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Assay of pyridinium crosslinks in serum using narrow-bore ion-paired reversed-phase high-performance liquid chromatography

Ian James and Claire Crowley

Department of Rheumatology, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ (UK)

David Perrett

Department of Medicine, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ (UK)

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ABSTRACT

The pyridinium crosslinks are important biomarkers of mature hard tissue collagen degradation. This paper describes an isocratic ion-paired reversed-phase high-performance chromatographic assay using narrow-bore columns and high-sensitivity fluorescence detection to enable for the first time the determination of pyridinium crosslinks in both serum and synovial fluid samples. Extracted freeze-dried acid hydrolysates were re-suspended in 20 m*M* pentafluoropropionic acid (PFPA). Separations were carried out using an Exsil 100 5-μm ODS2 column (100 mm × 2.1 mm I.D.) eluted with 10 m*M* PFPA in water at 0.15 ml/min and detected using a Jasco 821-FP detector (xenon lamp: excitation 290 nm, emission 400 nm). Fluorescent response was linear from 269 to 8620 fmol for pyridinoline (Pyr) and 85 to 2710 fmol for deoxypyridinoline (dPyr). The limits of detection were 28 and 57 fmol, respectively. The coefficient of variation for extraction and analysis of normal serum was 7.96% for Pyr and 6.30% for dPyr ($n = 6$). The mean ± S.D. concentration of Pyr in normal serum was 3.26 ± 0.83 n*M*.

INTRODUCTION

Collagen is the major structural protein in the body and the principal protein of bone. Once synthesised, collagen is assimilated into the extracellular matrix and arranged in a fibrillar manner in register with molecules already present [1]. The inherent strength and stability of the collagen matrix is primarily due to hydrogen bonding and the formation of inter- and intra-molecular crosslinks between adjacent α (I) chains [2].

The pyridinium crosslinks are derived from adjacent lysines and hydroxylysines within collagen following a series of enzymic and non-enzymic reactions, and are the predominant non-reducible crosslinks in mature skeletal collagens [3]. Pyridinoline (Pyr), first isolated from bovine tendon hydrolysates [4], is the major pyridinium crosslink and is widely distributed in skeletal tissues [5,6]. Deoxypyridinoline (dPyr), a minor crosslink and analogue of Pyr in which lysine is substituted for hydroxylysine, was subsequently isolated by Ogawa *et al.* [7]. dPyr is found predominantly in bone and dentin and is almost totally absent in cartilage [5]. Due to the slow turnover of dentin [8], dPyr is considered to be bone-specific.

In clinical use the pyridinium crosslinks are

Correspondence to: Dr. I. James, Department of Rheumatology, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ, UK.

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considered to be specific markers of mature collagen degradation and osteoclast function [9]. Both Pyr and dPyr are naturally fluorescent, enabling their sensitive detection. However, due to their low levels in most biological fluids clinical analyses have been limited to urine. Nevertheless 24-h crosslink excretion has been shown to correlate strongly with radioisotopic measures of bone resorption [10]. Urinary concentrations, unlike hydroxyproline, are unaffected by diet [11] and it is unlikely that circulating pyridinium crosslinks are further catabolised [12]. However, there appears to be diurnal [13–15] and longitudinal [16] variations in crosslink excretion.

Initially crosslinks were assayed using amino acid analysis with ninhydrin detection [6]. Robins [17] raised a polyclonal antibody to Pyr which showed little cross-reactivity with dPyr, however, the sensitivity and specificity for Pyr were limited due to the apparent hydrolysis-dependent interconversion of diastereoisomers of Pyr [5,18]. Subsequently gradient ion-paired reversed-phase high-performance liquid chromatography (RP-HPLC) methods were developed to quantify pyridinium crosslinks in tissue hydrolysates and hydrolysed urine extracts [19,20]. Initial gradient methods although providing resolution of dPyr from Pyr were inappropriate for routine application. Isocratic methods which were subsequently developed gave increased sensitivity and sample throughput [21–24]. Presently, urine hydrolysates are cleaned up using a manual cellulose extraction step prior to HPLC analysis [25], which removes the majority of polar fluorophores. Recently this extraction procedure has been automated [26,27].

