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Automated on-line solid-phase extraction and high-performance liquid chromatographic analysis of total and free pyridinium crosslinks in serum

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Abstract

An automated on-line solid-phase extraction procedure for free and total pyridinium crosslinks in serum, with HPLC analysis, is described. The pyridinium crosslinks either following hydrolysis in 3 *M* HCl or free in neat serum were extracted using a Gilson Aspec XLi system onto extraction cartridges containing an octylsilane/cation exchanger sorbent. Up to 525 μl of serum could be loaded onto the extraction cartridges. After washing, the crosslinks were eluted with 400 μl of 100 m*M* sodium formate pH 5 and 380 μl of this was concentrated on a RPB guard column eluted with 100 m*M* HFBA. The crosslinks were backflushed and separated on a 5-μm ODS analytical column eluted with 30 m*M* HFBA with 18% MeCN at 1 ml/min and detected by their native fluorescence (excitation 295 nm, emission 400 nm). The use of a high sensitivity fluorescence detector was essential. Recoveries were 95–100% with a limit of detection ($S/N=2$) of 109 pmol/l (p*M*) for pyridinoline (Pyr) and 143 p*M* for deoxypyridinoline (dPyr). The inter-assay R.S.D. was 9% for pyridinoline and 10.8% for deoxypyridinoline. The throughput of the system was up to 50 samples per day. © 1998 Elsevier Science B.V.

Keywords: Sample preparation; Pyridinoline; Deoxypyridinoline

1. Introduction

The pyridinium crosslinks, pyridinoline (Pyr) and deoxypyridinoline (dPyr), are widely regarded as sensitive markers of skeletal collagen degradation as they only form in ''mature'' assimilated skeletal collagens and consequently their presence in biological fluids is assumed to reflect bone resorption [1,2]. While Pyr is ubiquitously distributed throughout skeletal tissue, dPyr is found predominantly in bone and is biochemically a more specific measure of resorption [3]. However Pyr is present at four times the concentration of dPyr and in most clinical situations their urinary concentrations are highly correlated. They are present at nanomolar concentrations in urine and serum and their biochemical characterisation and recent clinical application has been facilitated by their native fluorescence. High-performance liquid chromatography (HPLC) techniques have been widely used for the analysis of pyridinium crosslinks in tissues, although an off-line prefractionation step on cellulose was necessary for the analysis of urine samples [4]. Several HPLC methods have been published. Recently immunoassays for both free and crosslink-containing peptides in urine have become available offering high sample throughput [5]. Automated methods for on-line extraction of urine samples with HPLC analysis have been developed from the manual cellulose procedure [6], using size-exclusion and column-

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switching [7] and recently using commercially available mixed mode solid-phase extraction (SPE) columns [8]. All give increased precision and throughput. However, the widespread clinical application of these urine assays has been hindered by large intra-individual variations of up to 40%, which effectively negates their use on an individual patient basis [9,10].

Intra-individual variations of serum marker of bone formation are considerably lower, approximately 15–20% [11] and consequently the development of automated sensitive serum assays for pyridinium crosslinks and crosslink containing peptides would appear to be a prerequisite for the routine clinical application of these markers. However, unlike urine, where samples can be diluted for analysis, serum samples require concentration, either pre or post extraction, to give a sufficient mass concentration of crosslinks to allow detection. Few HPLC methods have been developed enabling the analysis of pyridinium crosslinks in serum, and none allow on-line extraction and analysis [5]. James et al. [12] using narrow bore columns with high sensitivity fluorometric detection were able to quantitate Pyr in normal subjects, and showed elevated levels of Pyr and dPyr in serum from patients with osteoarthritis and Paget's disease. With minor modifications to this assay Walne et al. [13] were able to quantitate both Pyr and dPyr in as little as 250 μl normal serum. We and others have investigated different sample preparation techniques prior to extraction, including ultrafiltration [14], acid/solvent precipitation of serum proteins [15] and matrix dilution [16]. These improve extraction efficiency, allow more accurate quantitation and enable samples to be concentrated more easily. In the limited studies to date, concentrations of Pyr and dPyr in normal serum are approximately 4 n*M* and 1 n*M*, respectively [13–18].

