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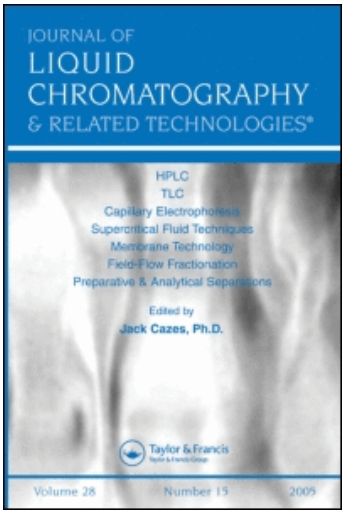

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ION EXCHANGE HPLC DETERMINATION OF PYRIDINIUM CROSSLINKS IN URINE AS MARKERS OF BONE RESORPTION

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ABSTRACT

The aim of this study was to improve the published method[1] for rapid and sensitive determination of the urinary pyridinoline as well as deoxypyridinoline, i.e. the crosslinking elements of bone and cartilage collagen. This method is based on the HPLC analysis of previously prepared urinary hydrolysates. Urine hydrolysate (6 N HCl, 110°C, 16 hours) was purified by selective fractionation using washing with a mixture of water -acetic acid-butanol (1-1-4 by volume) on small disposable columns filled with microgranular cellulose. Liquid chromatography was based on ion-exchange counteractions and was properly performed with the use of a strong cation exchanger as a stationary phase and with a buffered solution at pH=3.35 as the mobile phase, by keeping the oven temperature at 48°C and with the injection of a volume of 10 µl. The time desired for HPLC determination alone didn't exceed 15 min and by using a fluorescence detector (Shimadzu RF 535) set at 297 and 400 nm for excitation and emmision wavelengths, respectively, it reached a sensitivity of about 200 femtomoles for both the crosslinks.

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The method developed was experimentally tried in testing excretion levels of both markers of collagen tissue breakdown in different groups of subjects. The results obtained are in high correlation with the assumed conditions in the investigated groups and, therefore, this procedure seems to be very useful and effective as a sensitive indication of bone mass resorption processes.

INTRODUCTION

Collagen is the major structural protein in the human body and the crucial protein of bone. Its molecular chains are formed intracellularly in osteoblasts, where, among others, triple helical structure originates and partial hydroxylation of lysine and proline occurs. Procollagen molecules subsequently leave intracellular space, their N- and C- terminal propeptides are cleaved and fibril formation can be initiated. It may be noted, that non-collagenous structure of collagen propeptides prevent arranging in fibriles inside osteoblasts on the one hand, while on the other hand, the intracellular enzymatic hydroxylation of some lysine residues is an important step for covalent binding of mature collagen molecules in the extracellular matrix. Nevertheless, once synthetized and excreted from osteoblasts, procollagen molecules are, after propeptide cleavage, aggregated into fibrils, later covalently bound, and thus an extracellular matrix is organized.[2] The inherent strength and structural stability of bone tissue produced in such a manner[3] is stabilised e.g. by hydrogen bonds, hydrophobic interactions, and, above all, by lysinonorleucine and pyridinoline (nonreducible) covalent inter- and intra-molecular crosslinks between adjacent α-chains.

Hydroxylysylpyridinoline (pyridinoline, PD) and lysylpyridinoline (deoxypyridinoline, DPD) are derived from three hydroxylysine or two hydroxylysine and one lysine residue, respectively, following a cascade of enzymic and nonenzymic reactions (Fig. 1)[4,5] and both originate exclusively in mature collagen tissues.[6] It is believed that, whereas, the major crosslink PD is widely distributed in almost all collagen tissues,[3,7] its minor analogue DPD is found practically only in bone and dentin. Due to the slow metabolism of dentin, the latter is considered as bone specific, where the ratio of PD to DPD is about 3.5 to 1.[8,9] In the course of life there is a continual connective tissue turnover, so that osteoblasts activity is responsible for bone formation, whereas, osteoclasts affect bone matrix resorption. If one of these processes predominates, some disorder in bone turnover may occur. In such a case, it is very important to monitor concentrations of bone breakdown markers.

