Journal of Chromatography, 574 (1992) 255–260
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6187

High-performance liquid chromatographic determination of 3-hydroxypyridinium derivatives as new markers of bone resorption

S. Kamel and M. Brazier

Laboratoire d'Hormonologie, CHRU, Hôpital Sud, 80054 Amiens Cédex and Laboratoire de Pharmacie Clinique, Faculté de Pharmacie d'Amiens, Amiens (France)

G. Desmet*

Laboratoire d'Hormonologie, CHRU, Hôpital Sud, 80054 Amiens Cédex (France)

C. Picard

Laboratoire de Pharmacie Clinique, Faculté de Pharmacie d'Amiens, Amiens (France)

I. Mennecier and Jl. Sebert

Service d'Explorations Osseuses, CHRU, Hôpital Sud, 80054 Amiens Cédex (France)

(First received August 19th, 1991; revised manuscript received October 18th, 1991)

ABSTRACT

Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are intermolecular cross-linking amino acids of collagen and their urinary excretion reflects bone resorption. An isocratic high-performance liquid chromatographic assay using a reversed-phase column with a prefractionation step and fluorescence detection was developed. The accuracy and reproducibility were assessed by loading experiments and by double analysis of urinary samples. The recoveries after various loads were above 90% for HP and between 87 and 94% for LP, with an intra-assay relative standard deviation (R.S.D.) of 5% for HP and 8% for LP. The inter-assay R.S.D.s were 8% for HP and 12.4% for LP. The fasting and 24-h urinary excretions of HP and LP were measured in 40 healthy subjects (mean age 35 years) of both sexes. There was no difference between males and females. Mean adult normal values were 33.6 ± 8.1 pmol/μmol creatinine for HP and 7.0 ± 2.5 pmol/μmol creatinine for LP in morning fasting urine and 29.9 ± 7.0 pmol/μmol creatinine for HP and 5.8 ± 1.9 pmol/μmol creatinine for LP after 24-h urinary collection. HP and LP excretions were significantly higher in morning fasting urine than in 24-h collections, in agreement with the physiological circadian rythm of bone resorption. This simplified and optimized procedure is a good method for the determination of pyridinolines and should be useful for the evaluation of bone resorption.

INTRODUCTION

The evaluation of metabolic bone disease uses biochemical indices of bone resorption. Urinary hydroxyproline has been widely considered as a marker of collagen degradation [1,2], reflecting bone resorption. However this amino acid is not specific for bone or for collagen and may also derive from collagen synthesis. Indeed, hydroxyproline is rapidly metabolized by the liver and

0378-4347/92/$05.00

other sources, such as various tissues, circulating components and diet, limit its specificity as an index of bone breakdown [3,4].

Urinary excretion of hydroxylysine glycosides has been proposed as a more sensitive index of collagen degradation [5]. However, the enzymatic hydrolysis of the glucosyl fractions [6] limits the specificity of this marker as an index of bone tissue breakdown. The measurement of tartrate-resistant acid phosphatase [7] derived from osteoclasts has been proposed as a new index of bone resorption and high plasma levels have been observed in Paget's disease and primary hyperparathyroidism [8]. The specificity and sensitiviy of this index needs to be further assessed, however. Indices of collagen degradation based on the release of cross-linking components have advantages over previous methods both in specificity and sensitivity.

Cross-links between adjacent collagen chains stabilize the extracellular matrix [9] and are released during the degradation of mature collagen fibrils. Two mature cross-linking amino acids of the 3-hydroxypyridinium family have been identified by their natural fluorescent properties in connective tissues, particularly cartilage and bone, but not in skin [10,11]. Hydroxylysylpyridinoline (HP) is the major mature component and derives mainly from bone and cartilage. Lysylpyridinoline (LP) is significantly present only in bone and dentine [12]. Other advantages of HP and LP are the lack of absorption from the diet and the absence of metabolization in the liver, both increasing their specificity and sensitivity as markers of bone resorption. Their urinary excretions have been measured by paper chromatography [13], fluorescence detection after high-performance liquid chromatography (HPLC) [14–16] or by enzyme-linked immunoassay [17]. The urinary excretion level has been reported in healthy subjects [18] and changes with age and sex have been studied [19,20]. The urinary excretion of HP and LP has been found to be elevated in patients with osteoarthritis [16], rheumatoid arthritis [20], Paget's disease and primary hyperparathyroidism [21]. The specificity for bone resorption was recently demonstrated by measuring the short-term decrease of HP and LP in patients with Paget's disease before and after treatment with aminopropylidene bisphosphonate (APD), a potent inhibitor of bone resorption [21].

