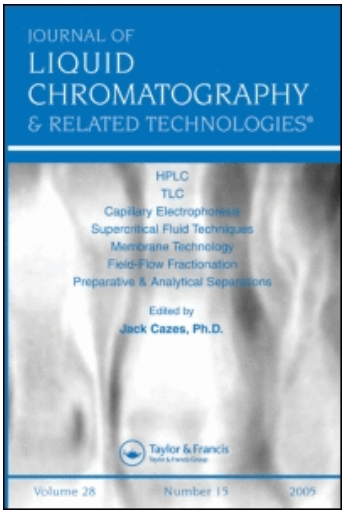

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PRE-FRACTIONATION WITH CATION EXCHANGER FOR DETERMINATION OF INTERMOLECULAR CROSSLINKS, PYRIDINOLINE, AND PENTOSIDINE, IN HYDROLYSATE

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ABSTRACT

A 3-hydroxypyridinium crosslink of mature collagen, pyridinoline (Pyr), is an essential substance in connective tissue except skin. A previous study of ours showed that the amount of Pyr in a hydrolysate of human articular cartilage could be used as an endogenous standard for the fluorometric analysis of pentosidine (Pen) [*J. Biochem.* 110, 714-718, 1991]. In the HPLC analysis of Pyr, partition chromatography utilizing CF-1 cellulose was usually employed to improve the resolution. However, this treatment seemed to be useless for Pen analysis. In this study, the usefulness of a cation exchanger, SP-Sephadex C-25 in the prefractionation of a hydrolysate is shown. The recovery of standards (mean ± SD) using CF-1 cellulose (n = 6) was 75.3 ± 3.7 % for Pyr and 11.5 ± 1.5 % for Pen, while that of standards using SP-Sephadex C-25 (n = 6) was 75.4 ± 2.3 % for Pyr and 71.1 ± 5.8 % for Pen. Using this method, age-related

changes in the content of Pen in urinary hydrolysates of normal humans (n = 60) utilizing fluorometry on HPLC under isocratic conditions were determined. This method is easy and useful for the study of intermolecular crosslinks, including Pyr and Pen in hydrolysates of tissues and humors.

INTRODUCTION

Pyridinoline (Pyr), characterized by Fujimoto et al. in bovine Achilles tendon collagen (1,2), is a trifunctional 3-hydroxypyridinium crosslink of mature collagen that is widely distributed in connective tissues except skin (3). Therefore, the determination of the Pyr content is a convenient method for the study of the presence of collagen in hydrolysates (4). On the other hand, deoxypyridinoline (D-pyr), characterized by Ogawa et al. in bovine femur insoluble collagen (5), is an analogue of Pyr and is thought to exist specifically in bone (4). Therefore, the determination of the D-pyr content has been used to understand bone function (4,6-10).

Pentosidine (Pen), characterized by Sell and Monnier in human dura mater collagen (11), is a bifunctional senescence crosslink and a condensation product of arginine, lysine and ribose, and is thought to accumulate in human tissues with age (11-13). They

also suggested that this accumulation correlated with disorders of pentose or hexose metabolism during aging or in diseases such as diabetes (12). A previous study of ours showed that highly sensitive detection of Pyr in tissue hydrolysates using fluorometry on HPLC could be used as an endogenous standard for Pen, instead of hydroxyproline (13).

In the HPLC analysis of Pyr content in hydrolysates found in tissues and humors, one can utilize partition chromatography involving treatments such as CF-1 cellulose powder (14). Although this method is useful for the purification of Pyr and D-pyr (Pyrs) and elastin crosslinks, desmosine and isodesmosine (15), we found that this method was not useful for the purification of Pen. In this study, therefore, we describe the use of a cation exchanger, SP-Sephadex C-25, for the prefractionation of hydrolysates before HPLC analysis and an application of this method to study age-related changes in the amounts of Pen in urinary hydrolysates of normal humans.

