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Note

Determination of hydroxylysylpyridinoline in tissues and urine by high-performance liquid chromatography[a]

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Collagen, the major structural protein of vertebrates, is an essential component of all connective tissues. One of its main functions is to impart mechanical strength and rigidity to tissue by means of the formation of collagen fibres of high tensile strength. The great strength associated with collagen fibres is mainly due to the extensive cross-linking of individual collagen molecules into three-dimensional polymers of very large size.

Hydroxylysylpyridinoline (HP) is one of the cross-linking amino acids involved in this process. It is based on a fluorescent 3-hydroxypyridinium ring derived from three residues of hydroxylysine. HP is most abundant in articular cartilage and nucleus pulposus [1] at concentrations greater than 1 mol/mol collagen, while its deoxy analogue, lysylpyridinoline (LP), is less abundant (less than 0.1 mol/mol collagen) and is present mainly in bone collagen. The determination of HP in tissues can give information about local variations in its distribution and changes in its content with tissue ageing and with the development of some diseases. HP may also serve as a specific urinary marker of collagen breakdown.

Previous methods for the determination of HP in tissues [1] and urine [2] are based on ion-pair reversed-phase high-performance liquid chromatography (HPLC) with gradient elution. Very recently, an isocratic ion-pair system was published [3]. We have found problems in ion-pair systems with long equilibration times and system peaks disturbing the baseline. Therefore, the aim of this work was to develop a simple, isocratic method based on ion-exchange chromatography.

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EXPERIMENTAL

Materials and reagents

HP is not commercially available and therefore it must be isolated from tissues for calibration purposes. In our method, HP was isolated from collagen type II according to Eyre *et al.* [1]; a small amount of HP for reference purposes was also kindly supplied by Prof. Eyre. All other reagents were of analytical-reagent grade and were purchased from Lachema (Brno, Czechoslovakia).

Apparatus

A series 410 LC Bio pump and an LCI-100 computing integrator were obtained from Perkin-Elmer (Norwalk, CT, U.S.A.). The column effluent was monitored with an SP-970 fluorimetric detector (Spectra-Physics, San Jose, CA, U.S.A.).

Chromatography

A 15 cm × 3 mm I.D. glass column packed with the strong cation exchanger Separon HEMA-BIO 1000 SB (sulphobutyl), 10 μm (Tessek, Prague, Czechoslovakia), was used. The mobile phase was 0.31 *M* sodium sulphate solution, the pH being adjusted to 1.8 with sulphuric acid. A flow-rate of 0.3 ml/min generated a pressure of 2.6 MPa. The column temperature was 50°C. The volume of the injection loop was 50 μl. The settings of the fluorescence detector were as follows: excitation wavelength, 297 nm; emission filter with cut-off, 389 nm; time constant, 10 s; sensitivity, 640; range, 0.2.

Sample preparation

Urine. After hydrolysis of 0.5–1 ml of urine in hydrochloric acid (6 *M*) for 16 h at 110°C, endogenous interferents were removed by partition chromatography on a small disposable column containing 1 ml of cellulose [2]. The column was washed with 3 ml of 1-butanol–water–acetic acid (4:1:1). Then the urine hydrolysate was applied to the column, which was washed with another 3 ml of the same mixture as above and HP was eluted with 3 ml of water. The effluent was evaporated under nitrogen at 60°C and the residue dissolved in 200 μl or the mobile phase.

Tissue. About 5 mg of tissue were directly hydrolysed in 6 *M* hydrochloric acid for 16 h at 105°C. After evaporation in a stream of nitrogen at 60°C the samples were dissolved in 1 ml of water and 0.5 ml was taken for hydroxyproline determination and another 0.5 ml for HPLC analysis. The content of collagen in the sample was calculated from the content of hydroxyproline, determined according to the method by Stegeman and Stadler [4].

RESULTS AND DISCUSSION

Chromatography

In order to establish optimum chromatographic and detection conditions, the effects of the separation conditions on column efficiency, retention time and detector response were investigated. The influence of column temperature on retention time and column efficiency was tested (Fig. 1). Owing to a better mass transfer, the column efficiency increases with increasing temperature and therefore analyses were performed at 50°C. Black *et al.* [2] reported a strong dependence of the fluorescence intensity of HP on temperature measured under static conditions. We found only a 5% increase in the peak height when the capillary from the column outlet was immersed in a constant-temperature bath (0°C). This discrepancy can be explained by the fact that the detection cell with its connection capillaries works like a thermostat and the mobile phase inside the fluorimetric detector was rapidly heated to the temperature of the detector cell. As usual with ion exchangers, the maximum column efficiency was obtained at relatively low flow-rates.

Under the final conditions, the retention time of HP is 8 min (Fig. 2). In order to remove retained compounds, the column is cleaned with ammonia solution (0.5 ml/l) at the end of the working day.

