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RAPID ASSAY FOR HARD TISSUE COLLAGEN CROSS-LINKS USING ISOCRATIC ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

Following a detailed study, a rapid and sensitive assay for the naturally fluorescent collagen cross-links pyridinoline and deoxypyridinoline has been developed using ion-pair reversed-phase high-performance liquid chromatography in the presence of 1-octanesulphonic acid (OSA). Pyridinoline and deoxypyridinoline were separated on an Exsil 100 ODS, 5-μm column (100 mm $\times$ 4.6 mm I.D.) using 25 m*M* sodium formate, 5 m*M* OSA and 1 m*M* ethylenediaminetetraacetic acid adjusted to pH 3.25, containing 20% (v/v) methanol. The mobile phase flow-rate was 1.5 ml/min. Compounds were detected by their natural fluorescence (xenon lamp; excitation wavelength 290 nm, emission wavelength 400 nm). Peak areas were linear to 25 pmol injected for pyridinoline and 20 pmol injected for deoxypyridinoline ($r = 0.99$). Intra-assay coefficients of variation for urinary extracts were 7.65 and 9.07% ($n = 10$), respectively. Limit of detection (signal-to-noise ratio = 5) was 200 fmol injected. Quantification of the cross-links in acid hydrolysates and human urine samples was possible in under 15 min.

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INTRODUCTION

The collagen matrices of most connective tissues are held together by small non-reducible trifunctional cross-linking moieties. In hard connective tissues such as cartilage, tendon and bone, the predominant cross-links are the naturally fluorescent pyridinium compounds, pyridinoline and deoxypyridinoline (M_r 429 and 413). Pyridinoline is widely distributed in hard tissues with high concentrations in cartilage whereas deoxypyridinoline appears to be specifically found in bone and dentine [1]. Interest has been shown in using these compounds as in vivo measures of mature bone and cartilage degradation, with potential application in the diagnosis and monitoring of osteoporosis, osteo arthritis and rheumatoid arthritis [2–6]. The cross-links are found in free and conjugated forms in urine at total levels of approximately 1 per 15 000 amino acid residues in urine. Due to the low levels of free cross-links, total levels are estimated post acid hydrolysis. Sensitive and specific methods for analysis are therefore necessary.

Several methods have been used for the quantification of pyridinoline. Originally pyridinoline was detected using amino acid analysis with ninhydrin [7]. Assay procedures were subsequently developed using molecular sieve [8] and ion-exchange chromatography [9]. An enzyme-linked immunosorbent assay (ELISA) for desmosines, elastin cross-links, was shown to be up to 30% cross-reactive with purified pyridinoline [10]. Robins [11] developed an ELISA for pyridinoline, which was later shown to have virtually no cross-reactivity with deoxypyridinoline.

Reversed-phase high-performance liquid chromatographic (HPLC) methods have been developed to quantify pyridinoline, using fluorometric detection [1,12]. Pyridinoline and deoxypyridinoline are highly polarised compounds at acid pH values and are poorly retained on octadecylsilane (ODS) reversed-phase columns. Published methods overcome this by using ion-pairing agents such as 1-octanesulphonic acid (OSA) [12] and N-heptafluorobutyric acid (HFBA) [1] to retain and resolve the analogues. All the methods have involved the use of gradient elution, and the solvents and ion-pair concentration have varied even in successive studies by the same group. However, only one [12] describes a method capable of resolving deoxypyridinoline from pyridinoline allowing the simultaneous quantification of both cross-links.

The possibility of increasing the specificity and sensitivity of an HPLC assay by using electrochemical detection was not applicable in this instance as the redox potentials of the 3-hydroxypyridine ring of pyridinoline were near anodic and cathodic limit potentials [13].

Gradient systems in general give a relatively low throughput of samples and are inappropriate for such an apparently simple separation as pyridinoline and deoxypyridinoline in routine clinical laboratories. There are other drawbacks with gradient methods: (i) equipment costs and complications are increased;

(ii) changes in ion-pair distribution with gradients and column washes necessitate re-equilibration; (iii) mobile phase is not recyclable.

We have partially purified and investigated in detail the chromatography of these compounds with a view to producing a rapid isocratic assay applicable to large-scale clinical analysis of cross-links in urine samples.