We have developed a simplified method for the simultaneous measurement of HP and LP in hydrolysed urine samples using ion-pair C_{18} reversed-phase HPLC. The chromatographic separation is coupled to a simple, fast prefactionation step of pyridinium components. We report here the analytical procedure and the level of the markers in a population of normal adults.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile of HPLC grade was purchased from Carlo Erba (Rueil Malmaison, France). Heptafluorobutyric acid (HBFA) (Aldrich, Strasbourg, France) was of Pierce sequanal grade. CF-1 cellulose (CF-1) was obtained from Whatman Biochemicals (Touzart et Matignon, Vitry/Seine, France). All solvents were of HPLC grade and chemicals were of analytical-reagent grade.

Urine specimens

Morning fasting 2-h (collected between 8 a.m. and 10 a.m.) and 24-h collections of urine were obtained on the same day from 40 volunteers (20 males, mean age 36.1 ± 5.4 years, and 20 females, mean age 35.4 ± 7.8 years on a gelatine-free diet. All were in good health, were receiving no medication and had not suffered previously from any bone or connective tissue disorder. After having measured the total volume of urinary collections, 10-ml aliquots were frozen and stored at −20°C until used for measurements, as it has been reported that prolonged storage at −20°C does not affect the measurement of HP and LP [18–21].

We also obtained 2-h or 24-h collections of urine from patients (n = 100) with various bone diseases in order to evaluate the reproducibility of double measurements.

Pyridinoline assay

Urine extraction. Urine (1 ml) was hydrolysed with 12 mol/l HCl (1 ml) for 3 h at 125°C in glass tubes. The hydrolysates were centrifuged at 2000 *g* for 10 min and the supernatant was mixed with pure acetic acid (1 ml), 1-butanol (4 ml) and CF-1 slurry (1 ml). CF-1 slurry was prepared as described by Beardsworth *et al.* [19].

Column preparation was carried out by adding 15 ml of CF-1 slurry to a Poly-Prep disposable column (Bio-Rad Labs., Paris, France), then washed with 10 ml of elution solvent [1-butanol–acetic acid–water (4:1:1, v/v/v)]. The hydrolysate–CF-1 slurry mixture was applied to the top of the bed of column for fractionation according to the method used for the purification of the elastin cross-links [22].

Columns were washed with 3 × 5 ml of the elution solvent, then with 1 ml of distilled water. The hydroxypyridinium cross-links were finally eluted with 2 × 6 ml of distilled water and collected in a 20-ml conical centrifuge tube. The eluate was freeze-dried and the dry residues were dissolved in HBFA (200 μl of a 1% aqueous solution), centrifuged for 10 min at 12 000 *g*. A 50-μl volume of the supernatant was used for HPLC analysis.

HPLC assay. The method was initially described by Eyre [15] and modified by Beardsworth *et al.* [19], Black *et al.* [16] and Uebelhart *et al.* [21]. Different gradient elution conditions were used by these workers for the separation of HP and LP. Here, we used an isocratic elution solvent on a Waters (St. Quentin en Yvelines, France) HPLC system equipped with an F 6000 A pump and a U6K automatic injector, controlled by a central computerized unit (NEC, APC IV). The reversed-phase column was an Altex Ultrasphere ODS (5 μm; 25 cm × 4.6 mm I.D.) protected by a Brownlee C_{18} guard cartridge (5 μm; 3 cm × 4.6 mm I.D.). HP and LP were detected by fluorescence of the eluted peak using a Shimadzu RF-535 spectrofluorimeter with excitation at 297 nm and emission at 380 nm. The eluent was 0.01 mol/l *n*-heptafluorobutyric acid–acetonitrile (91:9, v/v). HP and LP separations were performed at a flow-rate of 0.8 ml/min.

