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Determination of pyridinium crosslinks in plasma and serum by high-performance liquid chromatography

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Abstract

A chromatographic method for the determination of pyridinoline (Pyr) and deoxypyridinoline (Dpyr) in serum and plasma is described. The analytical procedure involved plasma or serum purification by ultrafiltration (20 000 relative molecular mass cut-off) under centrifugation at 2500 *g* for 4 h, as an innovative step. Analysis was done by isocratic high-performance liquid chromatography with fluorescence detection. The linearity of the method was tested from 0.6 to 15 pmol/ml and 0.12 to 3 pmol/ml for Pyr and Dpyr, respectively. The detection limit was 60 fmol/ml for both crosslinks. Except for Dpyr in plasma (coefficient of variation 19.9%), intra-assay variation was always below 10% in serum and plasma. The method has been applied to the quantification of crosslinks in serum and plasma of healthy volunteers and also in mouse and rat plasma. Serum proved to be the most suitable biological fluid for the systemic measurement of these compounds in humans and under the experimental conditions used, contained an average of 3.62 ± 0.65 and 0.7 ± 0.18 pmol/ml Pyr and Dpyr, respectively.

1. Introduction

The first step in collagen crosslink biosynthesis is the oxidative deamination of certain lysine and hydroxylysine residues by the enzyme lysyl oxidase. The aldehydes formed can subsequently react with each other or with other lysine or hydroxylysine residues to form difunctional derivatives or the trifunctional fluorescent pyridinium crosslinks [1]. Pyridinoline (Pyr), the major pyridinium compound, is formed by three hydroxylysine amino acid residues, whilst the less abundant form deoxypyridinoline (Dpyr), comprises two residues of hydroxylysine and one of lysine. The tissue distribution of Pyr and Dpyr seems to be specific, considering that their major sources are bone and cartilage. The highest concentration of Pyr is found in cartilage, and much less is found in bone. Dpyr was initially thought to occur exclusively in bone and dentine, but subsequent analysis detected significant amounts also in the aorta and ligaments. Nevertheless, the turnover of collagen in these tissues is very low, and urinary Dpyr can thus be considered a specific marker for bone collagen degradation [2].

In clinical practice, pyridinium crosslinks are measured in urine as an index of bone resorp-

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tion, which is altered in diseases such as osteoporosis, primary hyperparathyroidism, hyperthyroidism, Paget's disease, metastatic bone disease, osteoarthritis and rheumatoid arthritis [2–4].

Probably because of their low levels in plasma and serum, Pyr and Dpyr have been analysed only in urine so far. Initially crosslinks were assayed by amino acid analysis and immunoassay techniques, but later on gradient or isocratic ion-pairing reversed phase HPLC methods with fluorescence detection were used [5–8].

HPLC methods and sample purification procedures have now been automated [9–11]. Yoshimura *et al.* [12] assessed Pyr and Dpyr in urine by a HPLC column-switching method which avoided the cellulose extraction step. James *et al.* [13] assessed Pyr and Dpyr serum concentrations in patients with Paget's disease but not Dpyr in normal subjects by developing an isocratic ion-pairing HPLC assay, using narrow-bore columns.

This paper describes a crosslink assay in plasma and serum that includes a clean-up procedure of serum and plasma by ultrafiltration before HPLC analysis. This method is also able to measure both pyridinium compounds in healthy subjects. Pyr and Dpyr plasma levels were compared in man, rats and mice.

2. Experimental

2.1. Chemicals

Hydrochloric acid (37%), *n*-butanol, acetic acid and *n*-heptafluorobutyric acid (HFBA), analytical grade, and acetonitrile, HPLC grade, were purchased from Merck (Bracco, Milan, Italy). Water, HPLC grade, was obtained with a Millipore Milli-Q apparatus (Waters, Vimodrone, Italy). SPE (solid-phase extraction) disposable cartridges prepacked with 200 mg of purified Whatman CF-1 cellulose and ultrafiltration tubes (20 000 relative molecular mass cut-off) were manufactured by Laboratori Solaria (Tolmezzo, Italy). Creatinine was determined using a Boehringer Mannheim test kit (Milan, Italy).

