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New marker of bone resorption: hydroxyproline-containing peptide High-performance liquid chromatographic assay without hydrolysis as an alternative to hydroxyproline determination: a preliminary report

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Abstract

A high-performance liquid chromatographic (HPLC) assay for a urinary hydroxyproline-containing peptide (hydroxyproline peptide, HypP) is described. This peptide represents about 50% of urinary hydroxyproline-containing peptides. Its concentration and total 4-hydroxyproline (Hyp) concentration evaluated in 325 urine samples have been shown to be closely correlated ($r = 0.972$; $y = 0.499x - 1.5$), which may indicate that the two markers provide the same information. The HypP assay, similar to Hyp assay, is carried out without hydrolysis of urine samples. After the blocking of primary amino acids by *o*-phthaldialdehyde (OPA) and derivatization of secondary amino acids by 9-fluorenylmethyl chloroformate (FMOC-Cl), the FMOC derivatives of HypP and 3,4-dehydroproline (internal standard) were separated on a strong anion-exchange column and detected fluorimetrically. HypP concentration was calculated by measurement of peak-area ratios of HypP and the hydroxyproline standard. The HypP/creatinine (mmol/mol) ratio in fasting urine samples from healthy adults was found to be 8.2 (S.D.=1.6, $n = 33$) in 27–44-year-old premenopausal women and 6.9 (S.D.=1.7, $n = 21$) in 28–49-year-old men.

Keywords: Peptides; Oligopeptides; Hydroxyproline

1. Introduction

The measurement of urinary 4-hydroxyproline (Hyp) excretion is the most commonly used routine clinical assay for bone resorption. In recent years galactosyl hydroxylysine and pyridinium crosslinks have been proposed as new biomedical markers of bone resorption. Their greater bone specificity has been emphasized. They are not metabolized in the liver [1,2], their variability among populations is small and their discriminatory power is better [1]. However, the galactosyl hydroxylysine HPLC procedure causes some difficulties due to the necessity of a long-lasting gradient [2]. Pyridinium crosslinks assay requires a laborious standard preparation [3].

Several HPLC assays for urinary Hyp have been published [4–9], among which some were simple [7–9] and were employed routinely in the laboratory [8]. Recently, micellar electrokinetic chromatography has also been used [10]. The influence of diet is

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avoided by measurement of the fasting urinary Hyp/creatinine ratio [11].

Most of the urinary Hyp is contained in peptides. Overnight hydrolysis of the Hyp-containing peptides is a tedious step indispensable in urinary Hyp assays.

Several studies have described urinary peptides [12–17]: polypeptides separated by gel permeation chromatography [12], dipeptides by ion-exchange chromatography [13,14] and Hyp-containing dipeptides identified by gas chromatography with mass spectrometry [16,17].

The aim of this study was to evaluate the capability of a specific urinary hydroxyproline-containing peptide as a bone resorption marker, an alternative to Hyp, by use of a simple and practical HPLC assay to determine its concentration in non-hydrolysed urine samples.

The concentration values of Hyp and hydroxyproline peptide (HypP) were determined in 325 randomly selected urine samples, using the previously described double derivatization with *o*-phthaldialdehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC-Cl) [18] and eluting the FMOC derivatives by anion-exchange chromatography as described in our previous study [8]. Peak purity was checked by reversed-phase chromatography. In order to characterize the hydroxyproline peptide, the FMOC-HypP peak fraction was collected, hydrolysed and analysed for the amino acids.

2. Experimental

2.1. Instrumentation

The HPLC apparatus consisted of a Type 114 and 116 solvent-delivery module, a Type 450 data system and an Omniscribe d-5000 recorder (Beckman Instrument International, Geneva, Switzerland), a Model 401/231 sample injector (Gilson Medical Electronics, Villiers-Le-Bel, France) and a Type LS 4 spectrofluorimeter (Perkin-Elmer, Beaconsfield, Bucks., UK). The Beckman Synchron CX3 clinical system was used for creatinine determination.