Using gradient HPLC methods the levels in serum are undetectable suggesting serum concentrations are approximately 1% that of urine. Robins *et al.* [27] were able to detect crosslinks in some osteoarthritis (OA) samples but not normals using a combination of gradient ion-paired RP-HPLC and immunoassay. Applying our isocratic method [21] we demonstrated the presence of Pyr in serum from normal and uraemic subjects [29].

This paper describes an improved isocratic ion-paired RP-HPLC assay using narrow-bore columns with high-sensitivity fluorescence detection and further demonstrates the possibility of measuring pyridinium crosslinks in biological fluids other than urine.

EXPERIMENTAL

Chemicals

Octanesulphonic acid (OSA), sodium formate, methanol, acetonitrile (all Hypersolv), butanol, glacial acetic acid, ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid (all Analar grade) were obtained from BDH (Poole, UK). Heptafluorobutyric acid (HFBA) was obtained from Aldrich (Poole, UK), and pentafluoropropionic acid (PFPA) was obtained from Sigma (Poole, UK). Cellulose (type CF-1) was obtained from Whatman (Maidstone UK). Standards of Pyr and dPyr were prepared from decalcified human tibia as described previously [21].

Equipment

The HPLC system comprised of an ACS (350/01) isocratic pump (Applied Chromatography Systems, Macclesfield, UK), a Jasco 821-FP fluorescence detector (xenon lamp, excitation 290 nm, emission 400 nm) and a 851AS autosampler (Ciba Corning, Halstead, UK). Data were integrated using Midas 22^+ (Vr. 5.22) PC-based software (Comus Instruments, Hull, UK) running in Microsoft Windows. Separations were performed on an Exsil 100 5-μm (100 mm × 2.1 mm I.D.) ODS2 narrow-bore column (Hichrom, Theale, UK).

Mobile phase composition

The optimum mobile phase consisted of 10 m*M* PFPA (1.05 ml per l distilled, deionised water). The column was eluted at 0.15 ml min^{-1}.

Patients and samples

Blood was collected by venepuncture into sterile plain vacutainers from normal females and patients with OA and Paget's disease. Blood was allowed to clot for 30 min and spun at 2000 *g* for 10 min. Serum was removed, aliquoted (1 ml) and

stored at −20°C until required. Synovial fluid from an OA patient with an knee effusion was spun at 13 000 *g* for 5 min, aliquoted and stored at −20°C.

Extraction of crosslinks from serum and synovial fluid hydrolysates prior to HPLC

Aliquots of serum/synovial fluid (1 ml) were hydrolysed in equal volumes of concentrated hydrochloric acid at 116°C for 16 h in 4-ml autosampler vials (Chromacol, London, UK) sealed with 150-μm PTFE discs. Hydrolysates were transferred to 2-ml capped Eppendorf vials and spun at 13 000 *g* for 5 min. Aliquots (1 ml) of clear supernatant were mixed with glacial acetic acid (1 ml), CF1 cellulose (1 ml) in butanolic eluent (4:1:1, butan-1-ol–water–glacial acetic acid), butan-1-ol (4 ml) and mixed prior to application to the extraction columns.

Extraction columns were prepared as follows: CF1 cellulose in butanolic eluent [4 ml, 10% (w/v)] was added to 8-ml extraction columns (HPLC Technology, Macclesfield, UK), stoppered with Whatman 1 cm diameter GF/D glass fibre discs. The cellulose was allowed to settle and then washed with 5 ml of butanolic eluent. Samples were loaded onto the columns, the sample tube washed with 5 ml of butanolic eluent and the washings applied to the columns. Interfering fluorophores were eluted with 15 ml of butanolic eluent. A glass fibre disc, cut from a Whatman GF/A sheet, was applied to the top of each cellulose bed. Following a further 5-ml wash with butanolic eluent, the crosslinks were eluted with distilled water (9 ml) and collected in 15-ml conical centrifuge tubes. After centrifugation (2000 *g*, 5 min) at 5°C the upper butanolic layer was removed. The lower aqueous layer containing the crosslinks was freeze dried-overnight. Lyophilized samples were re-suspended in 100 μl of 20 m*M* PFPA and spun at 2000 *g* for 5 min to remove any cellulose debris. The clear supernatant was transferred to an HPLC vial prior to analysis. Analyses were performed using 20-μl injections.