For improved sensitivity and full assay automation several steps require optimisation, including sample preparation, hydrolysis conditions, sample loading conditions and absolute recovery of analyte through the extraction and analysis procedure. This paper details an automated SPE procedure for serum pyridinium crosslinks, enabling the on-line extraction and analysis of free and total crosslinks in as little as 250 μl of sample, using commercially available SPE cartridges and a Gilson Aspec XLi system.

2. Experimental

2.1. Materials

Heptafluorobutyric acid (HFBA), was obtained from Fluorochem (Old Glossop, UK). All other chemicals and solvent were of HPLC grade and obtained from BDH–Merck (Poole, UK). One ml Isolute Confirm HCX SPE cartridges, packed with 50 mg mixed-mode (octylsilane–benzenesulphonic acid) sorbent were obtained from Jones Chromatography (Hengoed, UK). Centrisart (M_r 20 000) ultrafiltration devices were obtained from Sartorius (Epsom, UK).

2.2. Preparation of Pyr and dPyr standards

Standards were isolated from demineralised human femur using CF11 cellulose partition chromatography and further purified by passage through a preconditioned preparative ODS cartridge. Standards were kindly calibrated against authentic material by Dr. S. Robins (Rowett Research Institute, Aberdeen, UK). Concentrated standards were stored frozen in 10 m*M* HFBA until required. Analytical standards were prepared by dilution in 3 *M* HCl for use with the ASPEC XLi system.

2.3. Preparation of samples for analysis of free and total crosslinks

Blood samples were obtained by venupuncture from healthy laboratory workers, allowed to clot and the serum isolated. Serum ultrafiltrate was used throughout and obtained by inverse centrifugation through 2.5-ml capacity 20 000 M_r cut-off Centrisart I devices at 2500 *g* for 1 h or until sufficient ultrafiltrate was collected. No correction factors were employed.

For determination of total pyridinium crosslinks, serum ultrafiltrate was hydrolysed in 3 *M* HCl (750 μl serum: 250 μl conc. HCl) at 116°C for 20 h, in 4-ml screw top vials sealed with PTFE lined phenolic caps. Hydrolysates were centrifuged at 13 000 *g* for 10 min to remove particulates prior to extraction.

For total serum pyridinium crosslinks, prior to extraction, hydrolysates were diluted 10-fold with 25 m*M* disodium tetraborate, pH 9.3, giving a final pH

of 0.6. For free serum crosslinks, samples were diluted 10-fold with 25 m*M* boric acid, pH 1.5. Urine samples could also be extracted and analysed after appropriate dilution of sample.

2.4. Chromatographic system

The chromatographic system comprised of a Shimadzu LC6A isocratic pump (Shimadzu Europe, Milton Keynes, UK), a Jasco 880 isocratic pump and 920 fluorescence detector (excitation 295 nm, emission 400 nm) (Jasco, Great Dunmow, UK). Samples were processed using a Gilson Aspec XLi equipped with two Rheodyne 7010 valves controlled by Gilson sampler software version 2.00 (Anachem, Luton, UK).

Extracts were first concentrated on a RPB guard column (100×3.2 mm I.D.) (Hichrom, Theale, UK), eluted with 100 m*M* HFBA at 0.45 ml/min. The concentrated analytes were then back flushed onto a 5-μm Techsphere ODS column (150×4.6 mm I.D.) (HPLC Technologies, Macclesfield, UK) eluted with 30 m*M* HFBA containing 18% MeCN at 1 ml/min, for analysis.

2.5. Automated extraction protocol for pyridinium crosslinks

All extraction steps were performed automatically using the Aspec XLi. Samples and standards were extracted sequentially on 1-ml Isolute Confirm HCX SPE cartridges.