EXPERIMENTAL

Gradient HPLC

The chromatographic system consisted of two LKB 2150 pumps, an LKB 2152 gradient controller (LKB-Pharmacia, Milton Keynes, U.K.) and a Shimadzu RF 535 dual-monochromator fluorometer (xenon lamp; excitation wavelength 290 nm, emission wavelength 400 nm) (Dyson Instruments, Hetton, U.K.). A stainless-steel column (100 mm×4.6 mm I.D.) was packed in house with 5-μm ODS-Hypersil (Shandon Southern Products, Runcorn, U.K.). Samples were loaded using a Rheodyne 7125 injection valve with a 20-μl loop (Anachem, Luton, U.K.). Output was recorded using a 0–10 mV potentiometric recorder.

Isocratic HPLC

The chromatographic system consisted of an ACS 350/04 quaternary gradient module (Applied Chromatography Systems, Macclesfield, U.K.), a Shimadzu RF 535 dual-monochromator fluorometer (xenon lamp; excitation wavelength 290 nm, emission wavelength 400 nm) (Dyson Instruments) and an Exsil 100, 5-μm ODS column (100 mm×4.6 mm, I.D.) (Chromtech, Wrexham, U.K.). Samples were loaded using a Rheodyne 7125 injection valve with a 20-μl loop (Anachem). Output was recorded using a 0–10 mV potentiometric recorder or integrated using a Midas 22 computer-based integration system (Comus Instruments, Hull, U.K.).

Chemicals

Ammonium acetate, ammonium chloride, hydrochloric acid and ethylenediaminetetraacetic acid (EDTA), all analytical reagent grade, and sodium formate, OSA and methanol, all HPLC grade, were obtained from BDH (Poole, U.K.). Powdered bovine articular cartilage was obtained from Sigma (Poole, U.K.). Water was glass-distilled and deionised prior to use. Whatman CF 1 and CF 11 cellulose powders were obtained from Jones Chromatography (Hengoed, U.K.). Sodium cellulose phosphate was obtained from 3M, Riker Labs. (Loughborough, U.K.).

Mobile phases

Isocratic system. The mobile phase consisted of 25 m*M* sodium formate, 5 m*M* OSA and 1 m*M* EDTA, adjusted to pH 3.25 with 6 *M* hydrochloric acid, plus 20% (v/v) methanol. The flow-rate was 1.5 ml/min. Eluents were recycled for up to one week.

Binary gradient system. Solvent A was 20 m*M* ammonium chloride, pH 3.50, containing 5 m*M* OSA. Solvent B was 5 m*M* ammonium chloride, containing 5 m*M* OSA adjusted to pH 3.50, plus 75% (v/v) acetonitrile. The gradient used was linear over 30 min (10% B to 40% B), then increased to 70% B for 15 min before returning to 10% B to allow the column to re-equilibrate with ion pair. The flow-rate was 1 ml/min.

Preparation of pyridinoline and deoxypyridinoline standards

Standard material was prepared from two sources: bovine articular cartilage which yielded predominantly pyridinoline and post-mortem human tibia which yielded both pyridinoline and deoxypyridinoline. The methods of isolation were modified from those of Black et al. [12].

Preparation of pyridinoline from bovine articular cartilage

Bovine articular cartilage (140 g) was stirred in 1.5 l EDTA (0.5 *M*) for a week at 4 °C. After freeze-drying, the cartilage was refluxed in 6 *M* hydrochloric acid for 16 h at 116 °C. The volume was reduced by distillation to 500 ml. This hydrolysate was mixed with 500 ml glacial acetic acid, a slurry of 50 g of CF 11 cellulose powder in 500 ml of butanolic eluent consisting of butan-1-ol–glacial acetic acid–water (4:1:1) and 2 l of butan-1-ol and stirred at room temperature for 30 min. The mixture was filtered and washed with 5 l of butanolic eluent to remove the bulk of the fluorescent hydrolysed components. Pyridinoline was eluted with distilled, deionised water (150 ml). The aqueous fraction was freeze-dried and resuspended in 10 ml of 0.05 *M* hydrochloric acid.