RESULTS

Selection of mobile phase for high-sensitivity analysis of serum crosslinks

Several mobile phases were evaluated for their applicability to the measurement of collagen crosslinks in serum. HFBA and OSA are the commonly used ion-pairs for the analysis of collagen crosslinks [19–21]. However, both are strongly hydrophobic in nature and elution of ion-paired collagen crosslinks complexes requires the addition of organic modifiers to the mobile phase. Using our published assay [21] with OSA, it was only possible to accurately quantify Pyr in serum from uraemic patients [29].

The use of narrow-bore columns increased sensitivity, but OSA and HFBA were then found to be inappropriate ion pairs. At the higher levels of sensitivity necessary to assay serum pyridinium crosslinks numerous trace fluorescent components eluted with or near to Pyr making peak identification and quantitation difficult. Although many variations on these HPLC conditions were investigated, no satisfactory conditions were obtained. Reduction in flow-rate from 0.5 to 0.15 ml min^{-1}, alteration of pH or percentage organic modifier, had little effect on resolution.

PFPA, a weak volatile ion-pairing agent, used previously for HPLC–mass spectrometric applications, was found to give the necessary resolution for serum analyses. However it was necessary to re-suspend samples in 20 m*M* PFPA prior to HPLC for reproducible chromatography. Inclusion of organic modifiers in the mobile phase had deleterious effects on the chromatography and were subsequently omitted.

Effect of PFPA concentration and flow-rate on retention and resolution of pyridinium crosslinks

The effect of increasing PFPA concentration (5–10 m*M*) on k' and resolution of Pyr from dPyr was investigated (Fig. 1). k' and resolution of dPyr from Pyr increased with PFPA molarity. Using 10 m*M* PFPA, Pyr was totally resolved from dPyr and other fluorescent interfering compounds. The effect of varying flow-rate on resolu-

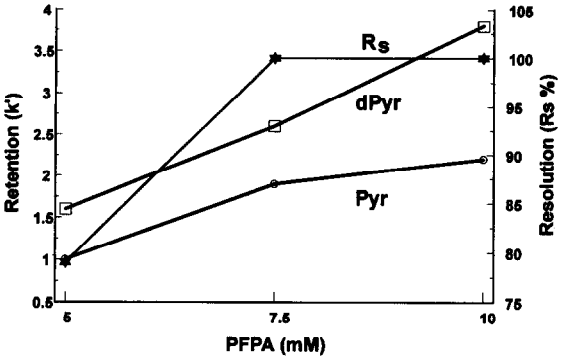

Fig. 1. Effect of PFPA concentration on retention (k') and resolution of pyridinium crosslinks at 0.25 ml min^{-1} flow-rate.

tion between 0.5 and 0.15 ml min^{-1} was investigated. A flow-rate of 0.15 ml min^{-1} was the optimum for the analysis. Analysis of standards took less than 10 min (Fig. 2). However, late-running fluorescent compounds in serum extracts necessitated an increased analysis time to avoid interferences.

Reproducibility, linearity and sensitivity

Stock standard (431 n*M* Pyr and 135 n*M* dPyr in 20 m*M* PFPA) was diluted serially with 20 m*M* PFPA. Regression analysis of peak areas against concentration of both Pyr and dPyr indicated a linear relationship over the range investigated (Pyr 269–8620 fmol, dPyr 85–2710 fmol per 20 μl injected, $r = 0.99$). The respective linearity equations were: response = 1.54(Pyr) − 0.082 (r^2 = 0.99) and response = 1.06(dPyr) − 0.087 (r^2 = 0.99). The limits of detection of the assay were 28 fmol for Pyr and 57 fmol injected for dPyr (signal-to-noise ratio = 2). The coefficients of variation (C.V.) of injected standards were less than 7% for Pyr and less than 13% for dPyr over the range investigated. Retention time C.V.s for repeated analysis of standards were 3.77% for Pyr ($n = 14$) and 3.31% for dPyr ($n = 10$). Pyr eluted at a mean (S.D.) of 4.85 ± 0.18 min and dPyr at 7.32 ± 0.25 min.