The results of HP and LP urinary excretion were given according to a comparison with two external standards injected in five different amounts and were expressed as pmol/μmol creatinine. The external standard was prepared as described by Uebelhart *et al.* [21] from fragments of human femoral bone and contained 1612 pmol of HP and 328 pmol of LP per 50 μl (determination kindly performed by Dr. P. D. Delmas, Inserm U 234, Lyon, France).

Creatinine (Crea) was determined in urine by a colorimetric procedure using an assay kit (Abbott).

Statistical analysis

The statistical significance of differences was determined using Student's *t*-test. In order to test the reproducibility of the technique at various concentrations, a Snedecor test [23] was applied to 100 double measurements of urinary specimens from patients with various metabolic bone diseases.

RESULTS

Chromatography

A chromatogram of a standard mixture consisting of 32.2 and 5.6 pmol (in 50 μl) of HP and LP, respectively, is shown in Fig. 1A. The linearity of HP and LP calibration graphs, in the range 0–322 and 0–65 pmol per 50 μl, respectively, was satisfactory, with correlation coefficients of 0.998 for HP and 0.999 for LP. Fig. 1B shows a chromatogram of a urine specimen after extraction and purification. As shown in Fig. 1, HP and LP separations are complete without any interfering peaks. With our system, HP and LP were eluted at about 11 and 12.5 min, respectively. The best resolution of the peaks was observed when using freshly prepared eluent (each day) and a pH of 2.

The accuracy of the total procedure, including hydrolysis, fractionation and HPLC measurement, was assessed by measuring the recovery of two known amounts of standards, one containing 322 and 65 pmol of HP and LP, respectively, and the other 1288 and 261 pmol of HP and LP,

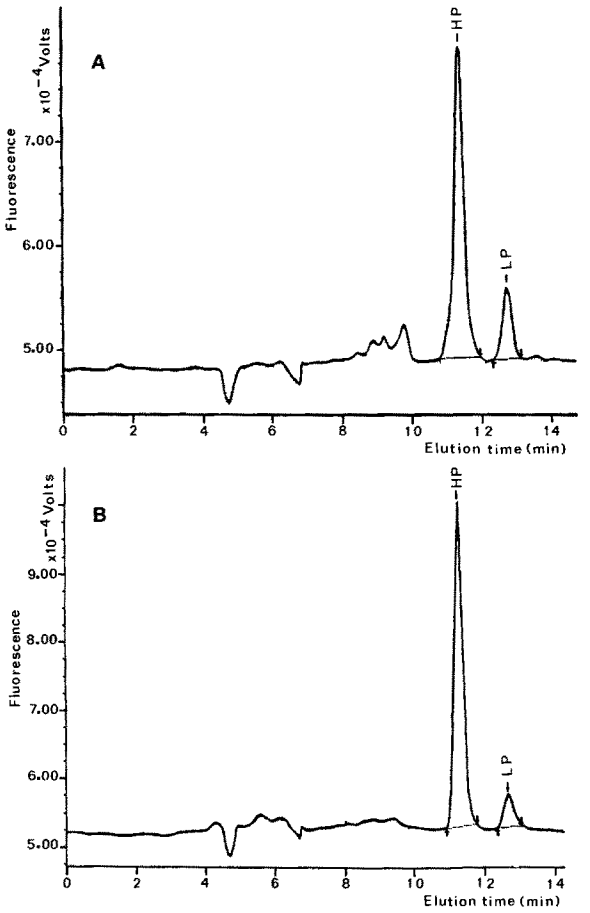

Fig. 1. Chromatograms of (A) standard mixture containing 32.2 and 5.2 pmol of HP and LP, respectively, and (B) human urine hydrolysate from a healthy subject.

respectively, added to urine before analysis. The recovery (± S.D.) of the two loads was evaluated from twelve experiments and the results are reported in Table I. The precision of the complete analysis, calculated as the intra-assay variation, is also presented in Table I.

The inter-assay precision was determined from twelve measurements performed on different days on the same non-hydrolysed urine sample. The mean (± S.D.) concentrations of HP and LP were 80.6 ± 6.5 and 9.2 ± 1.15 pmol/μmol creatinine, respectively, and the relative standard deviations were 8% for HP and 12.4% for LP.