SPE cartridges were conditioned with 0.5 ml methanol and 1.5 ml of 5 m*M* sodium formate, pH 2.75, prior to application of dilute acid hydrolysate or sample at 1 ml/min. A further 0.5 ml of 5 m*M* sodium formate was dispensed onto the cartridge. After washing with 2 ml of 5 m*M* sodium formate, pH 2.75, containing 40% (v/v) methanol followed by 2 ml of 5 m*M* sodium formate at 6 ml/min, any residual wash phase was removed by purging the cartridge with air. The retained pyridinium crosslinks were eluted at 0.5 ml/min with 400 μl of 100 m*M* sodium formate adjusted to pH 5 with conc. HCl and collected into tubes containing 40 μl 1 *M* HFBA. Extracts were mixed by repeated aspiration and dispensing and 380 μl injected into a holding loop on Rheodyne valve A. Following a series of valve switches the extract was transferred from the holding loop to valve B where the crosslinks were concentrated on the RPB column prior to back flushing onto the analytical column. The initial valve settings and system configuration is shown in Fig. 1. The full Gilson Aspec programme can be located at the following Internet address: Http://www.mds.qmw.ac.uk/medicine/Aspserum.htm

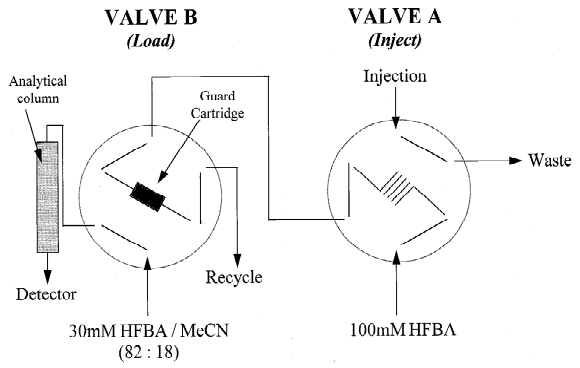

Fig. 1. Initial Rheodyne valve and system configuration.

2.6. Additional requirements for automated extraction of serum pyridinium crosslinks

Trace fluorophores in the HFBA, present to varying levels depending on synthetic batch, made its further purification essential. HFBA (500 g) was glass distilled at 121°C at atmospheric pressure and aliquots were passed through a wetted C_{18} SPE cartridge prior to use. In addition, the concentration column was washed with analytical mobile phase during the extraction procedure so as to prevent any build up of trace fluorophores.

2.7. Data handling and statistical analysis

All chromatographic data was collected using a personal computer based integration system, BORWIN (version 1.21) (Jasco). All statistical analysis was performed using ARCUS PRO STAT (Medical Computing, Cambridge, UK).

3. Results

Due to the low concentration of pyridinium cross-

links in urine and the trace levels in serum several investigations were carried out to maximise sample loading and absolute recovery through the extraction procedure, including sample ultrafiltration, modification of hydrolysis conditions and trace enrichment prior to analysis.

3.1. Hydrolysis conditions

The hydrolysis of samples in 3 *M* HCl enabled more sample to be applied to the SPE cartridge. We found no significant difference between absolute yield of crosslinks measured after hydrolysis in 3 *M* or 6 *M* HCl. In addition we investigated the time course of hydrolysis in 3 *M* acid on yield of pyridinium crosslinks. Aliquots of pooled serum were hydrolysed in 3 *M* HCl at 116°C for up to 22 h and timed samples extracted and analysed for crosslinks. The maximum yield of Pyr and dPyr occurred after 12 h with no deleterious effects on extended hydrolysis.

3.2. Ultrafiltration of serum

The removal of proteins from serum prior to hydrolysis or extraction gives cleaner chromatograms allowing better peak quantitation [14]. Ultrafiltration provided a rapid means of isolating low-molecular-mass molecules and peptides from serum. Kamel et al. [19] found >80% of crosslinks in urine were present either in free form or conjugated to peptides <10 000 molecular mass. We found no significant differences for samples spiked with isolated standard whether filtered using 100 000 or 20 000 molecular mass cut-off devices.

3.3. Elution profile of pyridinium crosslinks from mixed mode SPE cartridges

For high sensitivity assays a balance must be struck between sorbent mass/packing format and final elution volume from the extraction cartridge. We found that 50 mg sorbent mass in a 1-ml column format gave adequate retention with the smallest eluate volume. Under the conditions described, over 90% of Pyr and dPyr standards (958 n*M* and 310 n*M*, respectively) were reproducibly recovered in the first 400 μl elution of the column with relative standard deviations (R.S.D.s) <3% ($n=3$).