Recovery of standard from extraction

Recovery was assessed using pooled normal serum diluted one-fold with standard (431 n*M* Pyr, 136 n*M* dPyr). Samples ($n = 6$) were extracted as detailed in the Experimental section. The mean ± S.D. recovery was 83 ± 6.6% for Pyr and 79.8 ± 5.0% for dPyr.

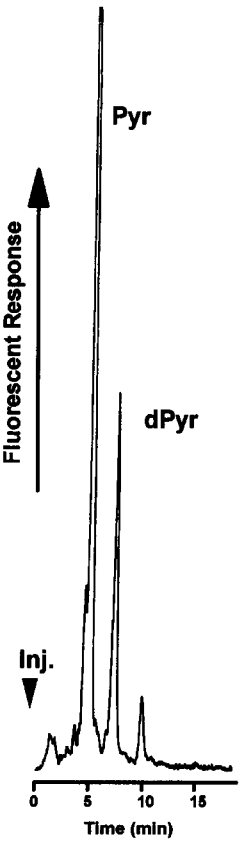

Fig. 2. Chromatogram of isolated bone standard (8.62 and 2.71 pmol per 20-μl injection for Pyr and dPyr respectively) analysed under final conditions. Detector settings were excitation 290 nm, emission 400 nm with 30-nm emission band width (xenon lamp), gain × 1000, attenuation × 32, standard response.

Intra-batch variation

The intra-batch C.V.s were assessed using both spiked and neat normal serum samples. The C.V.s in spiked serum samples were 7.96% for Pyr and 6.3% for dPyr ($n = 6$). In normal serum only Pyr could be accurately quantified and its C.V. was 15.2% ($n = 5$).

Analysis of crosslinks in normal and clinical samples

Pyr levels were determined in serum ($n = 7$)

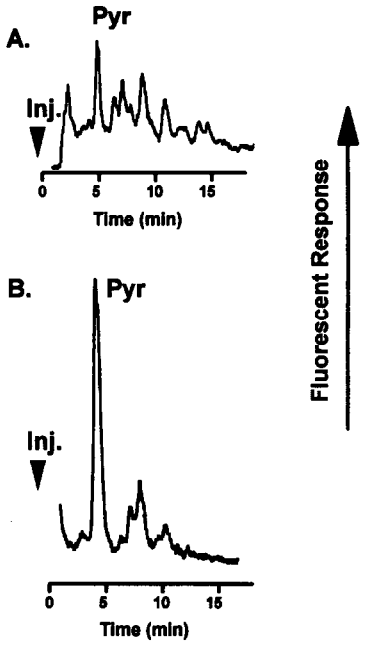

Fig. 3. Chromatograms illustrating (A) normal serum resuspended in 100 μl of 20 m*M* PFPA, Pyr equivalent to 260 fmol per 20-μl injection, and (B) same sample spiked with authentic Pyr and dPyr. Other conditions as Fig. 2.