Pyridinoline was further purified using sodium cellulose phosphate powder. The sodium cellulose phosphate (5 g) was swollen in distilled water (20 ml) and added to a 20-ml syringe barrel stoppered with glass wool to a depth of 5 cm. The column was washed with 10 ml of 1 m*M* hydrochloric acid prior to adding 1 ml of sample at pH >4. The column was washed with increasing concentrations of hydrochloric acid. Fluorescence was monitored using an Aminco Bowman fluorometer (excitation wavelength 330 nm, emission wavelength 425 nm). Pyridinoline was eluted with 10 ml of 6 *M* hydrochloric acid.

Preparation of pyridinoline and deoxypyridinoline from human tibia

Post-mortem human tibia was decalcified in 8% (v/v) formic acid in 10% (v/v) formal saline for several weeks. After freeze-drying, 112 g of bone were refluxed in 1500 ml of 6 *M* hydrochloric acid for 16 h at 116 °C. The volume was reduced by distillation to 500 ml. This hydrolysate was mixed with 500 ml

glacial acetic acid, a slurry of 50 g of CF 11 cellulose powder in 500 ml of butanolic eluent and 2 l of butan-1-ol and stirred at room temperature for 30 min. The mixture was filtered and washed with 5 l of butanolic eluent to remove the bulk of the fluorescent hydrolysis components. Pyridinoline and deoxypyridinoline were eluted with 1 l of distilled deionised water. The aqueous fraction was freeze-dried and resuspended in 10 ml of 0.05 *M* hydrochloric acid.

These prepared standards were kindly quantified by Dr S.P. Robins against authentic material using a described gradient method [12].

Quantification of cross-links in urine

Aliquots of urine (1 ml) were hydrolysed in an equal volume of concentrated hydrochloric acid at 116°C for 16 h in sealed ampoules. Hydrolysates (0.5 ml) were mixed with 0.5 ml of glacial acetic acid, 0.5 ml of CF 1 cellulose in butanolic eluent and 2 ml of butan-1-ol prior to application to the column. Columns were prepared from 5-ml Eppendorf combitips stoppered with glass wool and filled to a level of 3 cm with 50% (w/v) CF 1 cellulose in butanolic eluent. Samples were loaded onto the column and washed with 10 ml of butanolic eluent. The cross-links were eluted with 5 ml of distilled water and collected in a conical centrifuge tube. After centrifugation (2000 *g*, 5 min) the lower aqueous layer containing the cross links-was carefully removed with a pasteur pipette and freeze-dried. The samples were resuspended in 200 μl of 0.05 *M* hydrochloric acid (pH 3) and filtered using a Zetapor 0.2-μm syringe filter (Anachem). Analyses were carried out with 20-μl injections.

RESULTS

All experiments were carried out with a mobile phase of 25 m*M* sodium formate, 1 m*M* EDTA and 5 m*M* OSA, pH 3.25, plus 20% (v/v) methanol at a flow-rate of 1 ml/min unless otherwise indicated.

Effect of OSA concentration on k' and resolution of pyridinoline and deoxypyridinoline

Experiments were carried out to determine the effect of OSA concentration on the retention during reversed-phase chromatography of pyridinoline and deoxypyridinoline. The concentration of OSA was varied from 0 to 7.5 m*M*. Prior to each experiment the column was equilibrated with OSA by pumping at 1 ml/min for 1 h.

The cross-links co-eluted frontally with concentrations of ion pair below 2 m*M*. Increasing the OSA molarity further resulted in increased retention (k') and resolution of deoxypyridinoline from pyridinoline (Fig. 1).

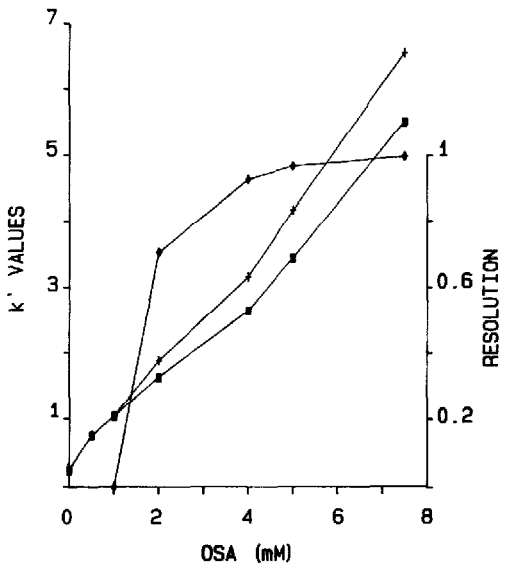

Fig. 1 Effect of octanesulphonic acid concentration (OSA) on resolution (♦) and k' of deoxypyridinoline (+) and pyridinoline (■). Conditions as described in Experimental.