3.4. Sample loading conditions

Due to the limited capacity of the sorbent, sample loading capacity was investigated by varying the volume of diluted pool serum hydrolysate added to the SPE column from 2.25 ml to 5.25 ml (i.e., 168.8 to 393.8 μl serum). A linear recovery of Pyr and dPyr was apparent over the range investigated (Pyr: $R^2=0.99$, dPyr: $R^2=0.96$, $n=2$). In addition the sample loading rate onto the SPE column was varied from 0.5 ml/min to 2 ml/min in 0.5-ml steps ($n=2$). With application rates >1 ml/min some loss of sample was noted (14% reduction in peak area for Pyr and dPyr at 2 ml/min when compared with application rates of 0.5 or 1 ml/min). The final sample loading conditions were 4.5 ml of diluted sample at a rate of 1 ml/min.

3.5. Reconcentration conditions

HFBA was used as an ion pair to retain and concentrate the pyridinium crosslinks on the RPB column. Although the HFBA was purified prior to use an additional 2.5-min wash of the concentration column with analytical mobile phase was built into the extraction protocol during the elution of crosslinks from the SPE cartridge, to remove any build up of potentially interfering fluorophores.

3.6. Rheodyne valve transfer conditions

Optimal transfer times of extracted sample from the holding loop on valve A to the concentration column were determined assuming a flow-rate of 0.45 ml/min. Reproducible recovery was noted from 60 to 90 s for Pyr and to 150 s for dPyr before sample breakthrough occurred. A optimised transfer time of 70 s was used throughout and the concentration column then back flushed with analytical mobile phase at a flow-rate of 1 ml/min.

3.7. Linearity and sensitivity

Fluorescent responses were linear for Pyr [2.6–37.2 n*M*, response=3.2 Pyr (n*M*) $r=0.99$] and dPyr

[0.85–12.1 n*M*, response=2 dPyr (n*M*) $r=0.99$] over the range investigated. The limit of detection (LOD) of the automated assay calculated using an extracted hydrolysed serum was 109 p*M* for Pyr and 143 p*M* for dPyr ($S/N=2$) under the conditions described in this paper.

3.8. Recovery of serum pyridinium crosslinks from mixed mode SPE cartridges

Recoveries were determined using serum pools spiked with isolated standard (final concentration Pyr 8.9 n*M*, dPyr 2.9 n*M*). Mean (S.D.) recoveries were 97.1 (2.6)% for Pyr and 98.5 (9.2)% for dPyr ($n=4$).

3.9. Reproducibility

The intra-assay reproducibility of the HPLC analysis alone, determined by direct injection of standards, ranged from 0.99 to 3% (990–110 n*M* Pyr, 320–36 n*M* dPyr). The intra-assay reproducibilities for automated SPE and analysis of total crosslinks in serum were 6.4% for Pyr and 7% for dPyr ($n=4$) and inter-assay reproducibilities were 9% and 10.8%, respectively ($n=10$). The intra-assay reproducibilities for automated SPE and analysis of total crosslinks in urine were 3.6% for Pyr and 4.3% for dPyr ($n=8$) with inter-assay reproducibilities of 3.6% and 6.5%, respectively ($n=10$).

3.10. Sample throughput using the ASPEC extraction technique

The system capacity would allow up to 108 samples to be analysed in a batch, comprising 12 standards, six pooled serum controls and 90 samples. Extraction (25 min) and analysis (20 min) resulted in total assay time for each sample of 45 min. Sequential on-line extraction would allow more than 50 samples to be analysed/24 h.

3.11. Analysis of normal serum

The mean (S.D.) concentration of total Pyr and dPyr in 13 normal serum samples was 2.32 (0.47) and 0.77 (0.16) n*M*, respectively. Mean (S.D.) free Pyr determined in the same samples was 1.12 (0.20) n*M*, hence 49.7 (11.4)% of Pyr was present in the free form. Free dPyr could not be found in normal adult serum. Inter-individual variations were 20.2% for total Pyr was 20.6% for total dPyr and 17.7% for free Pyr. Representative chromatograms of 3 *M* HCl controls, isolated standards, hydrolysed, neat and spiked serum following automated extraction are shown in Fig. 2.

