An enzyme-linked immunoassay for the collagen cross-link pyridinoline

Simon P. ROBINS

Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K.

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An inhibition immunoassay method for the determination of pyridinoline was developed with the use of microtitre plates coated with a pyridinoline–gelatin conjugate and rabbit antisera directed against pyridinoline linked to bovine serum albumin. The sensitivity of the assay is about 2 pmol of pyridinoline, and the presence of related pyridinium and lysine-derived compounds does not significantly interfere with the procedure. Its application to tissue and human urine samples is described.

Pyridinoline is a 3-hydroxypyridinium compound derived from hydroxylysine (Fujimoto *et al.*, 1977, 1978) that is present primarily in bone, cartilage and tendon, but also in those tissues where hydroxylation of telopeptide lysine residues is extensive, such as scar tissue (Moriguchi & Fujimoto, 1979). This compound has been proposed as a trifunctional cross-link in mature collagen (Fujimoto, 1980).

The present paper describes a sensitive immunoassay method for the determination of pyridinoline that was developed to facilitate analysis of the distribution and changes with age in the amounts of this component in small tissue samples. The procedure also provides a unique index of the degradation of certain forms of mature collagen by analysis of physiological fluids, and its application to the analysis of human urine is described.

Experimental and results

Materials

Polyoxyethylene sorbitol monolaurate (Tween 20), 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide, pig skin gelatin and alkaline phosphatase coupled to goat anti-rabbit antibodies, together with the enzyme substrate *p*-nitrophenyl phosphate (Sigma 104), were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Polystyrene 96-well microtitre plates (flat-bottomed) were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and sodium cyanoborohydride was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Isolation of cross-link compounds

Pyridinoline was isolated from decalcified long-bones of 11-year-old sheep by ion-exchange chromatography with 67 mM-sodium citrate buffers on sulphonated cross-linked polystyrene resins maintained at 60°C. Final purification was achieved by

chromatography with pH 4.25 buffer (Robins, 1982), and the sample was desalted by using a column (1.6 cm × 90 cm) of Bio-Gel P-2 with 0.1 M-acetic acid as eluent. The structure of the compound proposed by Fujimoto *et al.* (1978) was confirmed by ¹⁴C n.m.r. and fast-atom-bombardment mass spectrometry (S. P. Robins, unpublished work). Desmosine, isodesmosine, merodesmosine and lysinonorleucine were isolated from bovine ligamentum nuchae, and reduced lysine-derived components were isolated from borohydride-treated Achilles-tendon collagen as described previously (Robins, 1982).

Preparation of anti-pyridinoline sera

The antigen was prepared by linking pyridinoline to bovine serum albumin. To a solution of the protein (10 mg) in water (0.7 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (100 mg) followed by an aqueous solution (0.3 ml) of pyridinoline (4.5 mg). After reaction for 3 h at room temperature, the solution was dialysed against six changes (100 ml) of distilled water and freeze-dried. Amino acid analysis and fluorescence measurements showed that the product contained 9.5 mol of pyridinoline/mol of albumin.

The pyridinoline–albumin conjugate was dissolved in phosphate-buffered saline (0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4), emulsified with an equal volume of Freund's complete adjuvant and then injected intradermally at multiple sites into New Zealand White rabbits. Each animal received about 150 µg of antigen in this and in subsequent booster injections given with Freund's incomplete adjuvant at monthly intervals. Useful titres were obtained after 4–5 months.

Preparation of coating protein for immunoassay

Pyridinoline covalently linked to gelatin was used

Fig. 1. *Inhibition immunoassay for pyridinoline*
Microtitre plates were coated with pyridinoline–gelatin in phosphate-buffered saline (0.6 µg/ml). The samples (0.12 ml) in phosphate-buffered saline were preincubated for 30 min in a separate microtitre plate with 0.12 ml of antiserum diluted with 0.1% Tween 20 in phosphate-buffered saline (final dilution 1:200). Samples were transferred to the washed coated plate and left to react for 1 h. After the plate had been washed, 0.2 ml of goat anti-rabbit antibodies linked to alkaline phosphatase (1:1000 dilution in 0.05% Tween 20 in phosphate-buffered saline) was added, and 1 h later the plate was again washed followed by the addition of 0.2 ml of a solution of enzyme substrate (1 mg/ml) in 50 mM-sodium carbonate buffer, pH 9.6, containing 1 mM-CaCl₂. The reaction was stopped after 30 min by adding 2 M-NaOH (0.1 ml), and the absorbance at 405 nm in each well was measured directly with a Titertek Multiskan instrument (Flow Laboratories). The results are expressed as percentages of a maximum value determined in the same microtitre plate by using phosphate-buffered saline alone at the pre-incubation step.

system (Table 1). Only desmosine showed any significant inhibition in the assay, but even this was less than 0.1% of that exhibited by pyridinoline. Glucose and galactose and their corresponding 2-amino derivatives exhibited no inhibition of the antiserum, although the amounts of these compounds remaining after acid hydrolysis are likely to be small; the products of acid hydrolysis of the hexoses in the presence of amino acids were also uninhibitory.