2.2. Apparatus and columns

The equipment used to assay Pyr and Dpyr extracts consisted of a Kontron 422 HPLC pump (Milan, Italy), a Shimadzu SIL-9A auto-injector and a Shimadzu RF-551 spectrofluorimetric detector (Opera, Italy), coupled with a Waters 820 Maxima Data System. The reversed-phase column was a Phenomenex ODS (5 μm, 25 cm × 3.2 mm I.D., Lab. Service Analytica, Bologna, Italy). The fluorimeter was set at an excitation wavelength of 295 nm and an emission wavelength of 400 nm. The eluent was 0.02 *M* HFBA–acetonitrile (77:23, v/v). The flow-rate was 0.5 ml/min. Ultrafiltration was performed with an Heraeus Minifuge T centrifuge (Cavenago Brianza, Italy) with a swing-out rotor (Model 2250).

2.3. Plasma, serum and urine specimens

Blood and 24-h urine samples were obtained from nine healthy male volunteers, 25 to 39 years of age, with no bone or joint disease history. Blood (20 ml) was collected by venipuncture into sterile plain Vacutainers and 10 ml were allowed to clot for 30 min at 37°C and then centrifuged at 2000 *g* for 10 min. The serum was removed and stored at −20°C. For plasma preparation the other 10 ml of blood were collected in heparinized tubes and centrifuged at 2000 *g* for 10 min. The plasma was stored at −20°C.

The total urinary volumes were measured and 10-ml portions were frozen and stored at −20°C until analysis. To compare the plasma crosslink values in man and animal, male and female CD Cobs rats ($n = 8$; weighing 275–300 g) and male CD_1 Cobs mice ($n = 20$; weighing 20–25 g) (Charles River, Calco, Italy) were killed to obtain plasma.

2.4. Preparation of Pyr and Dpyr standard

Pyr and Dpyr standards were prepared from 10 g of rat femur. The femur were disarticulated and cleaned with care from their soft tissues. Bone was powdered and then triturated and hydrolysed with 6 *M* HCl for 72 h at 105°C. The

hydrolysate was evaporated to dryness, reconstituted with 1–10 ml of butanolic mixture (see below) and applied to a CF1 column (2 × 120 cm). The column was washed with *n*-butanol–acetic acid–water (4:1:1, v/v; butanolic mixture) and eluted with water. The eluate was freeze-dried and analysed by HPLC [14].

2.5. Extraction and chromatographic analysis of biological samples

Urine

Urine samples (0.25 ml) were hydrolysed in an equal volume of 37% HCl at 105°C overnight.