The global intra-assay precision was assessed with the Snedecor test from double analysis of 100 urine samples from patients with various bone diseases with a concentration range from 15.8 to 382.2 pmol/μmol creatinine for HP and from 2.2 to 51.2 pmol/μmol creatinine for LP. The standard deviations were 0.25 and 0.08 pmol/μmol creatinine, respectively.

Finally, the limit of detection was evaluated at 4 pmol/ml of urine.

Excretion of HP and LP in normal adults

The excretions of HP and LP (pmol/μmol creatinine) in 40 healthy adults are shown in Table II. The fasting and 24-h urinary excretions of HP and LP are not different in men and women. As also shown in Table II, the excretions of HP and LP are significantly higher in the fasting urine samples than in the 24-h collections.

DISCUSSION

The urinary excretion of pyridinolines, which reflects collagen degradation, has been proposed as a biochemical marker of bone resorption [17–21]. Urinary analysis by HPLC needs a prelimi-

TABLE I

RECOVERIES OF TWO AMOUNTS OF HP AND LP ADDED TO A URINE SPECIMEN AND PRECISION OF THE TECHNIQUE EXPRESSED AS INTRA-ASSAY VARIATION (n = 12 EXPERIMENTS)

Compound	Load (pmol)	Recovery (mean ± S.D.) (%)	Urinary concentration (mean ± S.D.) (pmol/ml)	Precision, R.S.D. (%)
HP	322	102.7 ± 7	432 ± 22.7	5.2
	1288	91.2 ± 8.9	1260 ± 56.7	4.5
LP	65	94.2 ± 4.5	78 ± 5.8	7.4
	261	87.4 ± 7.5	240 ± 19.5	8

TABLE II

EXCRETIONS OF HP AND LP (MEAN ± S.D. AND RANGE) FROM 40 HEALTHY ADULTS (20 MEN, 20 WOMEN) IN FASTING AND 24-h URINES

Sex	Fasting urine			24-h urine		
	HP (pmol/μmol Crea)	LP (pmol/μmol Crea)	HP/LP ratio (%)	HP (pmol/μmol Crea)	LP (pmol/μmol Crea)	HP/LP ratio (%)
Men	33.4 ± 8.6	7.6 ± 2.6	4.7 ± 1.2	29.5 ± 7.3[a]	6.0 ± 1.9[b]	5.1 ± 1
	17.6–45.3	2.2–12.1		15.4–41.4	2.2–9.3	
Women	33.6 ± 7.8	6.3 ± 2.3	5.7 ± 2.0	30.5 ± 6.9[a]	5.7 ± 1.9	5.6 ± 1.4
	16.9–51.1	2.7–11.8		15.5–41.2	3.0–9.1	

[a] Significance of the difference between fasting and 24-h urine: $p < 0.05$.
[b] Significance of the difference between fasting and 24-h urine: $p < 0.01$.

nary ion-exchange procedure on a cellulose column. This fractionation step before HPLC measurement allows the elimination of many fluorescent components present in urine. No appropriate internal standard is known at present, although pyridoxamine was used previously [16]. The modifications described in this paper lead to improved pyridinium extraction and separation after urinary hydrolysis. Indeed, the extraction of pyridinoline is almost complete, above 90% for HP and between 87 and 94% for LP over a wide range of concentrations, including very high ones. From these results, it seems that the extraction of HP is more important than that of LP. In addition, our chromatographic conditions allow a good separation of HP and LP, better than that previously reported by other workers using gradient elution methods [16,21] or isocratic conditions [24]. Finally, no wash-out step between injections is necessary, probably because a more acidic eluent is used. In spite of its simplicity, the precision of this technique seems satisfactory. The reproducibility of HP and LP measurements is better than that reported in previous chromatographic studies [16,21]. However, the absolute differences between double measurements may sometimes be important, which emphasizes the need for an internal standard or for the development of other techniques such as immunoassay procedures. Robins has recently developed an inhibition immunoassay method [17] and measured urinary pyridinoline excretions from patients with various bone diseases. However, it is not possible to measure HP and LP separately with this method.