4. Discussion

By optimising the sample preparation and extraction conditions we have been able to develop an automated, sensitive and reproducible on-line SPE method for the analysis of pyridinium crosslinks in normal serum. Conventionally total pyridinium crosslinks have been determined in samples after hydrolysis in 6 *M* HCl [4]. By hydrolysing urine or serum ultrafiltrate in 3 *M* HCl we were able to minimise analyte dilution and load more concentrated samples onto the SPE cartridges without breakthrough [8]. Under the conditions described in this paper approximately 90% of ultrafiltered crosslinks are reproducibly eluted in the first 400 μl elution step. However, due to the trace levels of crosslinks in serum, the further concentration of extracted crosslinks was essential prior to analytical separation. Using trace enrichment methods, 350 μl of extracted sample was concentrated on-line prior to analysis which gave an absolute recovery of 72% for total neat serum or 54% for hydrolysed serum, applied to the SPE cartridge. Further purification of HFBA was essential to remove trace fluorophores which otherwise concentrated on the enrichment cartridge.

Although the recovery of added standards to serum was >95%, values obtained for normal controls were approximately 50% of those found when using the manual cellulose extraction procedure with unfiltered serum [13]. Preliminary investigations comparing acid precipitation of serum proteins with ultrafiltration suggest that a proportion of free and peptidic crosslinks remain bound to serum proteins which may account for this discrepancy.

The extraction procedure itself relies on the cationic and hydrophobic properties of the pyridinium crosslinks, although the exact mechanism by which the crosslinks are retained is complex. With the

