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Note

Urinary peptides in rheumatic diseases
Separation by reversed-phase high-performance liquid chromatography

KATEŘINA TELLEROVÁ*

Research Institute for Rheumatic Diseases, 128 50 Prague 2 (Czechoslovakia)

PAVEL ŠPAČEK

Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, 162 06 Prague 6 (Czechoslovakia)

and

MILAN ADAM

Research Institute for Rheumatic Diseases, 128 50 Prague 2 (Czechoslovakia)

Urinary peptides are products of protein breakdown. Many of them contain hydroxyproline and are therefore thought to be derived from structural collagen. The presence of hydroxyproline-rich peptides in urine is brought about by the resistance of the triple-helical structure of collagen to common liver proteases. The concentration of urine hydroxyproline-rich peptides is considered to be a good indicator of collagen catabolism [1, 2].

The methods used for isolation and fractionation of urinary peptides are based mainly on liquid chromatography using an ion exchanger or molecular sieve or their combination. They are, however, rather tedious and slow and, therefore, are unsuitable for the determination of a large number of samples.

We therefore decided to test high-performance liquid chromatography (HPLC) for this purpose. Our attention was focussed at first on the higher-molecular-weight peptides (mol. wt. higher than 4000). There exist practically no data in the literature in this respect [3—6].

The diagnosis of osteoarthrosis is based on X-ray and clinical examination and no more precise method is available for determining the intensity of the osteoarthrotic process. However, as already mentioned, we suppose that more

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specific indicators of the metabolism of articular structures (especially of collagen) are present in the fraction of higher-molecular-weight hydroxyproline-containing peptides.

MATERIALS AND METHODS

HPLC instrumentation

The equipment used consisted of a reciprocating piston pump (Milton Roy, St. Petersburg, FL, U.S.A.), a "stop-flow" injector (home-made), a chromatographic cartridge-type column-system CGC [7] (a heavy-walled glass tube 150 mm × 3.5 mm I.D. inserted into a jacket made of a light alloy which, owing to the chemical reinforcement of the glass surface, can withstand pressures of up to 80 MPa) packed with silica (mean particle diameter 5 μm) chemically modified with octadecyltrichlorosilane [8] (Separon SI C 18, Laboratory Instrument Works, Prague, Czechoslovakia), UV variable-wavelength detector (Cecil Instruments, Cambridge, Great Britain) and a potentiometric recorder (Servogor 220, Goerz, Austria). The column showed 9700 theoretical plates for toluene as a test substance in methanol—water (70:30, v/v) as the mobile phase at a rate of 1 ml/min.

HPLC procedure

Twenty-four-hour samples of urine were obtained from eight healthy subjects (controls), from fifteen patients with different stage of osteoarthrosis, and from nine patients with rheumatoid arthritis of medium or high activity. Urines containing protein were excluded. A 25-ml volume of each urine was fractionated on a Bio-Gel P-4 column (Bio-Rad Labs., Richmond, CA, U.S.A.), 400 × 28 mm, equilibrated in and eluted with 0.01 *M* aqueous sodium chloride. The peaks were monitored at 220 nm (Pye-Unicam UV spectrophotometer SP 8-200). The first eluted peak (35 ml) containing peptides of higher molecular weight was used for further study. Samples were lyophilised and stored at −20°C.

A phosphate buffer (0.01 *M* KH_2PO_4, 0.1% H_3PO_4) with 25% methanol (p.a., Lachema, Brno, Czechoslovakia), pH 2.63, served as the mobile phase for isocratic HPLC separation of urinary peptides. Mobile phases with lower (over 15%) as well as higher (up to 40%) methanol contents have been also tested. However, under these conditions the separation was poor because the peptides were either heavily sorbed (in the case of lower methanol content) or eluted at very similar retention times (in the case of higher methanol content).

The eluted peptides were monitored at 210 nm, and the operating pressure was 3.2 MPa at a flow-rate of 0.12 ml/min. Samples were prepared by dissolving the lyophilised substances (approximately 1.5 mg in 30 μl of distilled water) immediately before injection; about 3 μl of this solution were injected. It was not deemed necessary to inject exactly the same volume of sample in each analysis, because the chromatograms were always evaluated in terms of relative concentrations (absorbances) of relevant components with respect to one that was used as internal standard; this procedure eliminated the necessity of absolute concentration measurements.

Peak evaluation

Five characteristic peaks of the chromatogram were selected for subsequent evaluation. One peak (the highest in all chromatograms) was designated P_0, and four other peaks showing the same retention volumes in all chromatograms were designated P_1 to P_4. The heights of individual peaks were measured and the relative values P_i/P_0 (i = 1, 2, 3, 4) were calculated. Thus, the relative heights of individual peaks could be easily compared irrespective of the injected volume and/or of the attenuation of the detector. It must be kept in mind that every peak corresponds obviously to a number of peptides with rather similar retention characteristics. The ratios thus obtained were evaluated statistically by Student's t-test and the results are presented in Table I.

TABLE I

STATISTICAL SIGNIFICANCE (t-TEST) OF DIFFERENCES BETWEEN THE GROUPS UNDER STUDY

	Controls vs. osteoarthrosis	Controls vs. rheumatoid arthritis	Osteoarthrosis vs. rheumatoid arthritis
P_1/P_0	$p > 0.05$	$p > 0.05$	$p > 0.05$
P_2/P_0	$p < 0.05$	$p > 0.05$	$p < 0.05$
P_3/P_0	$p < 0.01$	$p > 0.05$	$p < 0.05$
P_4/P_0	$p > 0.05$	$p > 0.05$	$p > 0.05$

RESULTS AND DISCUSSION

Chromatograms of the higher-molecular-weight fraction contain several peaks; five major and typical peaks were evaluated. The reproducibility of the method was good: the variation of relative peak heights did not exceed 3%. Prolonged use of a column packed with silica in a mobile phase containing inorganic buffer often leads to contraction of the bed, resulting in impaired separation efficiency and shortened retention times. This could be observed also in our study, but the decrease in retention times never exceeded 4%. The evaluation of individual peaks was not impaired by this effect.