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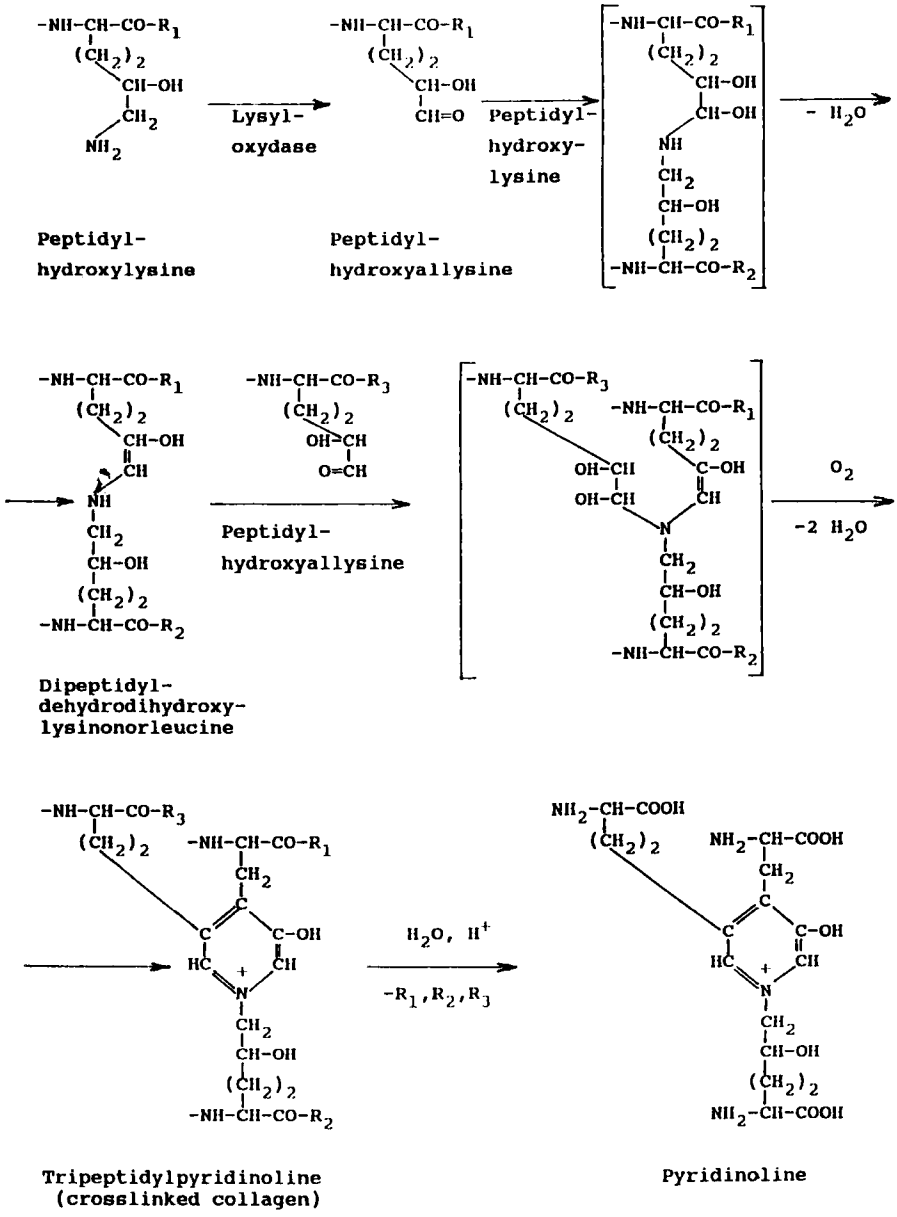

Figure 1. Possible mechanism of pyridinoline formation (schematically).

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For such purposes urinary hydroxyproline determination is often used, but this determination is not specific enough as a bone collagen catabolism marker. Moreover, its urinary level depends on the diet and the presence of C1q and it is of a relatively lower sensitivity, especially in "slow losers" (only a slightly increased bone breakdown). This, as well as the possibility of its catabolism,[10-12] are the reasons why pyridinium crosslinks PD and DPD became still more useful in bone resorption monitoring, particularly in connection with some bone disorders such as osteoporosis, osteoarthrosis, Paget's disease, bone malignancies, etc. Both the markers are present in urine, partially in free form, partially bonded in various molecular weigh peptides.[13] Both are naturally fluorescent, enabling their sensitive detection, are unaffected by diet[14] and are not further catabolised.[15] However, due to their low levels in serum, clinical analyses are practically limited only to urine, i.e. UPD and UDPD determination.