Effect of pH on ion pairing

The pH of aqueous buffer was varied between 3.50 and 2.75 by the addition of 6 *M* hydrochloric acid. As expected, the ion pairing of pyridinoline and deoxypyridinoline was pH-dependent. At pH 3.50, pyridinoline and deoxypyridinoline were retained and almost totally resolved (R_s=0.95). With decreasing pH, retention increased and resolution was total (Fig. 2). Lowering the pH to 2.75 retained the standards excessively. Decreasing the pH from 3.50 to 2.75 was accompanied by a decrease in peak height, but the fluorescent response (peak area) was constant for both compounds.

Effect of buffering salts

The effect of buffering salts on the chromatography was investigated in the presence of 5 m*M* OSA adjusted to pH 3 and 20% (v/v) methanol. In general, ammonium salts such as acetate and formate were effective counter-ions, and for a good separation of the cross-links low-molarity buffers (5 m*M*) were required but these are disadvantageous when working with biological extracts. Sodium salts such as citrate and formate are less effective counter-ions. Sodium formate (25 m*M*) could be used without deleteriously effecting the separation. Trisodium citrate, as expected, had greater eluting ability than sodium formate, but still resolved the cross-links (R_s 0.6) compared with similar molarities of ammonium formate (R_s 0). Sodium formate (25 m*M*) was chosen

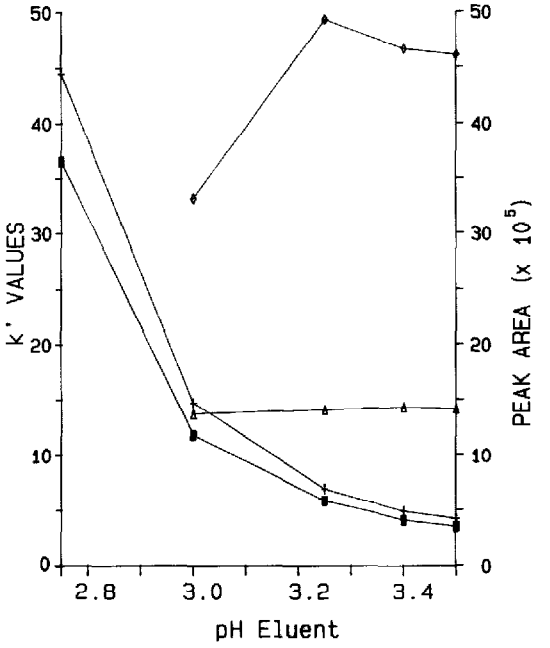

Fig. 2. Effect of pH on k' and peak area of pyridinoline (■,◇) and deoxypyridinoline (+,△) standards from human tibia. Conditions as described in Experimental.

because of its good buffering capacity and, as it is relatively volatile, its applicability to work with mass spectrometry.

Effect of EDTA

The EDTA concentration was varied between 0 and 5 m*M*. As little as 0.25 m*M* EDTA had a marked effect on the chromatography. There was a sharp decrease in the retention (k') of both pyridinoline and deoxypyridinoline on the column; however, resolution of deoxypyridinoline from pyridinoline was increased due to decrease in peak broadening (Fig. 3). Increasing the EDTA concentration to 5 m*M* had little further effect on resolution but reduces retention of the cross-links.

Excitation and emission spectra of pyridinoline and deoxypyridinoline

Since fluorescence spectra are lamp-, pH- and buffer-dependent, the excitation spectra were determined with emission set at 400 nm and emission spectra with excitation at 290 nm. Excitation and emission spectra were obtained by adjusting respective monochromators in 5–10 nm steps either side of published excitation and emission maxima [1,12].