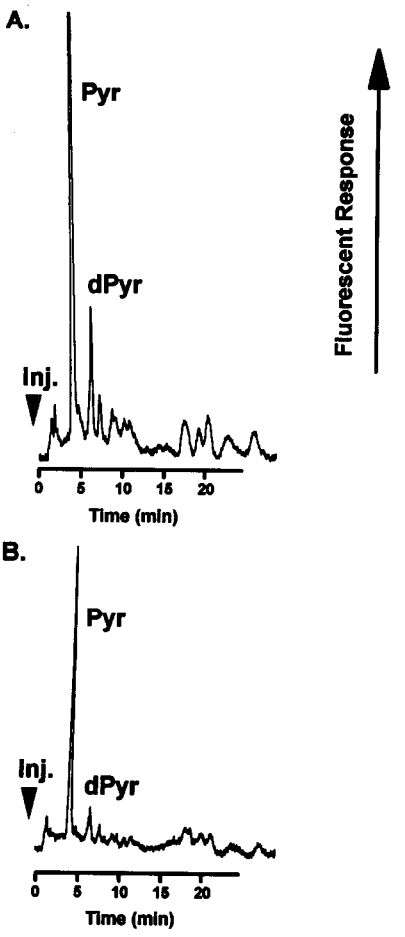

Fig. 4. Chromatograms illustrating collagen crosslinks in a patient with Paget's disease pre- and post-treatment with 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (APD). Samples were re-suspended in 100 μl of 20 m*M* PFPA. (A) Pre-treatment levels of Pyr and dPyr were 8.9 and 2.16 pmol per 20 μl injected, respectively. (B) Post-treatment levels of Pyr and dPyr were 2.2 and 0.56 pmol per 20 μl injected, respectively. Integrator sensitivity constant. Other conditions as Fig. 2.

from normal women of 53.5 ± 5.4 years (mean ± S.D.). The mean (± S.D.) level of Pyr was 3.26 ± 0.83 n*M*, ranging from 2.09 to 4.56 n*M*. dPyr was undetectable in normal serum, although further improvements to the assay may make this possible. Chromatograms showing the separation of Pyr and dPyr in a typical normal serum and the same serum spiked with authentic material are shown in Fig. 3.

Paget's disease is a metabolic condition where the rate of bone turnover is accelerated. Chromatograms of a Pagetic's serum, pre- and post-treatment with 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (APD) are illustrated in Fig. 4. Serum concentrations appear to be considerably elevated compared to normal controls and dPyr is detectable in some samples, even after treatment.

OA is characterised by an age-related progressive degeneration of articular joints. The concentrations of crosslinks in serum and synovial fluid of OA patients probably represent more subtle long-term changes in bone metabolism and disease activity. In two patients with OA, the concentrations of Pyr in serum were elevated compared with normal women (19.38 and 8.22 n*M*). In addition Pyr was present in significant quantities (55.6nM) in synovial fluid taken from another OA patient. dPyr was undetectable in OA serums and synovial fluid. Representative chromatograms are shown in Fig. 5.

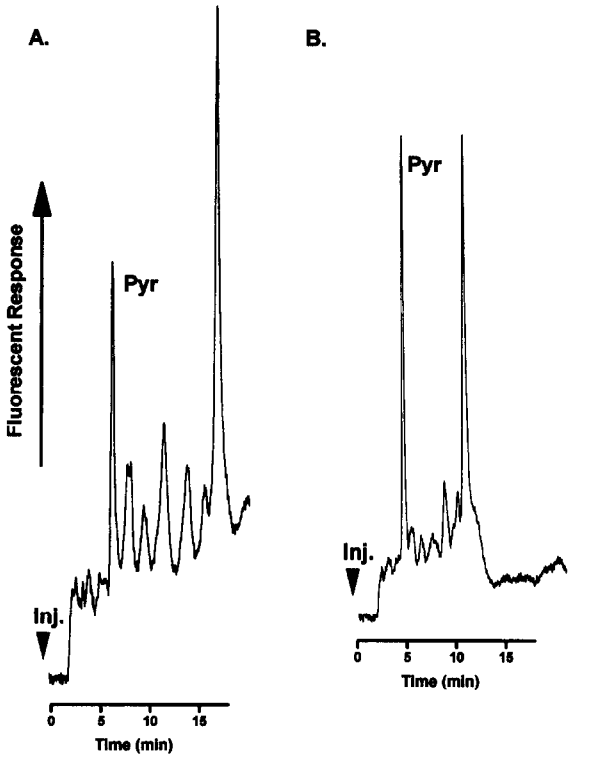

Fig. 5. Chromatograms of extracted osteoarthritis serum (A) and synovial fluid (B). Samples were resuspended in 100 μl of 20 mM PFPA. Pyr was present in OA serum at 1.94 pmol and in synovial fluid at 5.56 pmol per 20-μl injection. Integrator sensitivity constant. Other conditions as Fig. 2.