MATERIALS AND METHODS

Reagents

The following reagents were obtained from Wako Pure Chemical Industries, Osaka, Japan: HPLC-grade

acetonitrile (MeCN); sequenation-grade n-heptafluorobutyric acid (HFBA); reagent-grade HCl. All the water used was purified using a Milli-Q, Water Purification System (Millipore Corporation, Bedford, MA, USA).

Specimens

Specimens of human urine were obtained from normal volunteers with no diet restrictions and receiving no medication affecting calcium metabolism. The number of volunteers was 60, aged 3 to 88 year-old (males 6, females 54). They also had no previous history of bone diseases, connective tissue disorders or diabetes. For the study of the variation in the crosslinks contents with age, urine samples were collected between 9 and 12 o'clock. Collected urine samples were stored at -20 °C until used.

Instruments

The HPLC system consisted of a Model CCPM pump (TOSOH, Tokyo, Japan), a Model FS-8010 spectrofluorometer (TOSOH), a Model AS-8010 autosampler (TOSOH), and a Model SC-8010 system controller (TOSOH).

A column (8 mm x 10 cm) prepacked with Radial-Pak C18, of 10 μm particle size, type 8C1810 μ (Waters Associates, Inc., Milford, MA, USA) was used.

Chromatographic Procedure

A mobile phase of MeCN/30 mM HFBA (27:73, v/v) was used. The flow rate was 1.0 mL/min. The volume of each sample injected was 160 μL. The emission/excitation wavelengths of Pyr and Pen were at 390/297 nm and at 385/335 nm, respectively. The minimum amounts of crosslinks detectable (signal-to-noise ratio, 2) were about 0.6 pmol for Pyrs and 1.6 pmol for Pen, per one injection under our experimental conditions. The value of Pen contents in urine samples is expressed per 1μM urinary creatinine or 1 M Pyr.

Preparation of Standard Solution

Pyrs were isolated from human cortical bone (tibiae of a 13 year-old). Method for purification and characterization were practically the same as that descrived before (16).

Pen was isolated from human articular cartilage. Methods for purification and characterization were shown in our previous paper (13).

The concentrations of Pyr and Pen were determined from absorbance. The values of the molar absorption coefficients of Pyr and Pen in 0.1 M HCl were taken to be 8,300 L/mol/cm at 295 nm (1) and 4,500 L/mol/cm at 325 nm (11), respectively.

Prefractionation of Hydrolysates using SP-Sephadex C-25

After thawing, each urine sample (2 mL) was mixed with an equal volume of conc. HCl and purged with argon. The mixture was hydrolyzed at 110 °C for 20 hours in a sealed glass tube. An aliquot of the hydrolysate (0.25 mL) was mixed with 15 mL of water and applied to an SP-Sephadex C-25 column (H+ form, 0.8 x 1.0 cm, Pharmacia Fine Chemical AB, Uppsala, Sweden) that had been equilibrated with water. We washed the column with 20 mL of 0.15 M HCl to improve the separation. The crosslinks were eluted with 5 mL of 1.0 M HCl. The eluate was evaporated under a vacuum, and the residue was dissolved in 200 μL of 1 % HFBA. The solutions were stored at -20°C prior to the HPLC analysis.

Prefractionation of Hydrolysates by CF-1 Cellulose

Prefractionation of the hydrolysate with CF-1 cellulose (Whatman Ltd., Kent, UK) before HPLC analysis was the same method as described by Black et al. (14).

Urinary Creatinine

Before hydrolysis, the content of urinary creatinine was determined enzymatically on an aliquot of a urine sample using a Shimadzu CL-20 clinical chemistry analyzer (Shimadzu, Kyoto, Japan).

Statistical Analysis

Data were analyzed using a StatView program on a Macintosh computer. The statistical significance was determined by Student's t test (unpaired). P values of less than 0.05 were considered significant.