Maximum excitation was 290 nm and emission 400 nm (Fig. 4). Results are expressed relative to wavelength of maximum fluorescence response (peak

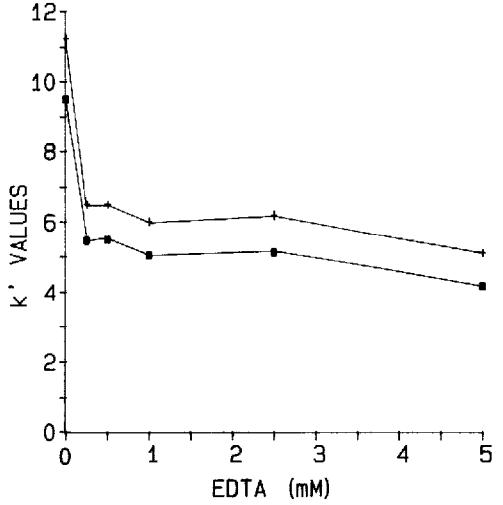

Fig. 3. Effect of EDTA on k' of pyridinoline (■) and deoxypyridinoline (+). Conditions as described in Experimental.

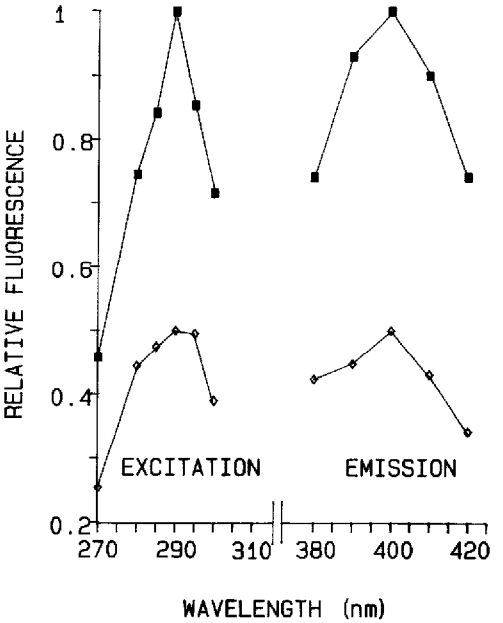

Fig. 4. Excitation and emission spectra of pyridinoline (■) from bovine articular cartilage and deoxypyridinoline (◇) from human tibia in column eluent. Conditions as described in Experimental.

area) and offset to clarify individual spectra of pyridinoline and deoxypyridinoline.

Other effects

Changes in molarity of sodium formate or methanol concentration lead to the expected variations in retention and resolution of pyridinoline and deoxypyridinoline without effecting column selectivity. Methanol was used at a concentration of 20% (v/v) in the system as it gave reasonable retention as well as speeding up the elution of normally frontally eluting fluorescent com-

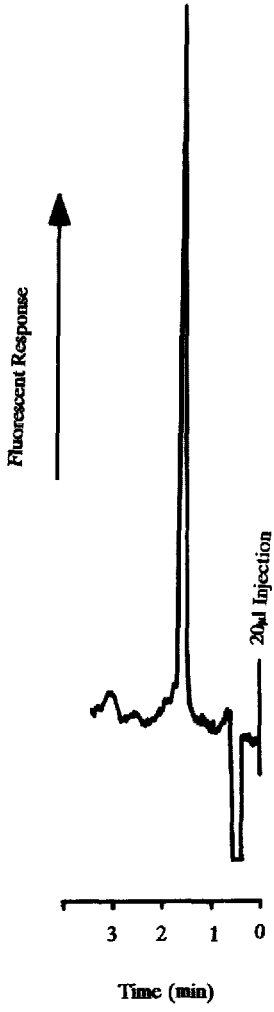

Fig. 5. Chromatogram of pyridinoline standard from bovine articular cartilage Conditions as described in Experimental. Detector setting, 4× (high sensitivity).

pounds. All investigations were carried out at room temperature, and the column eluent was not cooled [12]. There were no changes in column selectivity when columns from different manufacturers such as Nucleosil, Techsphere, ODS-Hypersil and Spherisorb were used. Resolution was improved with 3-μm material but at the expense of back-pressure. In general, increases in flow-rate decreased both retention times and resolution.