Linearity, sensitivity, precision and accuracy

The calibration graph was obtained by weighed linear regression (weighing factor $1/y^2$) and was linear in the range 0.025–2 μg/ml ($r = 0.99998$). The detec-

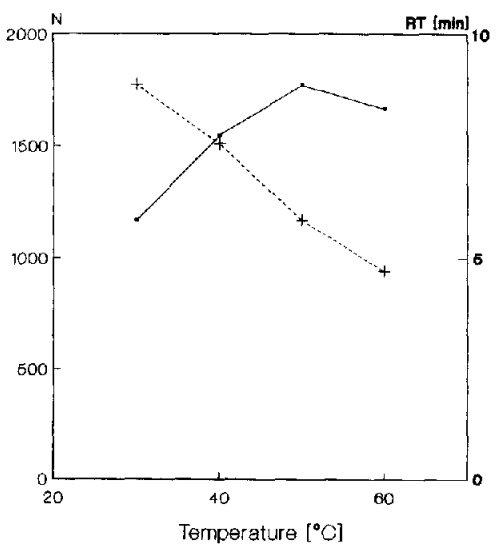

Fig. 1. Effect of column temperature on (□) column efficiency and (+) the retention of time of HP.

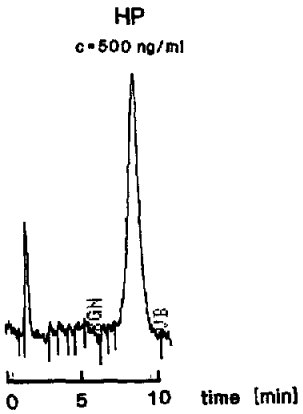

Fig. 2. Chromatogram of HP standard (25 ng). HP concentration, 500 ng/ml.

tion limit was 20 ng/ml (*i.e.* 1 ng or 2.5 pmol injected) at a signal-to-noise ratio of 2:1. The precision and accuracy of the method are given in Table I. Both peak heights and peak areas yielded similar values of precision and accuracy; for routine quantification peak heights were chosen, because they are less sensitive to baseline disturbances.

Applications

The chromatograms of the hydrolysates of rat articular cartilage and of human urine are shown in Figs. 3 and 4, respectively. The peak of HP was completely separated from endogenous interferents. Owing to the lack of a reference standard, we were not able to identify the peak of LP positively, although in some fractions of prepared standard we observed a second peak eluting after HP. The same peak was also present in some urine samples. In human urine we found

TABLE I

PRECISION AND ACCURACY OF HYDROXYLYSYLPYRIDINOLINE ANALYSIS

Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Relative standard deviation (%)	Accuracy (%)
0.025	0.024	4.7	2.2
0.05	0.053	6.4	6.4
0.125	0.122	3.2	2.5
0.25	0.242	1.5	3.4
0.5	0.492	1.8	1.6
1	1.004	3.7	0.4
2	2.084	6.2	4.2

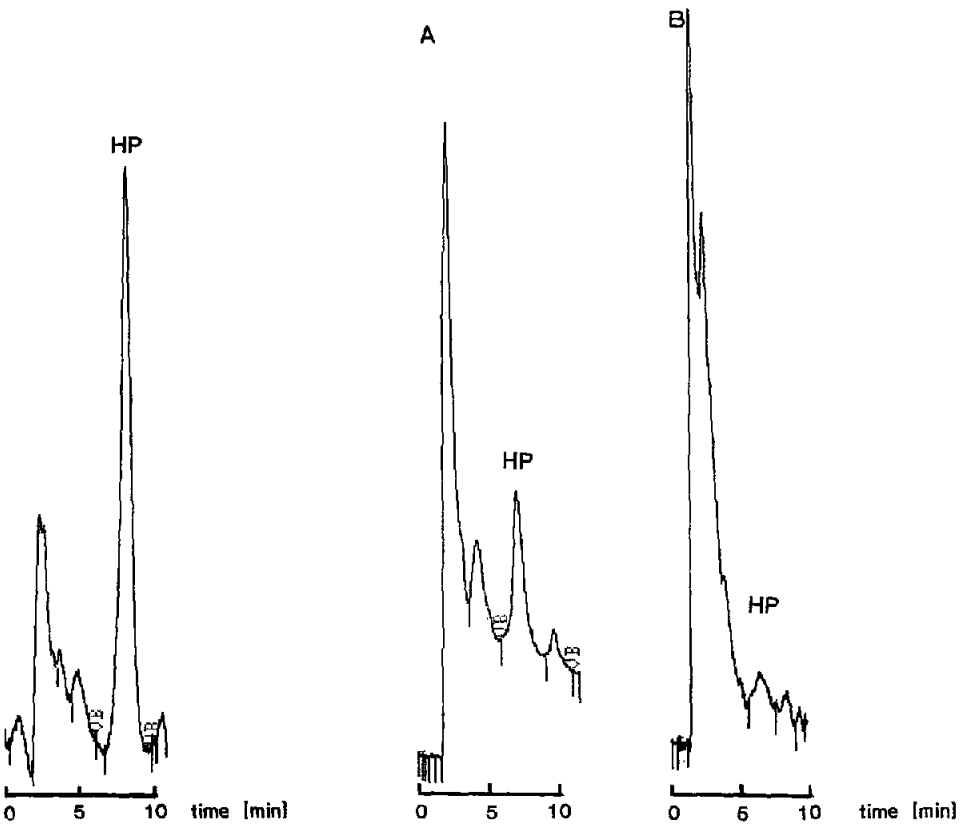

Fig. 3. Chromatogram of hydrolysed rat articular cartilage.

Fig. 4. Chromatograms of human urine hydrolysate. (A) Patient with osteoarthrosis; (B) healthy control.

increased pyridinoline levels in patients with rheumatoid arthritis and osteoarthrosis in comparison with healthy controls (Fig. 4). The detailed results will be published elsewhere.

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