2.2. Materials

Iodoacetamide, *o*-phthaldialdehyde, 9-fluorenylmethyl chloroformate, 3,4-dehydro-DL-proline, trans-4-hydroxy-L-proline, DL-proline, glycine, sarcosine, DL-serine and the Pro-Hyp dipeptide were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, acetone and 2-propanol were HPLC-grade; all other chemicals used were of analytical grade. The analytical column containing the strong anion-exchanger Partisphere 5 SAX (110 × 4.7 mm I.D., 5-μm particles) and AX guard system were obtained from Whatman (Clifton, NJ, USA); the Superspher 100 RP-18 endcapped (250 × 4.0 mm I.D., 4-μm particles) reversed-phase column and the LiChrospher 100 RP-18 (4 × 4 mm I.D. 5-μm particles) guard column for comparative purposes were obtained from Merck (Darmstadt, Germany).

The Hyp working standard was 500 μmol/l Hyp solution in 0.05 mol/l hydrochloric acid. The working standard for HypP was 125 μmol/l Hyp solution in 0.05 mol/l hydrochloric acid. A 125 μmol/l Pro-Hyp dipeptide solution was prepared in 0.05 mol/l hydrochloric acid. The internal standard for the Hyp and HypP determination was a 60 μmol/l 3,4-dehydroproline solution in 0.05 mol/l hydrochloric acid. A sodium hydroxide–borate mixture was prepared by dissolving 1.44 mol sodium hydroxide and 0.48 mol boric acid to 1 l with water. The 0.4 mol/l potassium borate buffer, pH 9.5, was prepared by dissolving boric acid and potassium hydroxide with water. A concentrated solution of ammonium formate buffer, pH 3.7, was prepared by mixing 2 mol formic acid, 1 mol ammonium hydroxide and water to 1 l.

2.3. Urine samples

After an overnight fast, first morning urine was discarded and the next urine was collected in a 10-ml tube containing 0.5 ml of 2.4 mol/l HCl. The samples were stored at 4°C and processed weekly.

Urinary creatinine was measured by the Jaffé rate method.

2.4. Procedure for the hydroxyproline peptide (HypP)

A modification of the procedure for hydroxyproline was employed. To a 20-μl aliquot of urine sample 100 μl of internal standard and 200 μl of 0.13 mol/l sodium carbonate solutions were added. Then 50 μl of 0.37 mol/l mercaptoethanol in

acetonitrile and 50 μl of 0.37 mol/l OPA in acetonitrile solutions were added. After a 1-min pause 50 μl of 0.75 mol/l iodoacetamide solution in acetonitrile were added. Exactly 30 s later, 100 μl of 2 mmol/l FMOC-Cl solution in acetone were added and, after mixing, 4 ml of water were added. The HypP working standard and the Pro-Hyp dipeptide solution were treated in the same way as the urine samples. A 10-μl portion of FMOC derivatives mixture was injected.

2.5. Procedure for hydroxyproline

The procedure proposed by Teerlink et al. [7] as a modification of the procedure described by Einarsson [18] was, in turn, slightly modified. To a 0.5-ml aliquot of urine 1.5 ml of 8 mol/l hydrochloric acid was added and the samples were hydrolysed at 110–115°C overnight in flame-sealed ampoules. To a 20-μl portion of the hydrolysate 100 μl of internal standard, 100 μl of sodium hydroxide–borate mixture and 100 μl of 0.4 mol/l potassium borate buffer (pH 9.5) solutions were added. The next steps are the same as for HypP derivatization. The Hyp working standard was treated in the same way as the urine samples except for overnight heating. A 10-μl portion of FMOC derivatives mixture was injected.

2.6. Ion-exchange chromatography

The same HPLC procedure was performed for the separation of HypP and Hyp FMOC derivatives using the Partisphere 5 SAX column and AX Guard System. Isocratic elution was carried out at a 1.2 ml/min flow-rate. The mobile phase was 20 mmol/l ammonium formate buffer, pH 3.7–acetonitrile–2-propanol (70:20:10, v/v/v). Fluorescence of the eluate was monitored at 260 nm for excitation and 330 nm for emission. The peaks of Hyp, HypP and internal standard FMOC derivatives were acquired by recorder and by computer for calculation.

2.7. Calculation of the molar concentration

Since we had previously [8] checked the linearity of the method for hydroxyproline in the range between 10 and 2000 μmol/l, the one-point calibration was used. The molar concentration of hydroxyproline was calculated on the basis of FMOC-Hyp/FMOC-IS peak-height ratios against the concentration of the Hyp working standard solution. The molar concentration of HypP was calculated on the basis of the FMOC-HypP/FMOC-IS peak-area ratios against the concentration of the working standard solution for HypP.