Application to tissue and urine samples

Dried tissue samples (0.1–1.0 mg) were hydrolysed in 6 M-HCl at 107°C for 21 h; freshly collected human urine samples (1.0 ml) were hydrolysed under similar conditions after the addition of conc. HCl (1.0 ml). Each hydrolysate was evaporated to dry-

Table 1. *Effect of relatively high concentrations of various compounds on anti-pyridinoline serum binding*
Each compound was tested separately at 150 nmol/ml under the standard assay conditions (see the legend to Fig. 1), and the inhibition of binding of the anti-pyridinoline serum was calculated by comparison with control samples containing buffer alone. Under the conditions used, pyridinoline gives 50% inhibition of binding at 0.15 nmol/ml.

	Inhibition of binding (%)
Desmosine	32
Isodesmosine	8
Merodesmosine	3
Amino acid mixture*	5
Dihydroxylysineonorleucine	5
Hydroxylysineonorleucine	5
Histidino-hydroxymerodesmosine	0
Lysineonorleucine	3
3-Hydroxypyridine	0
Pyridoxine	2

* A standard mixture (Calbiochem–Behring Corp.) containing 18 natural amino acids including lysine and hydroxylysine, each at 150 nmol/ml.

ness *in vacuo* at 50°C, the residue was dissolved in water (0.5 ml) and a portion (0.05 ml) was removed for hydroxyproline determination (Firschein & Shill, 1966). The remainder was diluted with 0.1 M-sodium phosphate buffer, pH 7.4, containing Phenol Red indicator, and, where necessary, the pH was adjusted to 7–7.5 by the addition of 2 M-NaOH. Portions (0.12 ml) of the samples were analysed directly in duplicate, with assays of pyridinoline standard solutions being included on each microtitre plate.

Analysis of 18-month-old bovine articular cartilage by immunoassay revealed a pyridinoline content of 2.8 pmol/nmol of hydroxyproline, compared with a value of 2.4 pmol/nmol of hydroxyproline obtained with a much larger quantity of the same sample by amino acid analysis.

The concentration of pyridinoline in the urine of a control group of eight individuals aged between 30 and 45 years was 0.85 ± 0.17 nmol/mg of creatinine. Addition of pyridinoline to a hydrolysed urine sample at 0.1 nmol/ml was detected with a coefficient of variation of 8%. Preliminary analyses of patients (aged between 57 and 82 years) with osteoarthritis have indicated a much higher urinary pyridinoline excretion at 3.91 ± 0.84 nmol/mg of creatinine ($n = 6$), and in a patient (aged 74 years) with Paget's disease of bone the urinary concentration of pyridinoline was 10.1 nmol/mg of creatinine. These differences in pyridinoline excretion do not appear to be merely due to the age differences, although a systematic investigation of the variations with age is not yet complete.

Discussion

The technique described provides a rapid, sensitive and highly specific assay for pyridinoline. The attachment of the molecule to a solid support for the enzyme-linked assay has some advantages over radioimmunoassay methods that have been developed for desmosine (King *et al.*, 1980; Harel *et al.*, 1980) in that the separation of bound and unbound antibody is simplified. The gelatin used for preparing the coating protein contained no pyridinoline, although its presence would not have affected the assay, as the antibodies recognize only free pyridinoline and not its peptide-bound form. The 'active' pyridinoline in the coating protein is attached to oxidized hydroxylysine itself and not to any carbohydrate moiety, as linking of pyridinoline to the glycoprotein mucin produced a conjugate that was unreactive towards the antisera, despite containing a much higher proportion of bound pyridinoline than the gelatin. The reason for this is unclear, but it may reflect masking of the antigenic determinant through reaction of two amino groups of the molecule with the dialdehyde oxidation products of the carbohydrate.

The presence in human urine of a 3-hydroxy-pyridinium compound with chromatographic and fluorescence properties identical with those of pyridinoline was reported by Gunja-Smith & Boucek (1981). The immunoassay method for pyridinoline, which does not require preliminary fractionation of the urine, provides a simpler and more-sensitive technique that is suitable for routine screening. The

increased urinary excretion of this compound in patients with certain bone and joint disorders suggests that measurement of pyridinoline may indeed have some clinical applications in the management of these conditions.

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