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Contemporarily, the most utilised methods for measurement of the pyridinium crosslinks concentration are based on ELISA, e.g.[13] as well as on HPLC methods. The ELISA method is very rapid and sensitive, but its selectivity is probably not sufficient each time.[16] HPLC is mostly employed in the well known, reverse phase modification, in the presence of ion-pairing agents, both with gradient[17] or (predominantly) with isocratic elution[18-20] as well. It seems, that ELISA can be very useful in screening the potentially at risk patient groups. But, for serious treatment of the selected subjects it is better to apply determination by the HPLC (which actually happens, as can be observed from the literature). The aim of this study is to improve the HPLC method for UPD determination,[1] previously published by our lab. It was based on ion-exchange chromatography (both crosslinks are special aminoacids). We wish to extend it for simultaneous measurement of both UPD and UDPD, apply it to selected groups of patients and to compare these results with the results of healthy subjects.

MATERIALS AND METHODS

Chemicals

Due to the fact, that both PD and DPD are not commercially available on the market, very often their isolation and purification is necessary, e.g. from cartilage.[3] In our case, both species are the kind gift of S.P. Robins, which enabled us to compare purity and verified the identity of our own prepared standards. n-butanol was partially obtained in HPLC grade from Fluka (Buchs, Switzerland), and partially together with other chemicals and solvents

from Lachema (Brno, Czech Republic). All were analytical reagent grade. Microgranular cellulose CC31 was purchased from Sigma-Aldrich (Prague, Czech Republic).

Patients and Samples

The utility of the modified and later described method was tested with fast urine samples in five different groups of subjects: 1) 28 controls (mean age 42), 2) 94 postmenopausal osteoporotic women (mean age 55), whose basal UPD and UDPD values were obtained before calcitonin therapy (Calsynar, Rhone Poulenc-Rorer). First control measurements were performed after 6 months of therapy and the second after 3 months, following the end of therapy. 3) 32 adolescents of both sexes (mean age 18), 4) 52 hemodialysed patients with chronic renal failure (mean age 51) and 5) 28 patients with blood malignancy (mean age 52). Urine samples were stored at -20°C in darkness until their later treatment (usually about 1 week), and it was found,[21] that under these conditions the tested stability of both crosslinks was 6 weeks and the extrapolated stability one can expect was about 25 years, respectively.

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Sample Preparation

Aliquots of 2 mL urinary samples (Fig.2) were hydrolysed with an equal volume of the concentrated HCl (36% by weigh) at 110°C for 16 hours under N_2 atmosphere. 0.5 mL of each, thus obtained hydrolysate was mixed with 2.5 mL of n-butanol - acetic acid mixture (4:1 by volume) and loaded into a disposable column filled with 1 mL (volume of the sediment) of CC31 microgranular cellulose, which was fixed in the equipment with individually controled solvent flow (SPE Vacuum Manifold, Supelco SA, Gland, Switzerland).

Each of of the columns were then slowly rinsed with 12 mL of the mixture of n-butanol - water - acetic acid (4-1-1 by volume) to remove most of balast substances (especially hydrochlorides of amino acids, which are in these conditions reversibly sorbed), after which partially purified pyridinium crosslinks (irreversibly absorbed at these conditions) were at the very end eluted from the cellulose by 3 mL of deionised water (see Fig.2). The later (water) fractions were than desiccated in the vacuum rotatory evaporator SpeedVac (Philadelphia, USA) to dryness and reconstituted in 0.2 mL of the HPLC mobile phase before loading into the HPLC column.