HP and LP urinary excretions in young normal adults (mean age 35 years) obtained with our chromatographic method are similar to those observed with other chromatographic techniques [16,20]. The normal HP and LP excretions measured in fasting or 24-h urines are *ca.* 30.0 and 6.0 pmol/μmol creatinine, respectively, with an HP/LP ratio of about 5. However, HP and LP excretions measured in fasting urine samples are significantly higher than those derived from 24-h urine collections. This is probably explained by a circadian variation as it is known that bone resorption is maximum during the night [25]. However, the statistical differences between fasting and 24-h urines remain very small and seem to be of negligible importance on a clinical basis. It seems possible, therefore, to use interchangeably the fasting and 24-h urines for the measurement of HP and LP, as proposed by some workers [26]. However, the effects of this circadian variation on the excretions of HP and LP should be evaluated in patients with bone disease, as differences between fasting and 24-h urines may be more important than in healthy subjects.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. A. Morel for skilful technical assistance and Mrs. B. Dame for typing the manuscript.

REFERENCES

1 K. I. Kivirikko, *Int. Rev. Connect. Tissue. Res.*, 5 (1970) 93.
2 J. Gielen, J. Dequeker, A. Drochmans, J. Wildiers and M. Merlevede, *Br. J. Cancer*, 34 (1976) 279.
3 P. C. Kelleher, *Clin. Chim. Acta*, 92 (1979) 373.
4 R. S. Bienkowski and C. J. Engels, *Anal. Biochem.*, 116 (1981) 414.
5 J. P. Segrest and L. W. Cunningham, *J. Clin. Invest.*, 49 (1979) 1497.
6 M. Sternberg and R. G. Spiro, *J. Biol. Chem.*, 254 (1979) 10329.
7 C. Minkin, *Calcif. Tissue Int.*, 34 (1982) 285.
8 K. H. W. Lau, M. E. Kraenzlin, T. Onishi and D. J. Baylink, in C. Christiansen *et al.* (Editors), *Osteoporosis 1987-2*, Osteopress ApS, Copenhagen, 1987, p. 682.
9 D. R. Eyre, *Annu. Rev. Biochem.*, 53 (1984) 717.
10 S. P. Robins, *Biochem. J.*, 215 (1983) 167.
11 D. R. Eyre and H. Oguchi, *Biochem. Biophys. Res. Commun.*, 92 (1980) 403.
12 D. R. Eyre, T. J. Koob and K. P. van Ness, *Anal. Biochem.*, 137 (1984) 380.
13 J. A. Gun, Z. Smith and R. J. Boucek, *Biochem. J.*, 197 (1981) 759.
14 D. Fujimoto, *Biochem. Biophys. Res. Commun.*, 109 (1982) 1264.
15 D. R. Eyre, *Methods Enzymol.*, 144 (1987) 115.
16 D. Black, A. Duncan and S. P. Robins, *Anal. Biochem.*, 169 (1988) 197.
17 S. P. Robins, *Biochem J.*, 207 (1982) 617.
18 D. Fujimoto, M. Suzuki, A. Uchiyama, S. Miyamoto and T. Inoue, *J. Biochem.*, 94 (1983) 1133.
19 L. J. Beardsworth, D. R. Eyre and I. R. Dickson, *J. Bone Miner. Res.*, 5 (1990) 671.
20 S. P. Robins, P. Stewart, C. Astbury and H. A. Bird, *Ann. Rheum. Dis.*, 45 (1986) 969.
21 D. Uebelhart, E. Gineyts, M. C. Chapuy and P. D. Delmas, *Bone Miner.*, 8 (1990) 87.
22 S. J. M. Skinner, *J. Chromatogr.*, 229 (1982) 200.
23 G. W. Snedecor, *Biometrics*, 8 (1952) 85.
24 A. Lichy, J. Macek and M. Adam, *J. Chromatogr.*, 563 (1991) 153.
25 S. Radom, M. Zulawski and E. Dahlig, *Clin. Chim. Acta*, 39 (1972) 270.
26 M. Arrigoni, G. Abbiati, S. Galimberti, A. Longoni and F. Bartucci, in B. L. Riggs *et al.* (Editors), *Osteoporosis 1988*, Vol. 2, Raven Press, New York, 1988, p. 598.