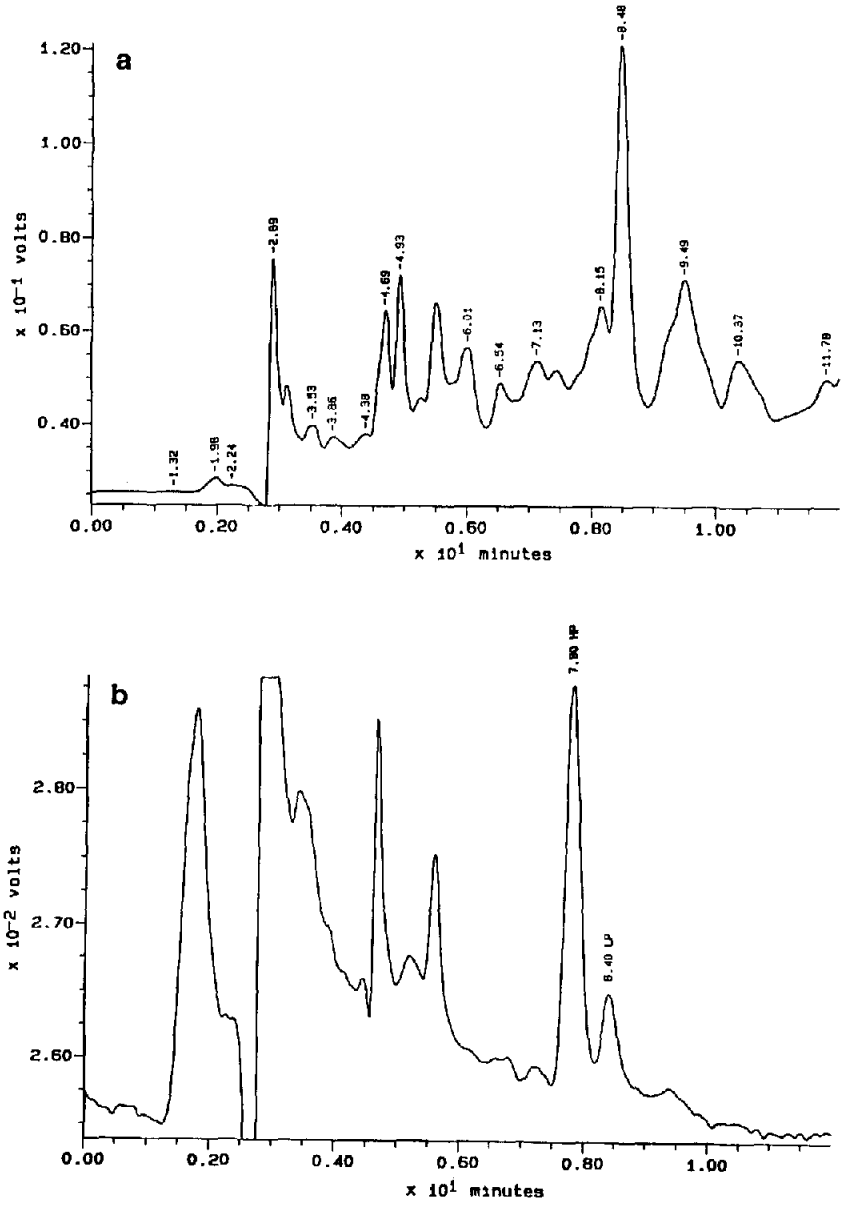

Fig. 1. Typical Pyr and Dpyr chromatograms obtained from (a) whole serum (without ultrafiltration), (b) plasma, (c) serum, and (d) 24-h urine of a healthy volunteer.