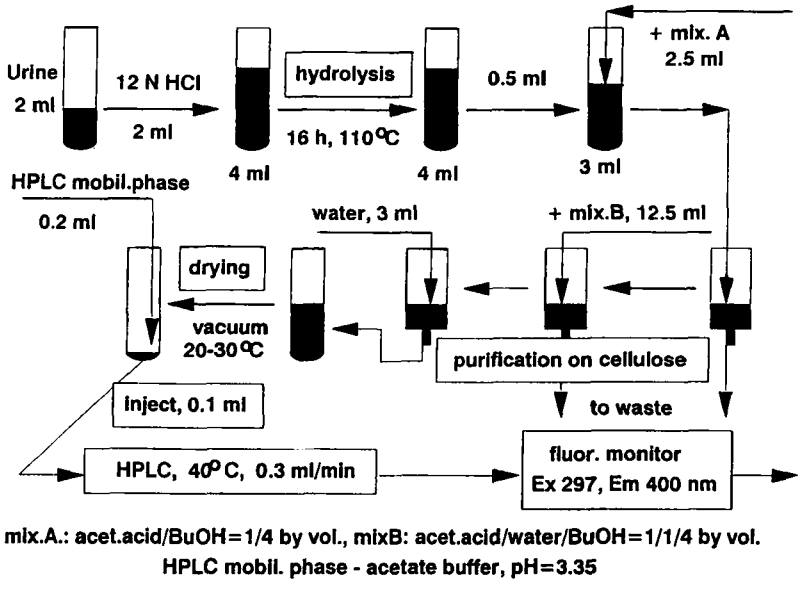

Figure 2. Overview of urine sample preparation.

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Apparatus

A Spectra-Physics HPLC liquid chromatograph (SP 8100) with autosampler (SP 8110) was equipped with a Spectra-Physics computing integrator Chrom-Jet (SP 4400) and connected with a fluorescence monitor (Shimadzu RF 535).

HPLC

Before each chromatography series (mostly at the beginning of a day) a standard sample (pooled and well stored sample) was applied on the HPLC column for verifying of the column performance as well as the retention times of both the relavant crosslinks, i.e. UPD and UDPD. The columns used were cartridge type glass columns (CGC, 150x3 mm) filled with SEPARON HEMA-BIO 1000 SB (poly hydroxyethyl methacrylate modified with sulphobutyl groups). As stationary phase, spherical particle size 10 μm, (TESSEK, Prague, Czech Republic) was used. Isocratic mobile phase consisted of part A and B in a ratio of 70:30 by volume. Part A was 0.3M acetic acid with pH adjusted to 3.35 with 4M NaOH, part B was 0.45 M Na_2SO_4. A flow of 0.3 mL/min

generated a pressure gradient of about 2.4 MPa along the column. The column oven was thermostated at 48°C and the injected sample volume loop was 10 µl. The fluorescence detector setup was 297nm and 400nm excitation and emission wavelengths, respectively.

RESULTS AND DISCUSSION

Due to the fact that the principal aim of this study was to elaborate a HPLC method suitable for simultaneous determination of both the crosslinks and to verify its usefulness in some groups of previously selected subjects, we shall only briefly comment on the results obtained. The clinical aspects will be considered in our future intended study.

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From the point of view of the time consumed and of the accuracy of the whole UPD and UDPD determination, it seems to be evident that the crucial step is foregoing separation and partial purification of hydrolysates (containing both crosslinks) on the cellulose-filled columns. Concerning this treatment, it is practically an independent chromatographic run, whose conditions should be adhered to. Even cellulose, which is used as the stationary phase, does not have adequate properties. However, it is usually employed (in our case as well). Unfortunately, under such conditions other naturally fluorescent substances eventually present in hydrolysate (e.g. desmosine and isodesmosine, the relevant crosslinks from mature elastin), can be adsorbed irreversibly on the cellulose surface. They can be the cause for the finding of a lot of undesirable peaks in the course of HPLC chromatography run. This could probably be the reason, why, when we work in commonly used ion-paired reverse phase HPLC mode, there is a greater occurence of interferring peaks close to the peaks which interest us. Therefore, we decided on an ion exchange principle, analogous to HPLC amino acid determination. Using fibrous cellulose, it was found[22] that this material was not ideal because of variations in packing density, which caused inconsistent elution of samples and large increases in pressure drop due to the elution of fine cellulose material after processing of several samples. This was the reason that often used fibrous cellulose (CF1) was substituted with microgranular cellulose CC31.[22]