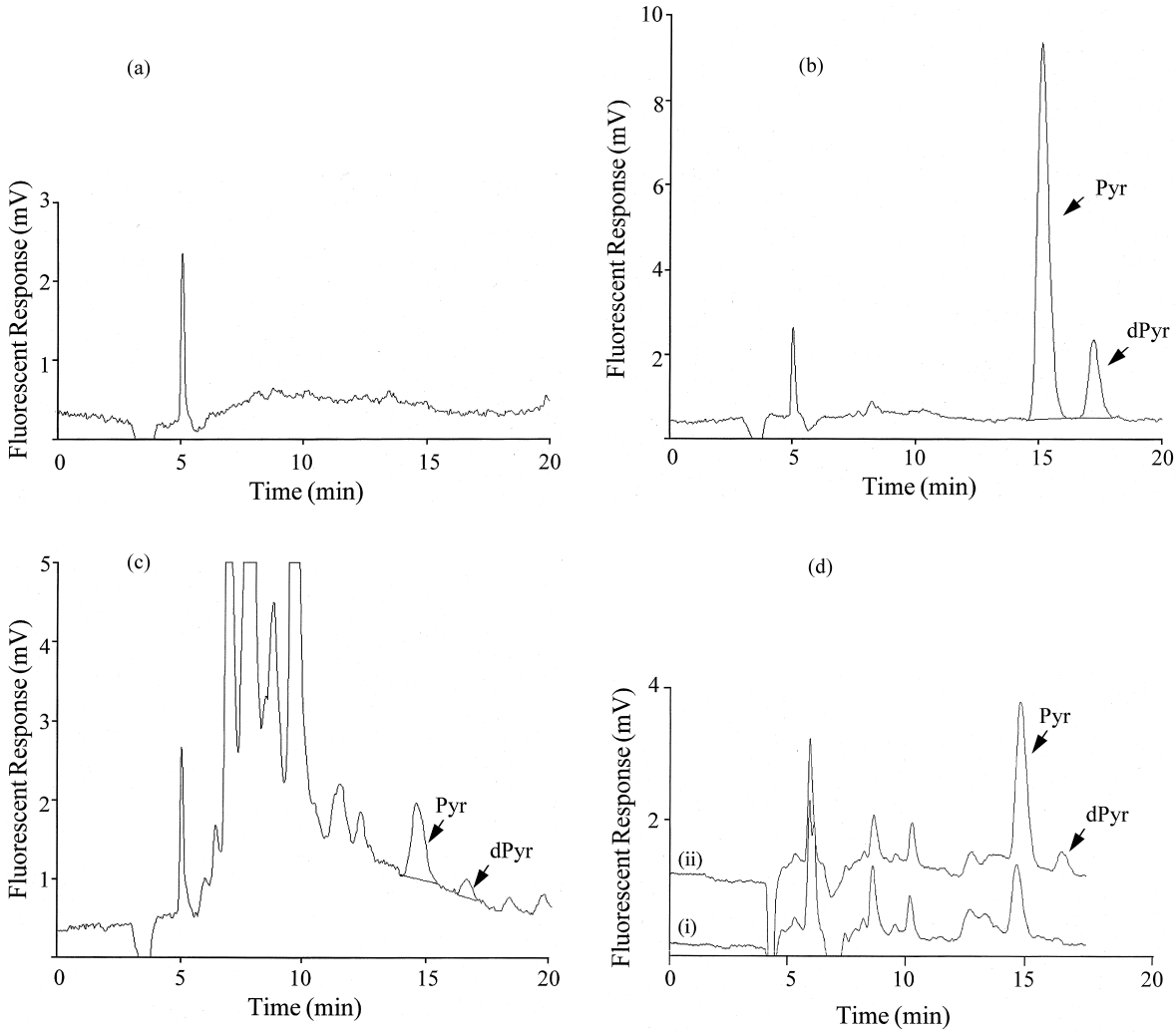

Fig. 2. Isocratic ion-paired reversed-phase HPLC separations of extracted samples. (a) 3 *M* HCl blank, (b) isolated pyridinium crosslink standard (Pyr 8.9 n*M*, dPyr 2.9 n*M*), (c) total serum crosslinks (Pyr 1.4 n*M*, dPyr 0.4 n*M*), (d) (i) free serum crosslinks (0.77 n*M*) and (ii) free serum crosslinks with standard spike. Extraction and analysis conditions as per Sections 2.4–2.6.

loading conditions employed (0.3 *M* HCl), the ionisation of the carboxylic acid groups of the crosslinks and residual silanols of the stationary phase are suppressed. The sulphonic acid residues of the stationary phase together with the positively charged amino groups and ring nitrogen of the crosslinks remain ionised and associate. Following washes to remove weakly ionic species and hydrophobic compounds the crosslinks are eluted by increasing the molarity to 100 m*M* sodium formate and the pH to 5. This causes the carboxylic acid of the crosslinks to ionise, internally neutralising their positive charge and enabling their elution from the extraction cartridge. Using the above conditions the majority of the remaining interfering fluorophores are retained by hydrophobic and ionic interaction.

This method, with its understood extraction mode, enables the measurement of free and total crosslinks in both serum and urine samples and represents a significant improvement over those methods currently available [5]. Sample throughput, assay sensitivity and reproducibility are sufficiently high to allow its use clinically. Few immunoassays have been specifically validated for use in serum [20].

Whether free, peptidic or total crosslinks should be measured remains a contentious issue. Crosslink containing peptides are produced locally at the site of osteoclastic bone resorption [21,22] while free crosslinks appear to derive from further processing of the resorbed collagen peptides in the osteoclast or during their subsequent circulation [16,23]. Recently several studies have highlighted the differential effects of treatment on the ratio of free and peptidic crosslinks in urine [24–26]. Whether similar differences are found in serum remains to be established, although Colwell and Eastell [16] have recently suggested a differential renal processing of peptidic crosslinks in high bone turnover states. Consequently HPLC analysis of serum or urine offers several advantages over immunoassay, primarily that a range of pyridinium crosslinks (Pyr, dPyr and glycosylated Pyr in peptidic or free form) can be separated and detected.

Serum analysis, although a transient measure, offers the potential of lower intra-individual variation with improved clinical differentiation and the comparison with other serum markers of bone formation at the same point in time. In addition sample collection is more reliable and data does not have to be creatinine corrected, hence avoiding the introduction of an additional variable. Preliminary data suggest serum crosslinks to be of clinical utility. Elevated serum crosslinks have been reported in arthritis [27], metastatic bone disease [28] renal failure [15,18,20] malignancy associated hypercalcemia and hyperparathyroidism [17]. Furthermore the administration of recombinant growth hormone (GH) to GH deficient patients caused a significant elevation in serum crosslinks (unpublished observations), whilst treatment of thyrotoxic patients with carbimazole resulted in a significant decrease of serum crosslinks [29]. Further studies are necessary to elucidate their utility in diseases such as osteoporosis where subtle changes in bone metabolism have significant long term effects on bone integrity. The availability of such specific high sensitivity assays are essential to fully evaluate these promising serum markers of bone resorption.

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