RESULTS

Pretreatment

For the pretreatment of hydrolysates in tissues or humors, a cellulose powder such as CF-1 cellulose is commonly used prior to the HPLC assay (6-10, 14). Figure 1 shows typical chromatograms of a hydrolysate of urine from a normal female (age 25); peaks of Pen and Pyrs are hidden in the background *without*

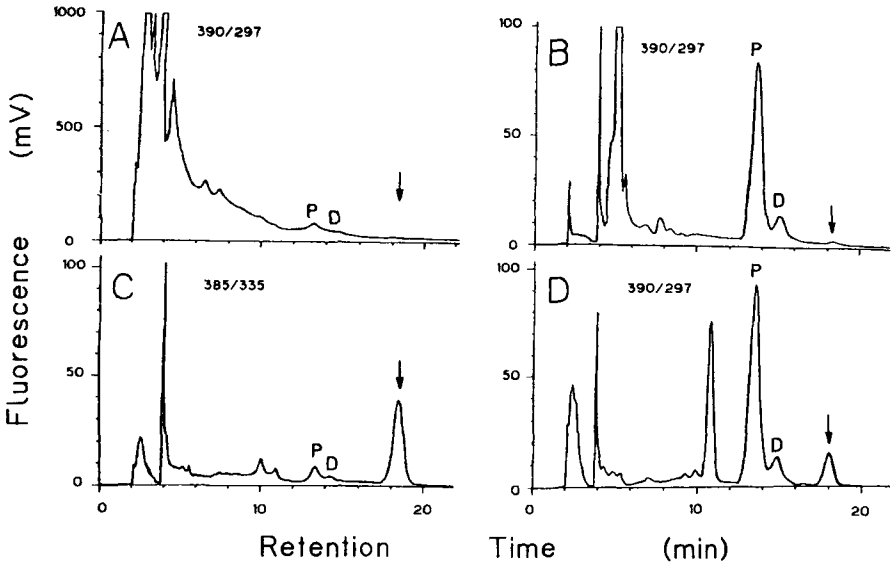

Figure 1. HPLC chromatograms of an urinary hydrolysate. Pretreatment: A, non; B, CF-1 cellulose; C and D, SP-Sephadex C-25. Emission/excitation wavelengths: A, B and D, 390/297 nm; C, 385/335 nm. Elution positions of authentic crosslinking amino acids: P, Pyr; D, D-pyr; arrow, Pen.

pretreatment (Fig. 1-A). As shown by Black et al. (14), the pretreatment of the hydrolysate with CF-1 cellulose was very effective in improving the separation of Pyrs, whose elution positions are indicated by characters of P; Pyr, and D; D-pyr in the Figure, from interfering substances (Fig. 1-B). However, we found that Pen, whose elution position is indicated by an arrow in the Figure, was also excluded by this treatment. On the other hand, Pen and Pyrs

were recovered well using pretreatment with a cation exchanger, SP-Sephadex C-25 (monitored wavelengths of emission/excitation at 385/335 nm, Fig. 1-C, and 390/297 nm, Fig. 1-D). The recovery of standards (mean ± SD) using CF-1 cellulose (n = 6) was 75.3 ± 3.7 % for Pyr, 93.9 ± 3.4 % for D-pyr and 11.5 ± 1.5 % for Pen, while that of standards using SP-Sephadex C-25 (n = 6) was 75.4 ± 2.3 % for Pyr, 73.1 ± 2.9 % for D-pyr and 71.1 ± 5.8 % for Pen. The coefficient of variation in the hydrolysate of urine using CF-1 cellulose (n = 6) was 3.1 % for Pyr, 10.3 % for D-pyr and 13.3 for Pen, while that of variation in the hydrolysate of urine using SP-Sephadex C-25 (n = 6) was 4.0 % for Pyr, 4.2 % for D-pyr and 8.2 % for Pen. We validated the adequacy of the capacity of SP-Sephadex C-25 for fluorophores, since the fluorescent response (integrated peak area) was linear to 5.0 nmol injected for Pyr or Pen, and 2.3 nmol injected for D-pyr (r = 0.99).