According to Student's t-test the relative decrease of the peak P_3 is the most significant; at the 1% level in the group of osteoarthrosis, and in this case also, peak P_2 is decreased significantly at the 5% level (Table I). It is important that there exist significant differences also between the patient groups of osteoarthrosis and rheumatoid arthritis. Generally, it can be said that the variation range of P_i/P_0 ratios was rather high for all peaks in the rheumatoid arthritis group and relatively small for P_2 and P_3 in the case of osteoarthrosis (Fig. 1). Examples of chromatograms for the studied groups are presented in Figs. 2–4.

The HPLC method used in this study enabled us to fractionate larger peptides separated previously by chromatography on the Bio-Gel P-4 column. Our preliminary results show that the determination of larger peptides could

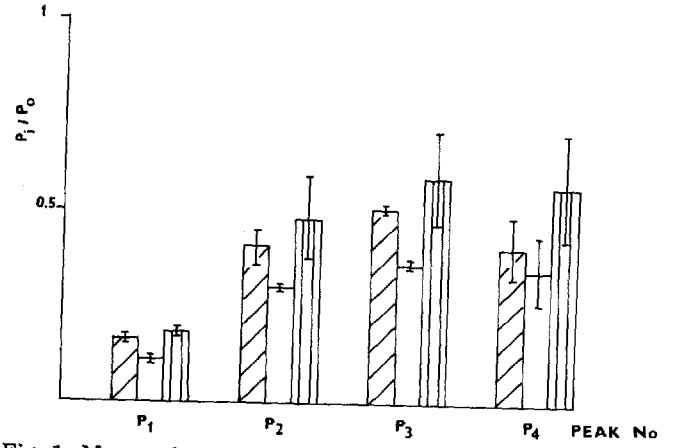

Fig. 1. Mean values and variation ranges of the relevant values P_i/P_0 for the three groups — osteoarthrosis (□), rheumatoid arthritis (▥), controls (▨).

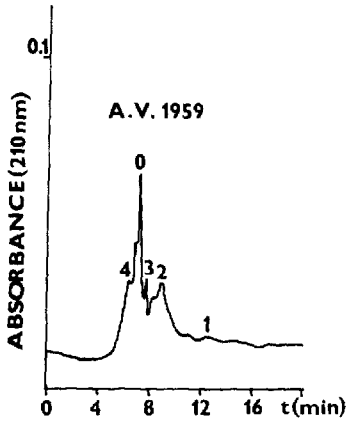

Fig. 2. Typical reversed-phase HPLC "metabolic profile" of urinary peptides of a healthy subject.

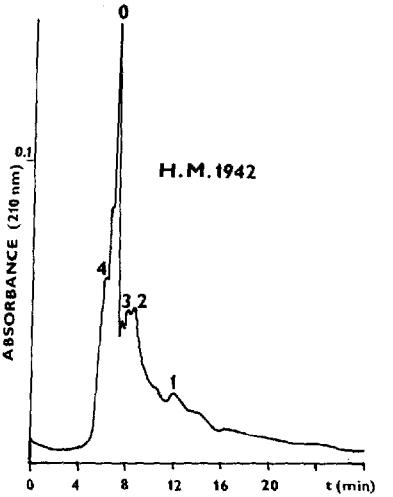

Fig. 3. Typical reversed-phase HPLC "metabolic profile" of urinary peptides of a patient with osteoarthrosis.

be a useful method in the treatment of degenerative joint diseases. We have found not only differences between controls and osteoarthrosis patients, but also between osteoarthrosis and rheumatoid arthritis patients. Further investigation of urinary peptide excretion in other rheumatic diseases is, however, necessary.

HPLC for the separation of urinary peptides was used also by Clark et al. [9]. These authors, however, were not able to find differences between urinary peptides in patients with osteoarthrosis and rheumatoid arthritis. The possible reason for the disagreement with our results could be explained by differences in the pretreatment procedure of the urinary peptide samples.

In comparison with the procedure of Szymanowicz and co-workers

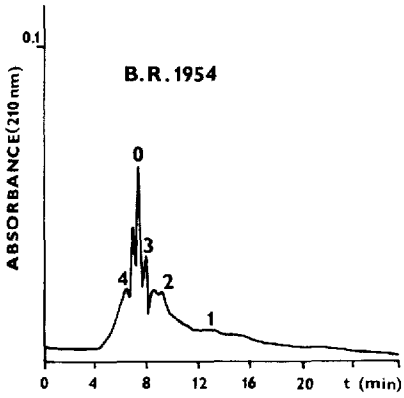

Fig. 4. Typical reversed-phase HPLC "metabolic profile" of urinary peptides of a patient with rheumatoid arthritis.

[10—12], our method is less sensitive and results in resolving several categories of peptides only. From the applicability point of view HPLC fractionation of urinary peptides is much more simple and can be used with large series of samples. On the other hand, Szymanowicz et al. [11] obtained better fractionation with smaller peptides using a combination of different liquid chromatographic methods, which according to our data are also fractionated rather well by HPLC [13]. This fraction could be also important, since according to data of Szymanowicz et al. [11] 75% of all peptides containing hydroxyproline are of lower molecular weight (up to 2000).

Investigation in this respect is in progress. We also hope that the use of a gradient may further improve the fractionation of larger peptides by HPLC. Finally, the analysis of the most important peaks seems to be necessary for elucidating their relationship to collagen.

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