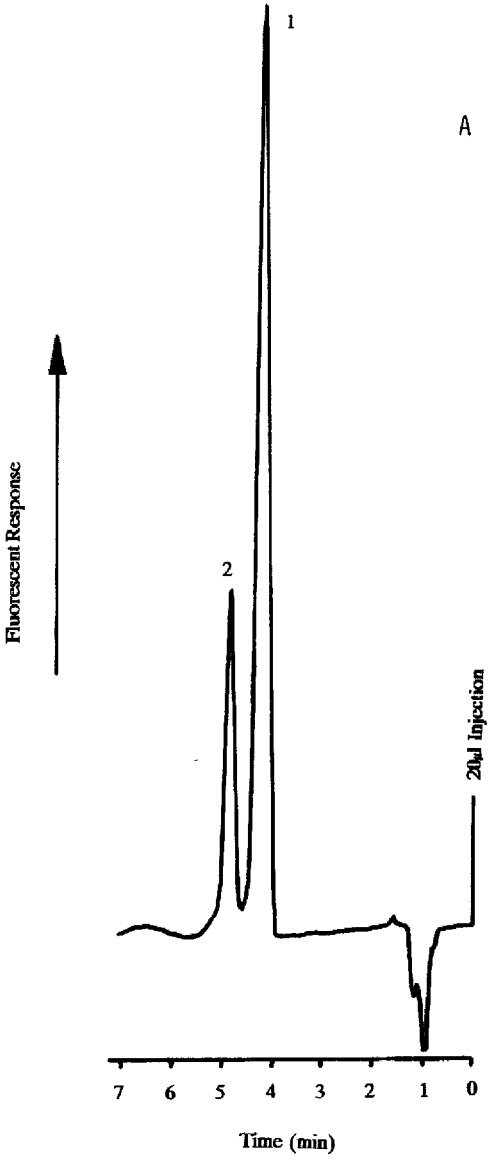

Fig. 6.

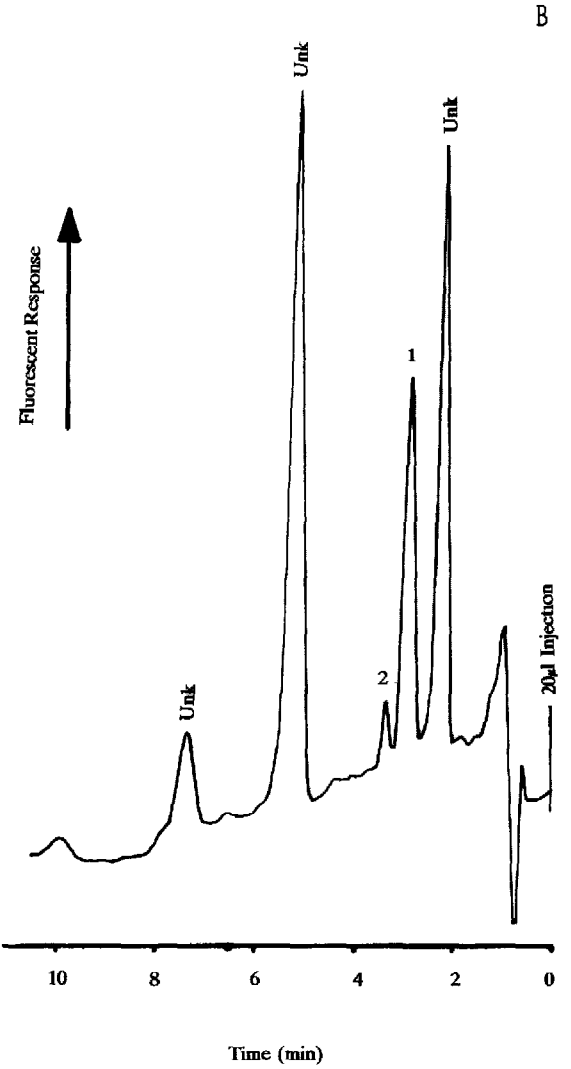

Fig. 6. Chromatograms of (A) pyridinoline and deoxypyridinoline standards from human tibia (detector setting, 16×, high sensitivity) and (B) pooled normal urine sample (pyridinoline and deoxypyridinoline are shown at levels of 5.04 and 0.92 pmol injected, respectively; detector setting 4×, high sensitivity). Other conditions as described in Experimental Peaks: 1 = pyridinoline; 2 = deoxypyridinoline.