Applications

The age-related changes of the contents of the crosslinks in the hydrolysates of urine from normal subjects were studied. We grouped the subjects into three groups by age; a children's group (C, 3-18 years,

Table 1. Mean Values for Excretion of Pyridinolines and Pentosidine in Urine of Normal Subjects

	Age (year)		
	Children (C) (3-18)	Adults (A) (22-67)	Old persons (O) (72-88)
Sex	F,M	F	F
n	10	41	9
Pyr/Cr (μmol/mol)	160.9 ± 94.6[a,b]	19.9 ± 5.1[c]	34.4 ± 8.3
D-pyr/Cr (μmol/mol)	50.6 ± 27.5[a,c]	4.4 ± 1.4[b]	5.8 ± 2.2
Pen/Cr (μmol/mol)	4.4 ± 1.4[d]	4.9 ± 1.2[c]	12.5 ± 4.6
Pen/Pyr (mol/mol)	0.03 ± 0.03[a,c]	0.26 ± 0.09[d]	0.40 ± 0.25
D-pyr/Pyr (mol/mol)	0.34 ± 0.08[a,c]	0.22 ± 0.07[b]	0.17 ± 0.04

[a] $p < 0.0005$ vs A.
[b] $p < 0.05$ vs O.
[c] $p < 0.0005$ vs O.
[d] $p < 0.005$ vs O.

both sex), an adult group (A, 22-67 years, females), an old persons' group (O, 72-88 years, females), and summarized mean values of the two of Pyrs or Pen per 1 mol of urinary creatinine in each group (Table 1). Values of the Pyr/Cr and D-pyr/Cr changed consistently with those of Pyrs shown by Beardsworth et al. (6), and the difference between C and A and between C and O in the ratios of Pyr/Cr were 8.1 and 4.7, respectively, and in the ratios of D-pyr/Cr were 11.4 and 8.7,

respectively. The value of Pen/Cr in O was significantly higher than in C and in A ($P < 0.005$ and < 0.0005, respectively), but the difference between C and A was not significant.

Fig. 2-A shows the age-related changes in the value of Pen/Cr. Furthermore, in Fig. 2-B, we show the change in the value of Pen/Pyr, because our previous study showed that the value of Pyr content could be used as an endogenous standard for the analyses of fluorophores in hydrolysates of tissues or humors instead of the amount of hydroxyproline (13). The pattern of each chromatogram was slightly different from the others. The values of Pen/Cr stayed constant in the subjects aged 3-67 years and began to increase beyond the age of 70 (Fig. 2-A), while those of Pen/Pyr in the subjects aged 3-18 stayed at a very low level and seemed to increase with age after the 20's (Fig. 2-B). In Table 1, we summarized these relations. A significant increase in the value of Pen/Pyr was detected in O, in contrast to C and A ($P < 0.0005$ and < 0.005, respectively), and in A in contrast to C ($P < 0.0005$). Moreover, we show that the value of D-pyr/Pyr changed significantly, as that of D-pyr/Cr did, while the difference between C and A and between C and O in the ratios of D-pyr/Pyr were 1.5 and 2.0, respectively.

