

HP (12.9 mg) was isolated as the monochloride–trihydrochloride form from bovine achilles tendon collagen (250 g) using Bio-Gel chromatography (repeated three times), reversed-phase HPLC (repeated twice) and gel permeation chromatography. The structure of HP was confirmed by elemental analysis and mass and NMR spectrometry. The molar absorptivity of HP at pH 2.0 was 5550 (297 nm) and that of 1-ethyl-3-pyridinol, which was used as a standard by Eyre *et al.* [2], was 3682 under the same conditions. These results show that HP concentration determined by Eyre *et al.*'s calibration, in which the molar absorptivity of HP at pH 2.0 and 297 nm was the same as that of 1-ethyl-3-pyridinol, was overestimated.

LP (0.47 mg) was isolated from chicken bone (4 kg) and it showed a molecular ion at m/z 416 in FAB-MS. These pyridinolines were used as the standards.

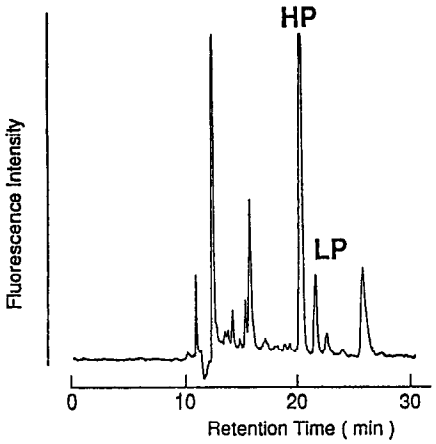

Fig. 4. Typical chromatogram of total HP and LP in rat urine using the column-switching system. Analytical conditions: precolumn (C1), Asahipak GS-220M (100 mm × 7.5 mm I.D.); mobile phase (L1), 0.03 *M* HFBA; flow-rate, 0.5 ml/min; connection time of C1 with C2, 4.7–5.9 min after injection; analytical column (C2), YMC A303 ODS (250 mm × 4.6 mm I.D.); mobile phase (L2), 0.03 *M* HFBA–acetonitrile (81:19); flow-rate, 1.0 ml/min; detection, fluorescence (detector 2), λ_{ex} 295 nm, λ_{em} 395 nm; concentration, HP 1.84 nmol/ml, LP 0.34 nmol/ml.

Determination of free pyridinoline (system A)

A number of methods for the determination of pyridinoline by ion-exchange chromatography [5] and reversed-phase HPLC using an ion-pair reagent [2–4] have been reported. Although strong cation-exchange chromatography has been found to be suitable for analyses for strongly dissociated compounds such as pyridinoline, we adopted reversed-phase chromatography with an ion-pair reagent considering the column efficiency.

n-Octanesulphonic acid [3,4] and HFBA [2,5–11] have been used as ion-pair reagents in the determination of pyridinoline. We used HFBA because the separation of pyridinoline from urinary substances with HFBA was superior to that with *n*-octanesulphonic acid.

As the structures of HP and LP are similar, the combination of a precolumn (C1) and an analytical column (ODS, C2) was investigated using HP. We selected a gel permeation column (GPC) packed with a porous polymer as C1. To avoid pressure variations caused by the column-switching, relief valves were used as shown in Fig. 2.

To adopt the heart-cut technique, the timing of column-switching was investigated. Pyridinoline was eluted from the GPC column between 2 and 3 min after injection. As a result, we set the connecting time of C1 with C2 for 1.2 min between 2.0 and 3.2 min after injection of the sample.

Fig. 3 shows a chromatogram of free pyridinoline in normal rat urine obtained with the proposed system. The retention times of HP and LP were 13 and 15 min, respectively, and the analysis of each sample including the clean-up procedure was completed within 20 min.

Determination of total pyridinoline (system B)

Pyridinoline is excreted in urine in both free and peptide-bound forms [1]. To determine the total pyridinoline, urine was hydrolysed with an equal volume of concentrated HCl at 110°C for 18 h. Many of the hydrolysates produced interfered with the analysis for pyridinoline. Hence for the determination of total pyridinoline, a more efficient clean-up is necessary.