2.8. Structural investigations of the HypP fraction

Four urine samples at elevated HypP concentration (180–330 μmol/l) were submitted to the derivatization procedure for HypP. Twelve separations of the FMOC-derivatives of each urine sample were performed, using 50-μl injections. The FMOC-HypP peak fraction was collected and pooled. The pooled FMOC-HypP fraction of each urine sample was evaporated to dryness in a centrifugal evaporator at 37°C and dissolved in 300 μl of 6 mol/l hydrochloric acid. A 200-μl aliquot of this solution was hydrolysed at 115°C overnight while the 100-μl remaining fraction was stored at 4°C. To attain the best comparison, both the hydrolysed and non-hydrolysed FMOC-HypP peak fractions of each urine sample were submitted to the derivatization procedure for Hyp, modified to increase the sensitivity by using 100 μl of 3 μmol/l 3,4-dehydroproline (internal standard), 50 μl of 2 mol/l FMOC-Cl, 1 ml of water. A 50-μl aliquot of final mixture was injected onto the anion-exchange column, obtaining a FMOC-Hyp peak from the hydrolysed and a FMOC-HypP peak from non-hydrolysed pooled fractions. The relative amounts of FMOC-HypP and Hyp were calculated on the basis of the FMOC-HypP/FMOC-IS peak-area ratios in the non-hydrolysed and FMOC-Hyp/FMOC-IS peak-area ratios in the hydrolysed peak fraction.

2.9. Reversed-phase chromatography of HypP

A 30-μl portion of the final mixture, containing the FMOC derivatives from the HypP derivatization procedure, was injected into the C_{18} Superspher reversed-phase column, preceded by the LiChrospher guard column. An isocratic separation (40 min), followed by a column washing (12 min), was carried out at a flow-rate of 0.8 ml/min with two mobile phases consisting of 20 mmol/l ammonium formate buffer, pH 3.7–acetonitrile–2-propanol (65:25:10, v/v/v) and (10:70:20, v/v/v), respectively. The fluo-

rescence was monitored and the HypP concentration was calculated as described above for the ion-exchange chromatography.

3. Results

The presence, in human urine, of a specific peptide containing about 50% of the total Hyp excreted, and its correlation with total Hyp, was verified on the urine sample of 325 fasting, randomly selected, men and women. Each urine sample was processed without hydrolysis to measure the HypP and, after hydrolysis, to measure total Hyp. Results are shown in Fig. 1. Fig. 2 shows a typical sequence of chromatograms from three human urine samples of the HypP determination (upper) and of the total Hyp determination (lower). The retention times of FMOC-HypP, FMOC-Hyp and FMOC-internal standard were 5.0, 5.6 and 6.6 min, respectively. The two other significant peaks, shown in the chromatograms from hydrolysed urine samples (lower), have the same retention times as the proline and sarcosine FMOC-derivatives, i.e. 3.9 and 4.4 min, respectively. No significant peaks were eluted after the internal standard.

Fig. 3 shows the separation of FMOC derivatives of Hyp and HypP from a urine sample containing a significant amount of free Hyp.

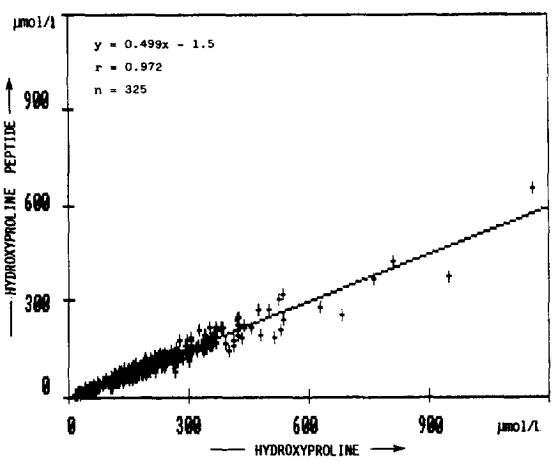

Fig. 1. Correlation diagram of the concentrations of hydroxyproline peptide (HypP) and total hydroxyproline obtained from 325 urine samples of fasting individuals by procedures presented.

Within-run precision was evaluated from repeated measurements of urine samples at two concentrations; between-run precision was evaluated by weekly repeated measurements of a urine sample stored in aliquots at −20°C. The results are summarized in Table 1.