DISCUSSION

The pyridinium crosslinks are the most specific and sensitive markers of bone resorption presently available. To date their measurement has been limited to urine primarily due to the low nanomolar concentrations in serum and absence of reliable immunoassays.

Robins *et al.* [28] were able to demonstrate the presence of crosslinks in OA serum but only when using gradient RP-HPLC in conjunction with immunoassay. Isocratic RP-HPLC methods increased throughput and by their very nature allow increased sensitivity. Using our published method we were able to demonstrate the presence of pyridinium crosslinks in serum of normal and uraemic patients, however, analyses of normal serum were carried out at the absolute limits of the assay [29]. Recently Lichy *et al.* [30] used cation-exchange chromatography with fluorescence detection, although the maximum sensitivity using this technique was insufficient to allow serum analysis. More sensitive gradient ion-paired RP-HPLC methods [19,20] allow accurate analysis in urine but sensitivity is reduced due to the baseline rise caused by the gradient.

The increases in sensitivity which have allowed the analysis of crosslinks in serum have been achieved by a combination of several improvements to the methodology. Instrumental increases are attributed to the use of narrow-bore HPLC columns, which has decreased on column analyte band dispersion and a higher-sensitivity fluorescence detector. Further increases in sensitivity have been achieved by modifications to the sample preparation procedure. The overall gain in sensitivity, routinely achievable, is approximately 30-fold compared to our previous methodology [21].

Even with these substantial increases in sensitivity OSA and HFBA were found to be inappropriate ion pairs, giving poor resolution of crosslinks from other fluorophores. The use of PFPA, a novel ion pair, enabled the resolution of Pyr from interfering fluorophores in normal serum. Using PFPA the retention and resolution of the pyridinium crosslinks can be sufficiently controlled by altering the molarity of ion pair, and elution can be achieved in reasonable time without the addition of organic modifiers to the mobile phase. Hence more hydrophobic compounds are retained on the column allowing the weakly ion-paired crosslinks to elute.

Standards containing Pyr and dPyr are well resolved, however, in normal serum only Pyr is quantifiable. Even though the Pyr peaks are relatively small they fall within the linearity of the standard curve and C.V.s are low. Pyr is easily detected in OA serum and synovial fluid. In Pagetic serum both Pyr and dPyr are detectable and easily quantified. Further increases in sensitivity through improvements in sample preparation are envisaged which may even permit the assay of free Pyr in serum.

The concentrations of Pyr in OA serum found using the present method are similar to those reported by Robins *et al.* [28]. The present results suggest that Pyr concentrations in OA serum are elevated some three- to four-fold compared with normal controls. Pyridinium crosslink concentrations in Pagetic serum are substantially elevated compared with normal controls and fall significantly following therapeutic intervention. In contrast to Robins *et al.* [28] who reported low to undetectable levels of Pyr in synovial fluid, in the single synovial fluid examined here the level was grossly elevated compared to plasma.

The analysis of serum pyridinium crosslinks offers the potential to diagnose patients with metabolic bone disease, boney metastases and common arthritis-related conditions as well as the evaluation and monitoring of potential therapies. Studies investigating metabolism, renal and hepatic clearance, diurnal and longitudinal variation are to be carried out, as do larger studies using well defined patient groups in order to validate the serum assay of pyridinium crosslinks for routine clinical application. Although diurnal and longitudinal variation may be more pronounced in serum samples compared with those in urine, serum samples are easier for a clinician to obtain and control, also analysis should not have to be corrected for creatinine as is required for random urines. Furthermore as most markers of osteoblast and osteoclast function are assayed in serum, the analysis of crosslinks in serum would allow direct comparison of markers of bone turnover in the same biological matrix and at the same point in time.

With the increased clinical awareness of the value of crosslink measurement a number of new approaches to their assay are being developed in addition to HPLC. Recently immunoassays for serum crosslinked type (I) peptides [31], and those for free pyridinolines [32] and crosslinked peptides [33] in urine have been developed whilst capillary electrophoresis with either fluorescence [34] or less sensitive UV detection [35] is offering a complementary separation technique. It remains to be seen if these new assays can match the sensitivity of the present assay towards the pyridinium crosslinks and be applicable to serum analysis.

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