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Short communication

Rapid screening of urinary proline–hydroxyproline dipeptide in bone turnover studies

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Abstract

In a recent report [J. Chromatogr. B 678 (1996) 165] a urinary hydroxyproline-containing peptide has been preliminarily suggested as a possible alternative to hydroxyproline (HP) determination in bone resorption studies. For this purpose a simple and practical procedure was developed for a rapid high-performance liquid chromatographic (HPLC) assay of the peptide in non-hydrolyzed urine samples. Hundreds of randomly selected urine samples were assayed for both the peptide and HP, the latter in hydrolyzed urine, and a high correlation between them was found. The promising results prompted us to search for the postulated biomarker of bone resorption in urine samples of postmenopausal women examined as osteoporosis suspects. As an alternative to the HPLC determination, an equally rapid procedure has been developed for the peptide assay using capillary gas chromatography (GC) with flame ionization detection (FID). By means of a solid-phase and a liquid–liquid phase extraction, involving ethyl chloroformate (ECF) as the derivatizing agent, two dipeptides and some urinary amino acids could be analyzed within 5 min. A high correlation between both HPLC and GC peptide assay was confirmed (r=0.944) and the compound was identified as proline–hydroxyproline (PHP) dipeptide. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Osteoporosis, losses of bone mass and destruction of bone architecture, belongs to a serious health care problem affecting especially the female aging population. Monitoring bone turnover markers in body fluids, e.g., degradation products of type I bone collagen in urine, can help in the diagnostics and therapy of the disease. Currently the pyridinium crosslinks, i.e. pyridinoline, and to collagen I more-specific deoxypyridinoline (DPD), became biomedical markers of choice. Their free fractions, comprising 40% of the total level in urine, are mostly determined using enzyme immunoassay (EIA) or HPLC–natural fluorescence detection. EIA is also often employed for measurement of N-terminal telopeptides (NTx), containing the mentioned cross-links, in urine or serum. In addition, due to a high content of 4-hydroxyproline (HP) in bone and tissue collagens, measurement of HP in hydrolyzed urine was established as a routine tool for bone resorption monitoring until recently. However, because of a

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lower specificity toward the bone collagen, diet influences and a necessity to hydrolyze urine, the HP assay declined in favor of the newly introduced markers [1,2].

In a recent study, presented by Mazzi et al. as a preliminary report [3], a HP-containing peptide was monitored in non-hydrolyzed morning urine samples of 325 randomly selected subjects using HPLC with fluorescence detection. Identical procedure and analytical conditions were employed for determination of HP in the same but hydrolyzed urine samples; the results were related to creatinine. To validate the approach, commercially available PHP-dipeptide and 3,4-dehydroproline were involved as standards; the former for a tentative identification of the HP-containing peptide, the latter as the internal standard. The study revealed a high correlation between HP and the peptide ($r=0.972$). Such findings justified a conclusion that the HP-containing peptide might replace HP as a marker of bone resorption, with the substantial advantage of using non-hydrolyzed urine.

Based on these promising findings we have initiated a project focused on a group of postmenopausal women to elucidate the role of the peptide in the field of bone disease [4]. Also other bone resorption markers like DPD and NTx have been assayed in urine samples of the subjects involved. The research still continues and preliminary results confirm the relevance of the project. In the course of the experiments an alternative method has been developed by us that employs capillary GC for determination of the urinary peptide. In this report we wish to describe the novel approach based on an immediate derivatization of amino acids with chloroformates in water containing media [5].

2. Experimental

2.1. Urine samples

Urine samples (overnight fast, second morning void of 7.00–8.00 a.m.) were collected from female subjects, mostly postmenopausal women with an average age of 56 years, who were classified as osteoporosis suspects and sent to our institutes for the DPD assay. The samples were stored at −20 °C and processed within a week. Urinary creatinine was measured by the common Jaffé rate method.

2.2. Materials

Standards of protein amino acids, i.e. phenylalanine, tyrosine, tryptophan, cystine plus homocystine, cystathionine, 3,4-dehydroproline [internal standard (I.S.) in HPLC screening], *p*-chlorophenylalanine (I.S. in GC screening) and HP, dipeptides PHP and glycine–proline, as well as the reagents, i.e. ethyl chloroformate (ECF), *o*-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC), iodoacetamide, pyridine, mercaptoethanol, ethanol, acetonitrile, chloroform, isooctane (2,2,4-trimethylpentane) and others were obtained from Sigma–Aldrich (Praha, Czech Republic).

Fifty microliter sorbent tips filled with cation-exchanger were supplied by Phenomenex (Torrance, CA, USA). Positive displacement PD-tips of 0.5 and 1.25 ml volume were obtained from Brand (Wertheim, Germany). Tapered polypropylene 1.1 ml reaction vials were purchased from Continental Laboratory Products (San Diego, CA, USA).