Several urine samples containing high and moderately high HypP values were diluted five- and ten-fold to check the linearity of the method in the range between 10 and 812 µmol/l. The ratio of observed/calculated HypP values was found to be 0.977 ± 0.043 (mean ± S.D., $n = 11$).

Signal-to-noise ratio was found to be 2 at a 5 µmol/l urinary HypP concentration, which represents the detection limit of the method.

The analytical accuracy of the HypP assay was tested by reversed-phase chromatography with 39 urine samples. The correlation ($r = 0.9957$, $y = 1.027x + 2.4$) of the urinary HypP concentration values obtained by the two methods are shown in Fig. 4. The retention times of FMOC derivatives of free Hyp, HypP and internal standard are 14.5, 18.6 and 36.8 min, respectively. Fig. 5 shows a typical chromatogram obtained by the reversed-phase chromatography from a non-hydrolysed urine sample.

The HypP and Hyp levels were determined on urine samples from 54 fasting healthy adult volunteers: 33 premenopausal women of 27–44 years and 21 men of 28–49 years. The values, expressed as the ratio of HypP/creatinine and Hyp/creatinine on a molar basis, are summarized in Table 2.

In the study of HypP the response of the FMOC-Hyp derivative obtained after hydrolysis, subsequent re-derivatization and appropriate dilution of the pooled FMOC-HypP peak fractions was identical to that of the non-hydrolysed HypP obtained from the corresponding volume of the same peak fraction. This result is in accordance with the "release" of one molecule hydroxyproline/molecule of HypP.

In addition to Hyp, other amino acids, such as proline, serine and glycine, were found in the hydrolysed peak fractions and identified by comparison of their retention times with those of the reference compounds.

The retention time of the FMOC-HypP peak was found to be identical to that of the FMOC-Pro-Hyp dipeptide, both in ion-exchange and in reversed-phase chromatography.

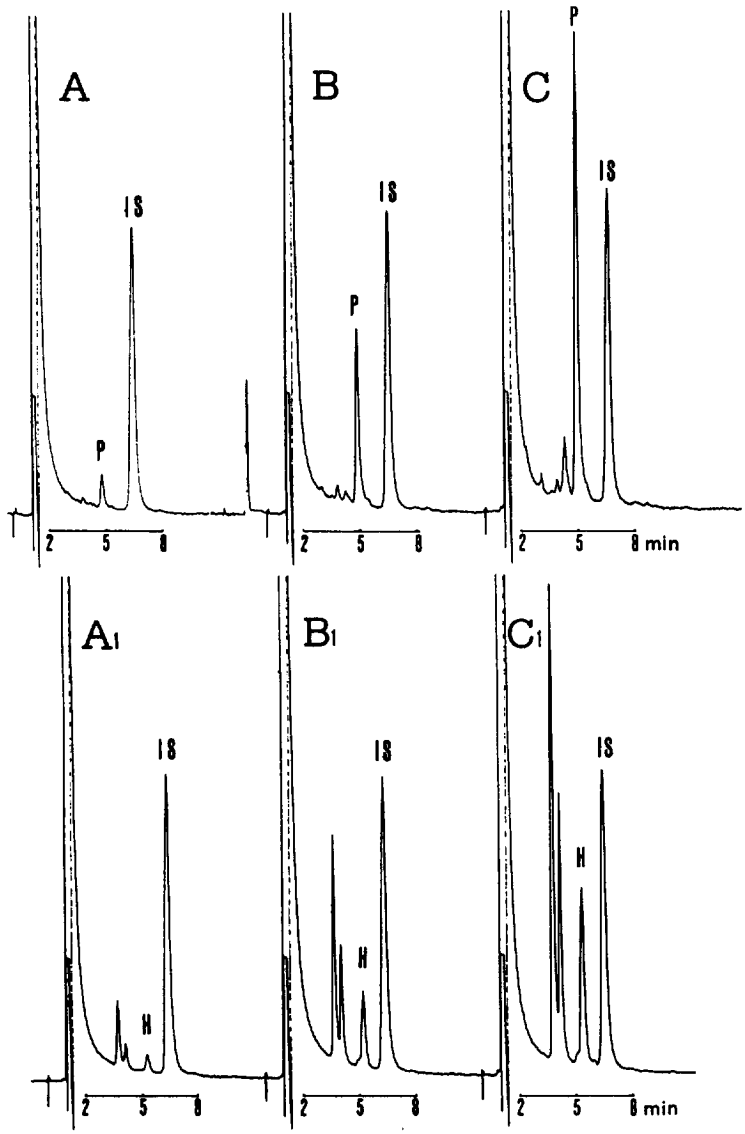

Fig. 2. Chromatograms obtained by ion-exchange chromatography, of the FMOC derivatives from three urine samples without hydrolysis (upper) and from the same urine samples after hydrolysis (lower, in the same order) at different concentrations. A, B, C: 20, 107, 300 μmol/l. A1, B1, C1: 42, 200, 539 μmol/l. Peaks: P, hydroxyproline peptide; H, hydroxyproline; IS, 3,4-dehydroproline.