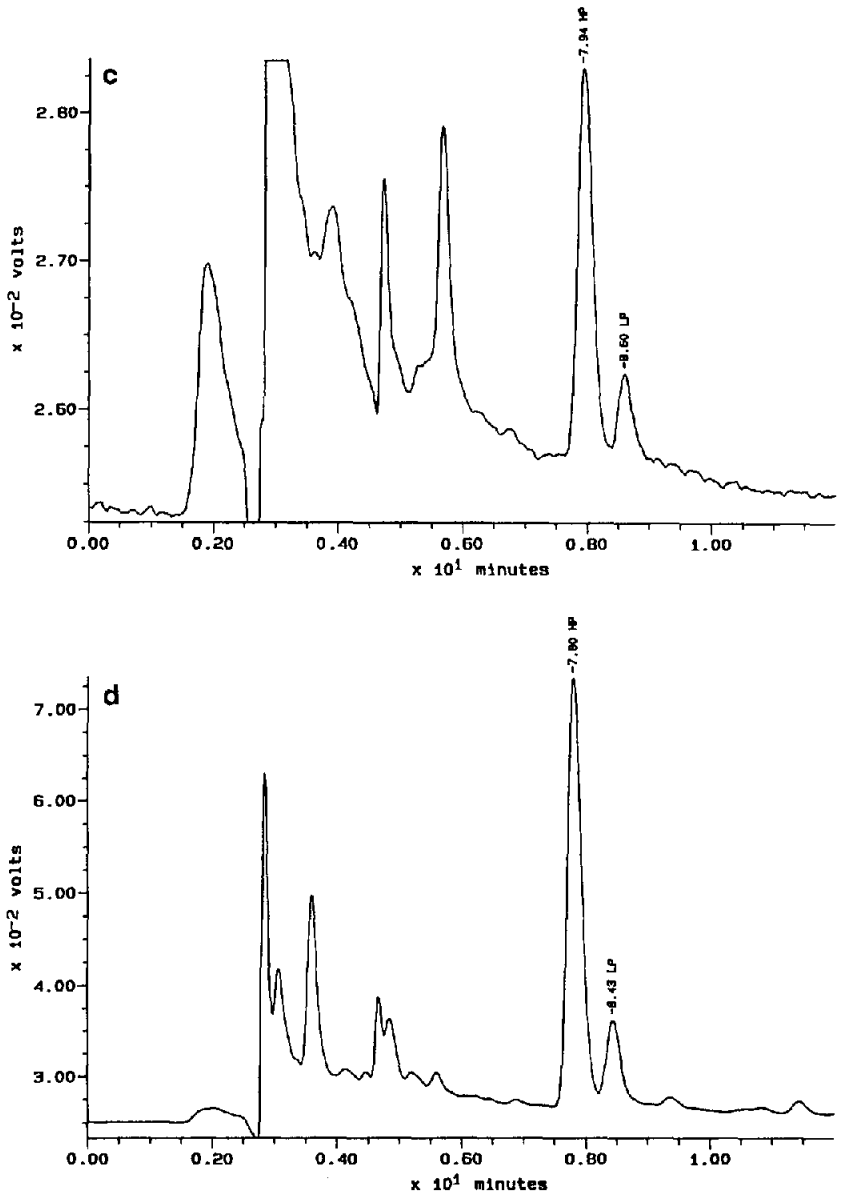

Fig. 1. (*Continued*).

Hydrolysates were mixed with glacial acetic acid (0.5 ml) and *n*-butanol (2 ml), and applied to the SPE CF-1 cellulose cartridges, previously conditioned with 5 ml of the butanolic mixture (see above). After washing with 25 ml of the butanolic mixture, samples were eluted with 5 ml of distilled water, dried and reconstituted with 200 μl of 1% aqueous HFBA solution. A 60-μl aliquot of the sample was injected and analysed by HPLC.

Plasma and serum

Before hydrolysis plasma and serum samples (2.5–5 ml) were purified by ultrafiltration, using centrifuge tubes with 20 000 relative molecular mass cut-off filters (4 h at 2500 *g*). This pro-

cedure removes macromolecules, such as proteins, that interfere with the analysis. Then, the ultrafiltrates were measured and hydrolysed overnight in an equal volume of 37% HCl (105°C), dried, reconstituted with 6 *M* HCl (0.5 ml), acetic acid (0.5 ml) and *n*-butanol (2 ml) and processed as described for urine. After drying the eluate, the samples were reconstituted with 150 μl of a 1% aqueous HFBA solution, and a 120-μl aliquot was analysed by HPLC.

3. Results and discussion

3.1. Chromatography

Fig. 1a–d shows typical chromatograms of plasma, serum and 24-h urine of an healthy volunteer, obtained using the proposed analytical method. The advantages obtained by ultrafiltration of the biological samples can be clearly observed when comparing the chromatogram of Fig. 1a (serum not ultrafiltered), with those of Fig. 1b and 1c. Ultrafiltration removes several peaks of compounds interfering with the analysis having retention times similar to those of Pyr and Dpyr. Also the baseline level decreases *ca.* 15-fold from 0.04 to 0.0026 V.

Fig. 2a,b shows the chromatograms of mouse and rat plasma.

Regardless of species and biological matrix, the chromatographic profiles were very similar, with much alike retention times. After ultrafiltration no peaks originating from hydrolysis products seemed to interfere with pyridinoline analysis.

Table 1 shows the main chromatographic data for the three biological fluids in man and for rat and mouse plasma. Chromatographic analysis of each sample was completed in 12 min.

3.2. Calibration and sensitivity

A stock solution was prepared containing micromolar concentrations of Pyr and Dpyr. Five-point calibration curves for Pyr (0.6–15 pmol/ml) and Dpyr (0.12–3 pmol/ml) were obtained by serial dilution of the stock solution with 1% HFBA in water.

The least-squares regression fit showed good linearity, passing through the origin for Pyr and Dpyr (correlation coefficient 0.999). The detection limit of the assay was 60 fmol/ml for both Pyr and Dpyr (signal-to-noise ratio 2).

Recovery was assessed using pooled normal serum added with standard (10 pmol/ml for Pyr and 2 pmol/ml for Dpyr). Samples ($n = 5$) were analysed as detailed in the Experimental section. The mean ± S.D. recovery was 101.5 ± 5.96 for Pyr and 91.2 ± 4.37 for Dpyr. Recovery calculation was done by subtracting the Pyr and Dpyr endogenous values determined in the same serum samples without standard addition.