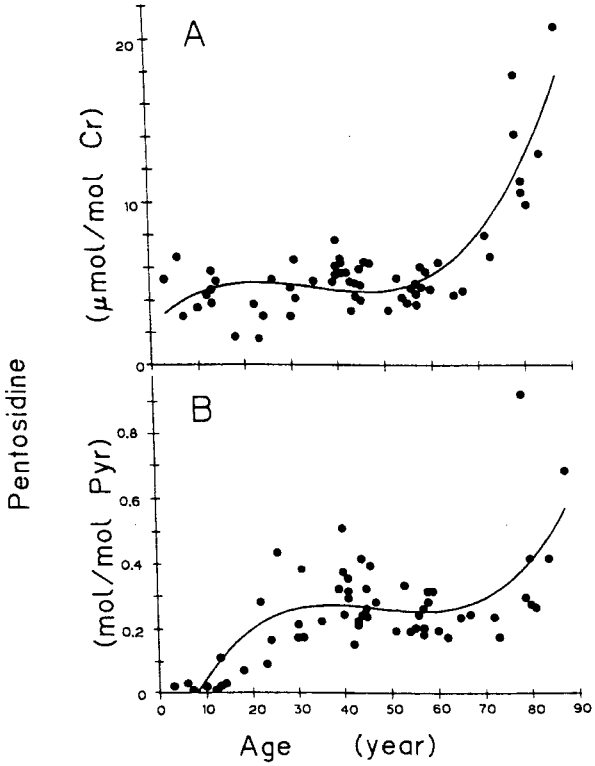

Figure 2. Relationship between the amount of urinary Pen and age. The contents of Pen per 1 mol of Cr (A) and per 1 mol of Pyr (B) as a function of age. The solid lines represent polynomial regression-line equations: A, Pen (μmol/mol Cr) = 2.371 + 0.292 x (age) - 0.010 x $(age)^2$ + 9.515 x 10^{-5} x $(age)^3$, r = 0.863, P = 0.0001; B, Pen (mol/mol Pyr) = -0.225 + 0.034 x (age) - 0.001 x $(age)^2$ + 5.393 x 10^{-6} x $(age)^3$, r = 0.710, P < 0.0005,

DISCUSSION

The pretreatment of hydrolysates using CF-1 cellulose is very effective in the analysis of Pyrs utilizing HPLC. However, in this work, we demonstrated that there is a limit to the use of that method in the study of Pen, crosslinks other than Pyrs (Fig. 1-B). It also demonstrated the usefulness of a cation-exchanger, SP-Sephadex C-25 in the pretreatment of hydrolysates (Fig. 1-C and -D). The application of this method in determining the urinary contents of Pyr in hydrolysates from normal humans showed that there were no essential differences between our results (Table 1) and those obtained previously using the method with CF-1 cellulose (6,8).

Most of senescence-crosslinks are known to accumulate in connective tissues, therefore, the content of the crosslinks in hydrolysates is usually expressed by the content of hydroxyproline (11-13,17). However, urinary hydroxyproline is influenced by diet and is metabolized by the liver, and only a small part of hydroxyproline produced by tissue catabolism is excreted in urine (18). Still more, the method for measuring hydroxyproline in hydrolysates is very complicated and not so sensitive. On the other hand,

Pyr is known to be excreted in urine without metabolization and not to be influenced by the gelatin load (19). Robins et al., and Body and Delmas showed that the concentration of Pyr in human-urine hydrolysates correlated significantly with that of hydroxyproline (8,10). A previous study of ours showed that the use of Pyr in tissue hydrolysates is more appropriate as a highly-sensitive endogenous standard than hydroxyproline (13). In this study, therefore, we show that this method is applicable to measure the change of urinary content of Pen in hydrolysates of normal humans. As shown in Table 1 and Fig. 2, two types of different results between the age-dependent changes of the values of Pen were obtained; the value of Pen/Pyr in C was significantly lower than that of Pen/Pyr in A or in O, while the value of Pen/Cr in C was significanltly lower that of Pen/Cr in O only. Exact meanings of these differences are still unknown, however, we think the value of Pen/Pyr is useful for the study of the role of Pen in human, because the accumulation site of Pen is known to be extracellular matrices (11), and Pyr is one of well studied indicative markers of collagen in those matrices (4,9).

In this study, we showed that all the values of Pyrs (i.e. Pyr/Cr, D-pyr/Cr and D-pyr/Pyr) were

significantly different each other. In the meantime, other workers have observed no significant variation of Pyr/D-pyr with age (6). We think one of causes of these divergencies may possibly be attributed to the different methods of the prefractionation.

In conclusion, the use of SP-Sephadex C-25 in the study of multifunctional, intermolecular crosslinks including Pen and Pyrs in hydrolysates of human tissues and humors is effective for the pretreatment prior to their assay by HPLC.

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