For GC assay two media were prepared and stored in refrigerator for a number of weeks:

Eluting/reaction medium consisted of physiological solution, ethanol and pyridine mixed in a volume ratio of 7:4:1.

Derivatizing/extraction medium consisted of a mixture of chloroform, isooctane and ECF in a volume ratio of 9:4:1.

2.3. HPLC assay

Sample work-up, requiring 20 μl of urine only for PHP and HP determination, followed exactly the protocol described in the earlier paper [3]. The whole process of double-derivatization and sample dilution could be accomplished within 5 min. The conditions of the subsequent analysis were modified to fit an inland HPLC column.

2.4. Procedure for GC assay

To 100 μl of urine or, alternatively, to 100 μl of amino acid standards (10 nmol each) in 1% aqueous urea, an equal volume of I.S. solution (0.1 m*M* *p*-chlorophenylalanine in 50 m*M* aqueous HCl) was added to the 1.1 ml polypropylene (PP) vial. The

sorbent tip was attached to the 1.25 ml PD-tip and after immersing the tapered end into the fluid the content was sucked slowly through the exchanger bed by moving the piston in the PD-tip a few millimeters up. After sucking the fluid into the sorbent completely, 100 μl of water–ethanol (2:1) were added (without removing the equipment from the vial) and sucked in by further moving the piston up, more rapidly than before, to allow a subsequent draining of the bed. The PD-tip was disconnected and its content discarded. The 0.5 ml PD-tip with a slightly elevated piston of about 1 cm from the orifice was now attached to the sorbent tip. After adding 200 μl of the eluting medium into the same working vial, the content was sucked through the resin bed into the barrel of the PD-tip. The elution can be carried out rapidly like the previous resin wash and the resin bed is further drained by pulling air into it. Thereafter, the content of the PD-tip was emptied into the same or another vial and subjected to derivatization of the eluted analytes. This was simply done by adding 150 μl of the derivatizing/ extraction medium, and vortexing the two-phase system without any closure of the vial for about 5–7 s. During this action the lower organic phase usually clears from the initially cloudy one. Following addition of 100 μl 1 mol/l aqueous HCl and a brief vortexing for about 2–3 s, an aliquot of the lower organic phase was injected into the GC instrument. Alternatively, 100 μl of the lower phase were transferred into a low-volume glass insert of an Autosampler vial.

2.5. Chromatographic conditions

LC-10AD pump (Shimadzu, Kyoto, Japan), L-7480 fluorometric detector Merck–Hitachi (Darmstadt, Germany) with 260/330 nm wavelength setting, and a 150×3 mm I.D. strong anion-exchange column (Separon SGX AX; Tessek, Praha, Czech Republic) were employed for the HPLC analysis. The mobile phase used consisted of 100 mmol/l ammonium formate buffer (pH 2.7), acetonitrile and isopropanol, mixed in a ratio of 2:1:1. Separation of the FMOC-derivatized analytes, i.e. proline, PHP, HP and the I.S. (3,4-dehydroproline) succeeded in this elution order at 0.5 ml/min flow-rate within 7 min.

GC analysis employed a GC-17A instrument with FID and a AOC-20i Autoinjector, both from Shimadzu. For the separation of ECF-treated amino acids and peptides, a 7 m×0.25 mm fused-silica capillary column Zebron-50 (0.25 μm film thickness; originally delivered in a length of 15 m) from Phenomenex was used. It was run in a range of 170–320 °C/min at 30 °C/min temperature rise under a constant velocity mode. Injector and FID temperatures were 270 and 320 °C, hydrogen served as carrier gas under head pressure of 20 kPa (corresponding to 1.1 ml/min or 46 cm/s column flow-rate). Split injection (2 μl) was performed at a doubled-pressure of 40 kPa applied for 0.2 min (1:10 split-ratio). A Siltek-deactivated split liner (no. 2086) with Siltek wool supplied by Restek (Bellefonte, PA, USA) was employed.

CSW software version 1.7 (DataApex Ltd, Praha, Czech Republic) was employed for a computer-assisted work-up of the chromatographic data.

3. Results and discussion

3.1. Comments to the procedures

Both the approaches of PHP screening in urine, the established one using HPLC with fluorometric detection and the novel one based on GC with FID, proved to be rapid and reliable. The HPLC-assay takes the advantage of two reagents in the same reaction medium, where OPA-thiol is used for blocking the primary amino groups while FMOC is used for treating the secondary ones [6]. Using a wavelength setting relevant to FMOC-derivatized secondary amino acids the OPA-treated primary ones pass the fluorescence detector unrecorded so that they do not interfere in the assay.