3.3. Inter- and intra-assay variation

The precision of the Pyr and Dpyr assay in the three biological fluids was evaluated on the basis of the within- and between-assay variation (Table 2). Pyr and Dpyr within-assay variation was evaluated testing five replicates of each biological fluid. The between-run precision was determined from the analysis of the same samples in three different experiments. For both crosslinks the coefficients of variation were under 10%, with the exception of Dpyr in plasma where the C.V.% was *ca.* 20%. In our opinion this may be due to a smaller efficiency of the ultrafiltration process when plasma is used instead of serum.

3.4. Pyr and Dpyr in healthy volunteers' plasma and serum

Pyr and Dpyr concentrations (pmol/ml ± S.D.) in plasma and serum of healthy volunteers ($n = 9$) are shown in Table 3. Crosslinks in plasma were lower than in serum (*ca.* 32 and 44%, respectively). The values in both biological fluids were nevertheless closely correlated (Pearson's correlation coefficient: Pyr, $r = 0.829$, $p < 0.01$; Dpyr, $r = 0.678$, $p < 0.05$). Serum is therefore the most suitable biological fluid for systemic measurement of these compounds.

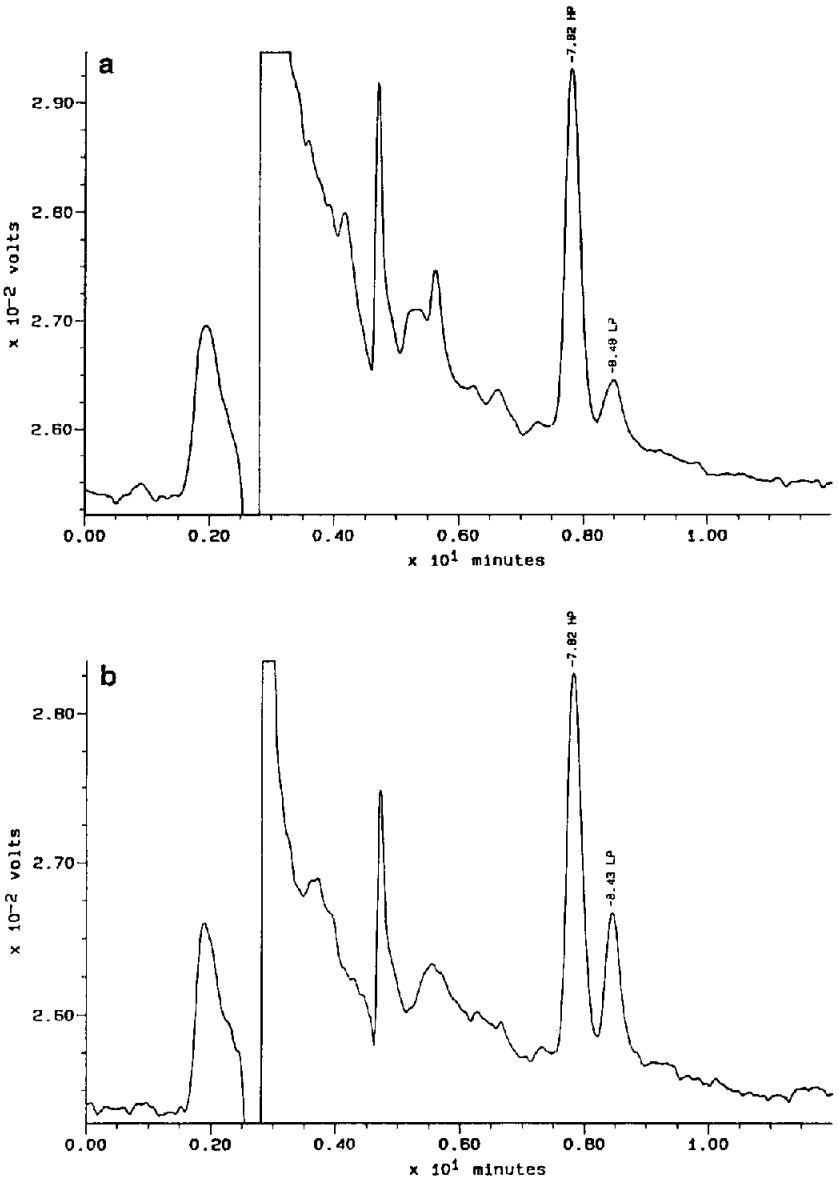

Fig. 2. Pyr and Dpyr chromatograms of mouse (a) and rat plasma (b).