The HPLC (alone) intra assay coefficient of variation (CV%) was 1.2% (obtained largely by use of the autosampler), estimated by 8 times multiple injecting of the stock standard urine sample previously elaborated on the only cellulose column in one step. The whole intra assay CV% was found to be 6.45%, determined by 5 times independent prefractionation of the same urine sample on 3 various cellulose columns, which can be considered as total uncertainty of the method elaborated. The inter assay CV% values differ

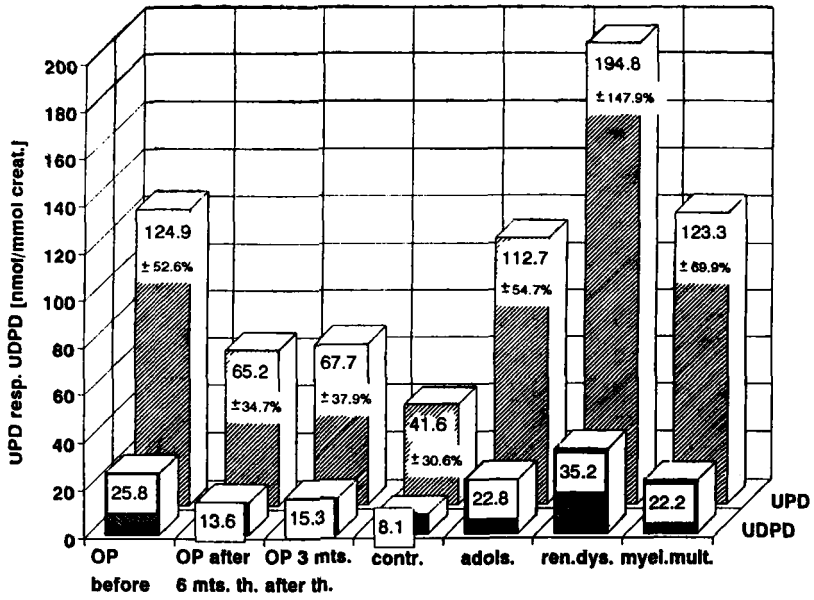

Figure 3. Results in some of the groups under study: UPD - mean values and coefficients of variation (CV%), UDPD - mean values only. Values are related to creatinine concentrations.

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significantly depending on the subjects in the groups under study, and are summarized in Fig.3, together with mean values of UPD and UDPD, both related to urinary creatinine. In this figure, one can see that, in the group of osteoporotic women (OP), there were significantly elevated values of the crosslinks before therapy which, after 6 months of calcitonin therapy (Calsynar, Rhone Poulenc Rorer), markedly decreased to almost half of their basal values and, even during the following 3 months this tendency remained practically the same. Sex dependence was not found in our study. Relevant values in the control groups (i.e. healthy, premenopausal younger women) are slightly lower. Nevertheless, one would of course take into consideration, that, menopause can frequently start very rapid osteoporosis, so called "fast losers", when every retardation may be very beneficial.

Elevated values in the group of adolescents (Fig. 3) are in high agreement with the presumption of higher bone turnover, i.e. that acceleration of newly formed collagen matrix, as a consequence of osteoblasts function on one hand, is balanced by raising the rate of bone resorption by osteoclast action on the

other hand. Significantly higher concentrations of both the crosslinks in the group of chronically dialysed patients (next to last column in Fig. 3) give evidence that PD levels were influenced by serious system disease secondary altering bone metabolism, and for similar reasons, UPD and UDPD values in group with blood malignancy were probably elevated as well (myeloma multiplex, last column in Fig. 3).

In conclusion, it can be stated that, the described modified method yields relatively rapid, sensitive (limit of detection is about 200 femtomoles), and reproducible results, suitable enough for monitoring of kinetic and intensity of bone resorption in various groups of patients. It should be noted, that very good correlation was found (not shown) between both markers (corr. coef. R about 0.95). Therefore, when the ELISA method is used for the crosslinks determination, only one of them is sufficient.

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