First, in the GPC column, the flow-rate of L1 was decreased from 1.0 to 0.5 ml/min, and the fraction containing pyridinoline introduced into the analytical column was subdivided. Next, an analytical column with twice the number of theoretical plates compared with the free pyridinoline analysis column was used. The connecting time of C1 with C2 was set at 1.2 min between 4.7 and 5.9 min after injection.

Fig. 4 shows a chromatogram of total pyridinoline in normal rat urine. The retention time for HP and LP were 21 and 23 min, respectively, and determination of the levels in one sample including the clean-up procedure was completed within 25 min.

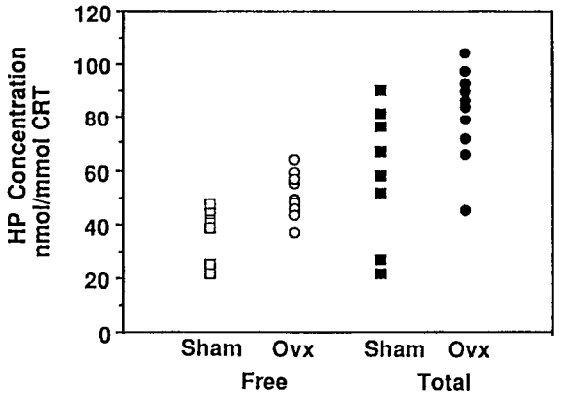

Fig. 5. Urinary free and total HP concentrations in ovariectomized (Ovx) and sham-operated (Sham) rats four weeks after operation.

Calibration

The calibration graphs for free HP and LP were obtained by analysing standard solutions over the pyridinoline concentration ranges 0.2–10 nmol/ml using system A and 0.2–2.6 nmol/ml using system B. The least-squares regression fit showed good linearity, passing through the origin for HP and LP (correlation coefficient = 0.999) using both systems. The detection limits of HP and LP using system A were 36 and 44 pmol/mol, respectively, and those using system B were both 44 pmol/ml at a signal-to-noise ratio of 3.

Recovery from urine

The recoveries of HP and LP from rat urine spiked with standard pyridinoline using system A were 95–98 and 100–103%, respectively, and the reproducibility was 1.5% ($n = 9$). The recoveries of pyridinoline from hydrolysed rat urine spiked with standard pyridinoline using system B were 100–109% and the reproducibility was 3.5% ($n = 5$).

TABLE I

URINARY FREE AND TOTAL HP CONCENTRATION IN OVARIECTOMIZED (A) AND SHAM-OPERATED (B) RATS FOUR WEEKS AFTER THE OPERATION

Group	n	HP concentration[a] (nmol/mmol creatinine)	
		Free	Total
A	10	50.7 ± 2.6[b]	81.4 ± 5.4[c]
B	10	39.0 ± 2.8	58.2 ± 7.0

[a] Mean ± standard error.
[b] $P < 0.01$.
[c] $P < 0.05$.

Application to rats with model bone disease

Urinary free and total HP concentrations in rats with bone model disease were determined. Urine was obtained from rats four weeks after ovariectomy or sham operation. Table I and Fig. 5 show the results. HP concentration is presented as the ratio to urinary creatinine concentration.

Both free and total HP concentrations in urine from ovariectomized rats were significantly higher than those from sham-operated rats. These results suggest that pyridinoline are useful biomedical markers for bone disease.

CONCLUSION

We isolated HP and LP from collagen and chicken bone and determined the molar absorptivity of HP at pH 2.0 and 297 nm. The results showed that HP concentrations determined according to Eyre *et al.*'s calibration method for HP were overestimated 1.52 times.

We have established on-line urine clean-up systems for the simultaneous determination of free and total pyridinoline by HPLC using the column-switching technique. The detection limits using these systems were 36 pmol/ml for free HP and 44 pmol/ml for free LP, and that using system B was 44 pmol/ml for total pyridinolines. The reproducibilities of systems A and B were 1.5 and 3.5%, respectively. Both the sensitivity and precision of the systems are good, and the analysis of one sample including clean-up is completed within 25 min. These systems can readily be applied to determine pyridinolines in urine samples automatically using an autoinjector. The systems offer a convenient, highly sensitive and useful method for the determination of pyridinolines. The determination of pyridinolines will be helpful in the diagnosis of bone diseases and the evaluation of the therapeutic efficacy of agents in their treatment.

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