3.5. Pyr and Dpyr in serum and urine

In clinical studies crosslinks have up till now been measured in 24-h urine or in 2-h fasting morning urine. Unexpectedly, our experimental results, particularly for Dpyr, showed no significant correlation between the concentrations of these compounds found in serum and urine (Pearson's correlation coefficient: Pyr, $r = 0.619$, $p = 0.075$; Dpyr, $r = 0.417$, $p = 0.689$), even though the Pyr/Dpyr ratios were close, 5.19 in serum and 5.5 in urine.

As an example of the utility of the method, Pyr and Dpyr mean plasma levels detected in man, rat and mouse are reported in Table 4. In all three species, Pyr concentrations range be-

Table 1
Pyridinoline (Pyr) and deoxypyridinoline (Dpyr) retention times in human plasma, serum and urine and in mouse and rat plasma

Biological fluid	Retention time (mean ± S.D.) (min)		C.V. (%)	
	Pyr	Dpyr	Pyr	Dpyr
Plasma (man)	7.78 ± 0.03	8.39 ± 0.05	0.41	0.56
Serum (man)	7.90 ± 0.04	8.56 ± 0.05	0.47	0.56
Urine (man)	7.81 ± 0.01	8.43 ± 0.01	0.07	0.14
Plasma (mouse)	7.82 ± 0.02	8.48 ± 0.02	0.65	0.72
Plasma (rat)	7.81 ± 0.02	8.43 ± 0.01	0.57	0.64

Table 2
Within-assay ($n = 5$) and between-assay (n = 15) variation in plasma, serum and urine

Biological fluid	Coefficient of variation (%)			
	Within-assay variation		Between-assay variation	
	Pyr	Dpyr	Pyr	Dpyr
Plasma	7.38	19.64	5.79	19.94
Serum	3.79	3.88	3.82	6.01
Urine	3.62	3.83	4.49	9.08

Table 3
Pyr and Dpyr concentrations in plasma, serum and urine of healthy volunteers

Compound	Concentration (mean ± S.D., $n = 9$)		
	Plasma (pmol/ml)	Serum (pmol/ml)	Urine (nmol/24h)
Pyr	2.38 ± 0.50	3.62 ± 0.65	704.92 ± 182.67
Dpyr	0.37 ± 0.16	0.70 ± 0.18	128.72 ± 39.87

Table 4
Pyr and Dpyr plasma levels in different species

Species	n	Concentration (mean ± S.D.) (pmol/ml)		
		Pyr	Dpyr	Pyr/Dpyr ratio
Man[a]	15	2.85 ± 0.80	0.48 ± 0.23	6.77 ± 2.18
Rat	8	4.10 ± 0.57	1.99 ± 0.53	2.13 ± 0.40
Mouse	8	3.54 ± 1.16	0.36 ± 0.07	9.83 ± 2.46

[a]Nine healthy volunteers plus six plasma bank samples.

tween 2.5–4.5 pmol/ml, whereas Dpyr concentrations in rat plasma were higher than in the other two species (also in urine–data not published).

4. Conclusion

Pyridinium crosslinks are the most specific and sensitive markers available for bone resorption. Their measurement to date has been limited to urine, mainly because low concentrations were expected in biological fluids like plasma and serum [8].

Recently James *et al.* [13] measured Pyr but not Dpyr in healthy volunteers' serum. Introducing a clean-up of serum/plasma by ultrafiltration as an innovative step, we quantified both Pyr and Dpyr concentrations in serum and plasma in healthy volunteers and in rats and mice.

Plasma and serum were ultrafiltrated using a special centrifuge tube with a maximum capacity

of 5 ml and fitted with a cellulose acetate membrane, which separates the macromolecules weighing more than 20 000 Da. The use of ultrafiltration produces a larger amount of purified biological samples (up to 3.8 ml), increasing the injectable concentrations of Pyr and Dpyr and lowering the detector baseline level. This method quantifies Pyr and Dpyr serum concentrations in healthy subjects and animals and should be able to establish the level of crosslinks in serum of patients, making it possible to study the relationship between the circulating levels of crosslinks and their urinary excretion.

5. References

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