All developmental work was carried out with both standards and pooled normal urine samples. An optimised chromatographic separation was devised that would provide a rapid, specific and sensitive isocratic assay with sufficient retention to avoid the more frontally eluting components found in urine and providing high resolution of deoxypyridinoline from pyridinoline allowing quantification of both in urine samples.

Final conditions

The mobile phase chosen consisted of 25 m*M* sodium formate, 5 m*M* OSA and 1 m*M* EDTA, adjusted to pH 3.25 with 6 *M* hydrochloric acid, plus 20% (v/v) methanol. The flow-rate was 1.5 ml/min. Excitation wavelength was 290 nm with emission at 400 nm (xenon lamp). Limit of detection (signal-to-noise ratio = 5) was 200 fmol injected (Fig. 5). The fluorescent response (integrated peak area) was linear to 25 pmol injected for pyridinoline and 20 pmol injected for deoxypyridinoline ($r = 0.99$). Chromatograms of standards prepared from human tibia and a typical urine sample are shown in Fig. 6. By varying the flow-rate, up to ten tissue hydrolysates could be analysed per hour, but because of frontally and late-eluting unknowns in urinary hydrolysate extracts, only four samples per hour could be analysed. Attempts to identify the unknown peaks against other naturally fluorescent compounds such as tryptophan, tyrosine and phenyl alanine have so far proved fruitless.

Reproducibility of the assay

Repeated injections of pyridinoline (bovine articular cartilage) and deoxypyridinoline (human tibia) standards gave coefficients of variation of 1.6 and 2.6%, respectively ($n = 10$). A pooled normal urine sample was repeatedly assayed for pyridinoline and deoxypyridinoline as described in Experimental. The mean ($\pm$S.D.) values were 2.25 ± 0.17 pmol injected for pyridinoline and 0.59 ± 0.05 pmol injected for deoxypyridinoline. Using ten aliquots of pooled urine, the intra-assay coefficients of variation were 7.65 and 9.07%, respectively. Still no suitable internal standard is available.

DISCUSSION

Urinary levels of hydroxyproline and hydroxylysine corrected for creatinine have been used as markers of in vivo collagen turnover in the monitoring conditions of increased bone turnover, such as the effect of weightlessness during space travel [14]. However, the technique is not specific due to the release of hydroxyproline during collagen synthesis and both hydroxyproline and hydroxylysine from the C1q component of complement [15]. As pyridinoline and deoxypyridinoline are present only in mature collagen, their concentration in urine provides a specific measure of degradation of insoluble collagen without interference from precursors or other proteins.

The relatively low levels present in urine have necessitated the use of highly specific and sensitive means of detection to allow quantification. Originally, pyridinoline was detected using cation-exchange chromatography with ninhydrin detection. Using an amino acid analyser without derivatisation it is possible to resolve both pyridinoline from deoxypyridinoline, monitoring for natural fluorescence (mercury lamp; excitation wavelength 365 nm, emission wavelength > 400 nm) (unpublished observation). However, when derivatised

with o-phthalaldehyde–3-mercaptopropionic acid [16], fluorescence is lost possibly due to interference with fluorophore formation or quenching of natural fluorescence. Reversed-phase HPLC has been used to separate these cross-links and their natural fluorescence has enhanced selectivity and sensitivity of detection [1,12]. Analysis of hydrolysed urine samples using reversed-phase columns with fluorescence detection reveals a host of polar frontally eluting compounds with similar fluorescence characteristics. Fractionation of the urine is essential both to remove interfering fluorophores and for rapid analysis. To date we have not been able to improve on the partition chromatography clean up of Black et al. [12].