The procedure developed for the GC determination employs a combination of a simple and fast solid-phase and liquid–liquid-phase extraction. The ability of chloroformates to derivatize polyfunctional acids in water-containing media nearly instantaneously [5] proved to be useful in treating eluent from the solid-phase extraction directly, without the need to evaporate the eluate. Thus, reaction medium for the subsequent chemical treatment can be advantageously used for elution of the captured amino compounds. The one-shot phase transfer derivatization is a modified version of that recently developed

for protein amino acids [7], tailored here for a selected group of amino acids and peptides. Concerning the composition of the organic phase, chloroform ensured effective extraction and isooctane aided in improving the derivatization yields. Such conditions suited perfectly not only for the two dipeptides, (glycine–proline was included as another product of bone collagen degradation [8]), but also for the aromatic and disulfidic amino acids and cystathionine (Fig. 1). However, without employment of mass spectrometry it could not be identified, whether some small peaks of the turbulent baseline belonged to glycine–proline or homocystine, the abundance of which fell below detection limits in common urine samples. Histidine, a highly abundant urinary amino acid, was also partly eluted from the exchanger under the chosen conditions and recorded (asterisk in Fig. 1).

3.2. Validation of the novel method

Regarding the solid-phase extraction, the uptake of amino compounds proved to be efficient enough to be largely independent of the flow velocity through the sorbent bed. Any lowered uptake was observed even when using a sucking rate shorter than 10 s, a rate commonly used. Since commercial cartridges used to be delivered with unnecessary large sorbent amounts, the sorbent tips proved to be more convenient and suitable for 100 μl or less of applied urine. Comparing responses of directly derivatized analytes with those obtained after passing the solid-phase extraction step recoveries of 97–108% were found for five of the analytes; a bit lower for tryptophan, cystathionine and homocystine, i.e. 89, 82 and 66%, respectively.

It was not of prime importance to find out whether conversion to the derivatives proceeded completely. However, comparable yields were obtained under conditions used for the class of protein amino acids [9] so that a nearly quantitative conversion can be expected. The Siltek-deactivated liner from Restek proved to be superior over some others tested.

Imprecision of the results in terms of coefficients of variation (C.V.), evaluated by means of five independently prepared standard samples, provided a value of 4.62% for PHP. The C.V. values for cystathionine, cystine and glycin–proline were close to that value, being lower for the aromatic analytes (2.77%) and higher for homocystine (7.34%). Repeated analyses of pooled urine samples with low and high PHP abundance gave values of 3.82±0.28 and 12.53±0.63 mmol/mol creatinine, respectively.

Reliability of the procedure was checked by spiking urine with 10–200 nmol PHP/ml urine (approximately 1–20 mmol/mol creatinine). Recoveries were between 90 and 114%, with an average value of 101%. Linearity was checked in the same range and provided regression data of $r=0.998$ and $y=0.0924x-0.011$. Detection limit of a still reliable quantitation of PHP with FID in 100 μl or less urine was about 10 μmol PHP/l urine.

The procedure proved to be sufficiently robust. The yields proved to be largely independent from the parameters of the two consecutive extraction steps. Selectivity with FID required prevention of any peak overlapping, while employment of mass spectrometric detection with selective ion monitoring would obviate this requirement.

3.3. Application into the field of bone collagen disorders

Regression analysis was employed to compare results obtained by the established HPLC method and the newly elaborated GC–FID determination of PHP. Second-void morning non-hydrolyzed urine samples of 133 subjects, mostly women after menopause, were screened as described in the Experimental section. With a reasonably high degree of correlation, (determination coefficient equal to 0.89), both the approaches afforded a reliable quantitation of the PHP dipeptide (Fig. 2). As apparent from the plot, there is a higher density of values in the lower concentration range. Using a cut-off value of 10, approximately two thirds of the subjects provided a mean and biological range (defined as 5 to 95th percentiles) of 6.30±1.90 with the LC and 6.69±2.23 mmol PHP/mol creatinine with the GC assay. The higher-level values were 14.52±3.50 with LC and 13.81±3.62 with GC, respectively. Significance of the findings for a possible screening of bone turnover disorders is currently under study.