4. Discussion

For the derivatization procedure, we have used the principle described by Einarsson [18] for selective determination of secondary amino acids, and that of Teerlink et al. [7] for the urinary Hyp determination, in which primary amines are blocked by reaction with OPA and mercaptoethanol, followed by labelling of secondary amines with FMOC-Cl. Iodoacetamide was added to neutralize the excess of mercaptoethanol. OPA derivatives may be co-eluted with FMOC derivatives, but their fluorescence does not interfere.

The sodium hydroxide–borate mixture, as well as the sodium carbonate solution, were used instead of sodium hydroxide and the borate buffer system as

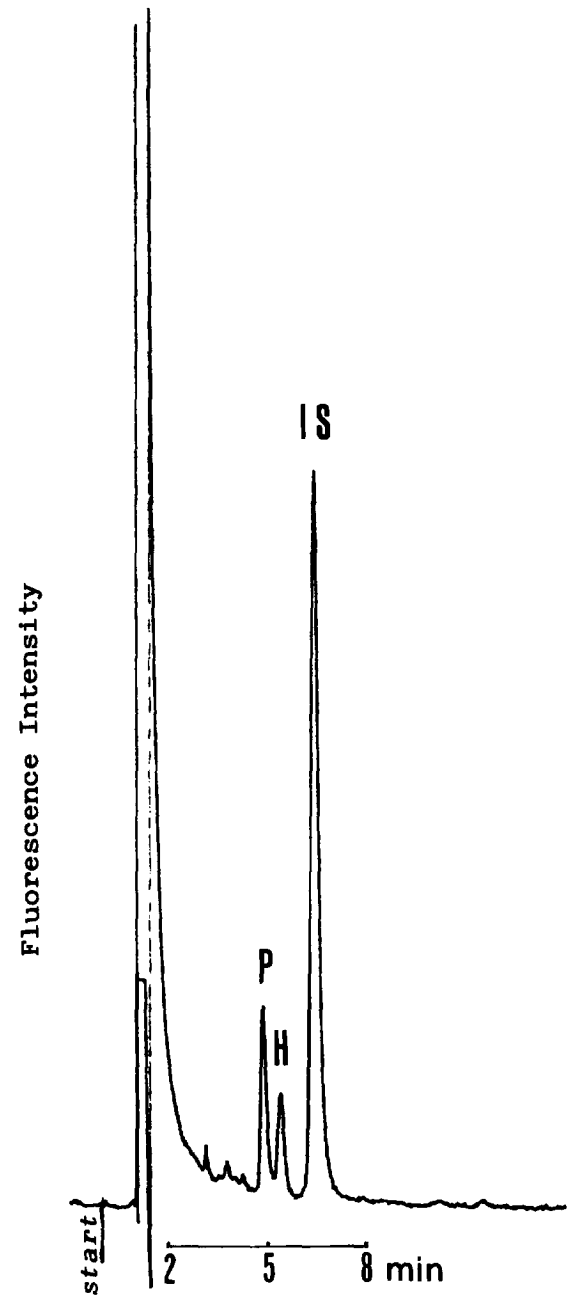

Fig. 3. Chromatogram obtained by ion-exchange chromatography of the FMOC derivatives from a non-hydrolysed urine sample containing hydroxyproline peptide (54 μmol/l) and free hydroxyproline (29 μmol/l). Peaks: P, hydroxyproline peptide; H, hydroxyproline; IS, 3,4-dehydroproline

Table 1
Hydroxyproline peptide determination precision study

	n	HypP (μmol/l)		C.V. (%)
		Mean	S.D.	
Within-run	10	20.3	1.4	7.0
	10	304.5	4.8	1.6
Between-run	10	124.8	7.7	6.2