Anionic ion-pairing agents such as OSA and HFBA have been used at acidic pH values to retain and resolve pyridinoline and deoxypyridinoline from any remaining frontally eluting compounds in urine. The ion pairing of the cross-links is pH-dependent, retention and resolution increasing as the pH becomes more acidic and the cross-links more strongly ionised. There was no marked effect of pH on fluorescence response (peak area) for pyridinoline or deoxypyridinoline.

All published HPLC systems have used gradient elution with ion pairing to retain and resolve the cross-links. In addition samples have been injected in excess ion pair, which was claimed to improve reproducibility [12]. We found no benefit and some disadvantages in injecting samples in this manner, since it disturbed ion-pair equilibrium in the column and prevented buffer recycling.

Isocratic analysis reduces costs and increases throughput, as re-equilibration of column with ion pair is not necessary. The column could be washed as required to remove retained compounds. The marked effect EDTA on k' values and resolution has not been previously reported. This was probably due to the suppression of metal ion interactions with pyridinoline and deoxypyridinoline and reduction in ion exchange with surface silanol groups of the packing material.

The fluorescence response (peak area) of pyridinoline prepared from human tibia, unlike that from bovine articular cartilage, decreased markedly with increasing acidity of eluent. This response was due to the resolution of an unknown compound from pyridinoline. The compound could not be detected using the gradient system described in Experimental, as shown in Fig. 7. Peak-area ratios for pyridinoline and deoxypyridinoline were 4:1. Using optimum chromatographic conditions ($R_s = 0.93$), pyridinoline/unknown/deoxypyridinoline area ratios were 5:3:2. Excitation and emission data showed similar spectra for all three compounds. Investigations are proceeding to isolate and characterise this compound.

In conclusion, a rapid and sensitive isocratic system has been developed which is clinically applicable and capable of resolving and quantifying both deoxypyridinoline and pyridinoline in under 15 min.

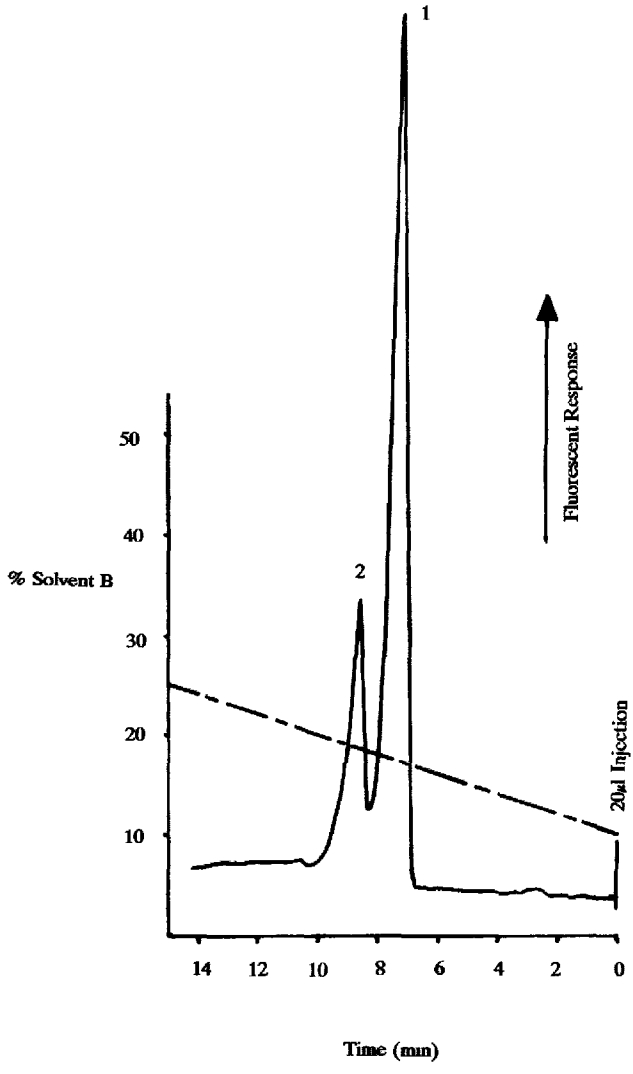

Fig. 7. Chromatogram of gradient analysis of pyridinoline (1) and deoxypyridinoline (2) standards from human tibia. Detector setting, 16× (high sensitivity). Other conditions as described in Experimental.

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