To summarize, the simple and cost-effective assay

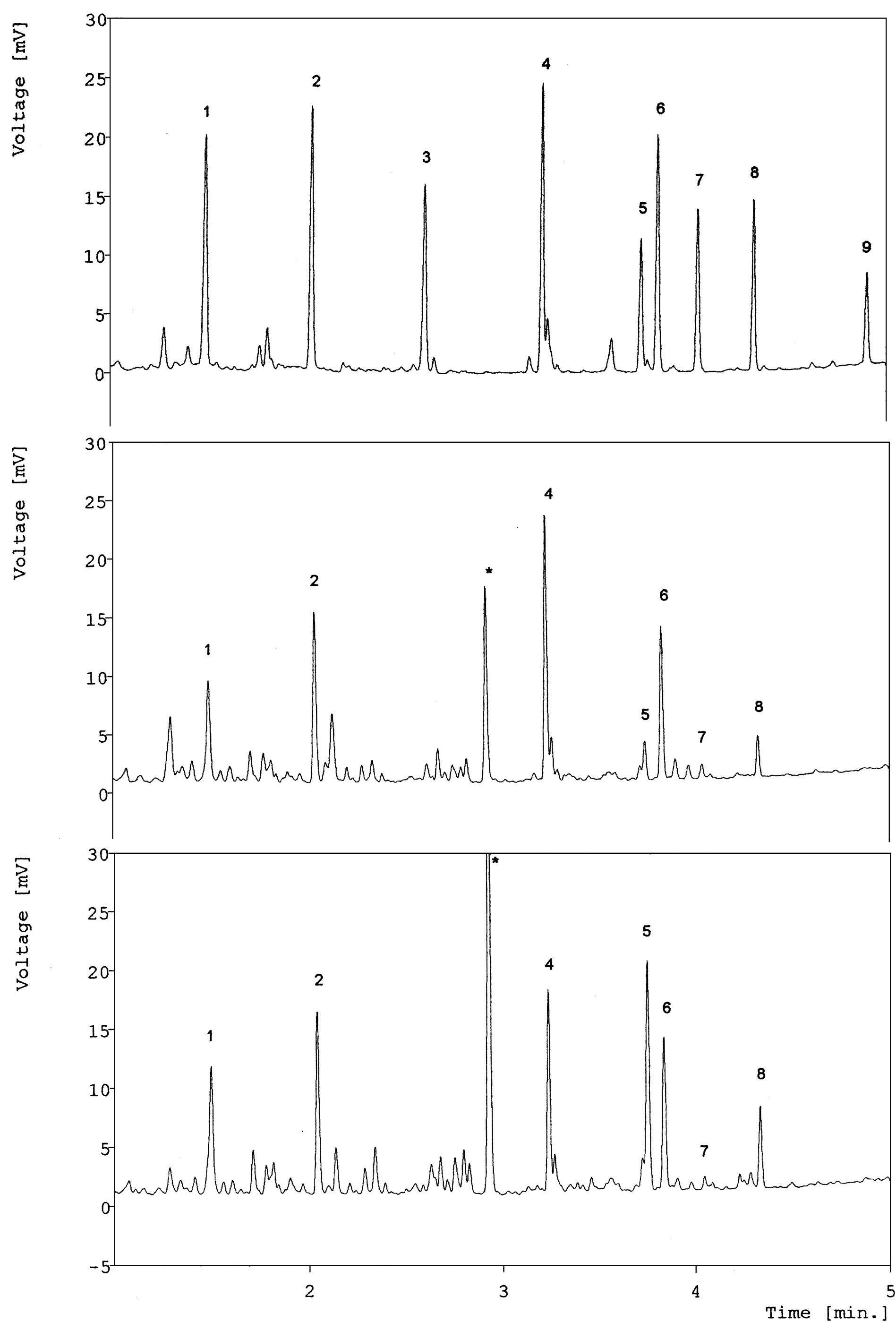

Fig. 1. GC–FID analysis of amino acids and dipeptides after a solid-phase extraction and phase-transfer derivatization using ECF. From top to bottom: equimolar mixture of standards (initial amount 10 nmol each) and urine samples (each 100 μl) with a lower and a higher abundance of PHP (i.e. 4 and 14 mmol/mol creatinine). Analytes: 1, phenylalanine; 2, *p*-chlorophenylalanine (I.S., 100 nmol/ml added to urine); 3, glycine–proline; 4, tyrosine; 5, PHP; 6, tryptophan; 7, cystathionine; 8, cystine; 9, homocystine.

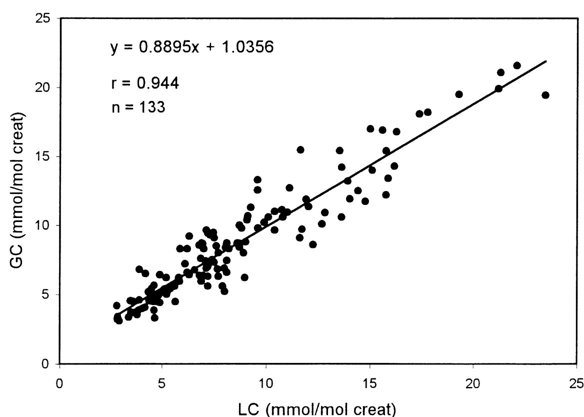

Fig. 2. Correlation diagram of PHP assay by HPLC and GC in morning urine samples of 133 subjects, mostly postmenopausal women (osteoporosis suspects).

of PHP in non-hydrolyzed urine samples using LC or GC could provide a mean for rapidly measuring bone resorption rate in the clinical routine, perspectively under full automation.

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