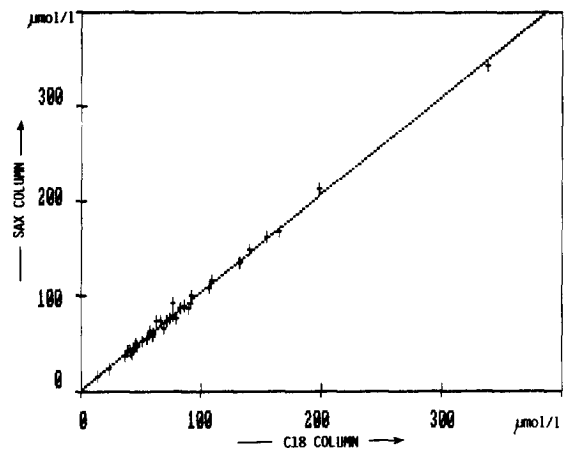

Fig. 4. Correlation diagram of the concentrations of hydroxyproline peptide (HypP) from 39 urine samples by separation on the ion-exchange as well as the reversed-phase C_{18} matrix.

alternative reagents to make easier dispensing or preparation.

Due to the lack of a HypP standard, we have been compelled to use the Hyp standard to obtain the HypP molar concentration. This may be a reasonable approximation because the responses of the FMOC derivatives are elicited only by the contribution of the FMOC moiety.

Analytical recovery of Hyp was previously tested [7,8]. However, recovery of HypP causes some difficulties due to the lack of an authentic synthetic standard.

The chemical composition of the hydroxyproline peptide still needs to be confirmed. Presumably it is the Pro-Hyp dipeptide, but further investigations, by

Table 2
Hydroxyproline peptide/creatinine ratios in the non-hydrolysed urine samples and hydroxyproline/creatinine ratios in the same urine samples after hydrolysis from healthy adults: premenopausal women 27–44 years, men 28–49 years

Group	*n*	HypP/creatinine (mean±S.D.) (mmol/mol)	Hyp/creatinine (mean±S.D.) (mmol/mol)
Women	33	8.21±1.61	15.81±2.45
Men	21	6.90±1.66	13.73±2.79
All	54	7.70±1.74	15.00±2.76

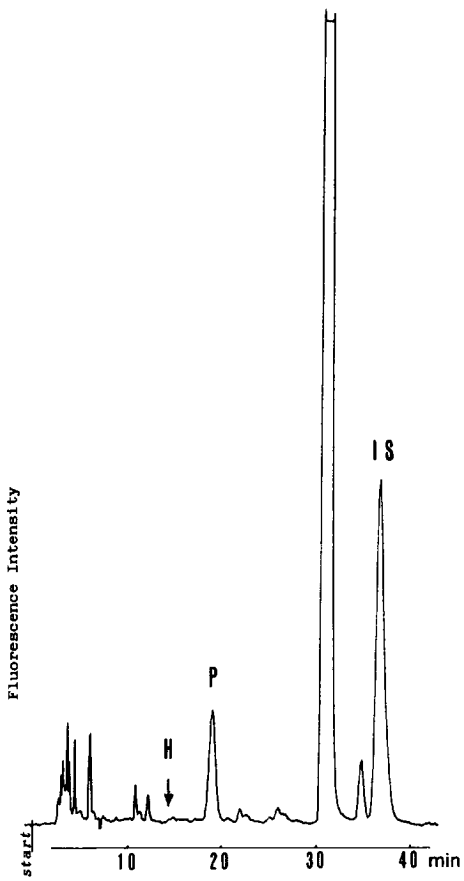

Fig. 5. Chromatogram of the FMOC derivatives, obtained by reversed-phase chromatography on a C_{18} matrix, from a non-hydrolysed urine sample. Peaks: P, hydroxyproline peptide (79 μmol/l); IS, 3,4-dehydroproline. H, retention time of hydroxyproline.

Edman degradation and mass spectrometry, have to verify its chemical formula.

The urinary excretion of total hydroxyproline has been shown to be a useful and precise measure of bone resorption rate in postmenopausal women with osteoporosis [19]. The measure of the urinary excretion of HypP provides the same but more direct information. The tedious and time-consuming hydrolysis step was avoided, thus allowing the possibility for a fully automated routine assay. Finally, if free Hyp is present in urine at a concentration higher than 10 μmol/l it can be measured in the same HPLC run as HypP.

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