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DOTTORATO DI RICERCA IN BIOCHIMICA
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TESI DI DOTTORATO DI RICERCA

A proposed new internal standard for free, glycosylated and total pyridinium crosslinks quantification in healthy women and children urine: validation of an HPLC-fluorescence method.

BIO/10

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SUMMARY

Emerging evidence supports the concept that biochemical markers are clinically useful as non invasive diagnostic tools for the monitoring of changes in bone and cartilage turnover in subjects affected by pathologies with destructive bone and joint diseases such as osteoporosis, osteoarthritis, rheumatoid arthritis and osteosarcoma. Epidemiological studies demonstrated that the measurements of different bone degradation products in urine or serum samples are higher in patients affected by the above reported pathologies compared with healthy subjects.

Among the different biochemical markers used for clinical purpose, the changing in urine concentrations of pyridinium crosslinks is usually measured. Pyridinium crosslinks are generally referred to the chemical compounds pyridinoline (Pyr) and deoxypyridinoline (D-Pyr), both considered specific markers of skeletal collagen degradation. They are released into the circulation during bone resorption as small peptides that can be further degradated into free crosslinks in kidney and excreted in urine in both free and peptides or sugars bound forms as the disaccharide glucose-galactose Pyr (GluGal-Pyr) and the monosaccharide galactose Pyr (Gal-Pyr), both formed from a post-translation modification of collagen. The first sugar bound form, reported absent in bone by some authors, is considered an important predictor of joint tissue degradation, whereas for the second one, mainly present in bone, no specific role has been found and it has never been measured.

Commonly, only the total Pyr and D-Pyr (free+bound forms) amounts, obtained from urine submitted to overnight acid hydrolysis, are measured as markers of bone resorption by high-performance liquid chromatography (HPLC) coupled to a fluorescence detector. However, the variability of literature results, depending on analytical problems (hydrolysis, solide-phase purification, recovery) and physiological factors (age, gender) renders the results from different analytical laboratories difficult to be compared. Moreover, results obtained from different analytical methods are often compromised by the lack of an adequate internal standard allowing to eliminate errors occurring during sample preparation and analytical measurement.

In spite of extensive literature, important points for crosslinks measurement have not been solved:

1) the choice of an adequate internal standard, required by many researchers working in this field, that allows the specificity of the analytical method and avoids errors occurring during all the pre-analytical steps;

2) the pure authentic reference standards, obtained by synthesis, to be used as primary calibrators necessary to improve the crosslinks quantification process;
3) the evaluation of crosslinks in their total (free + bound forms) and/or free forms. In fact the assessment of total urinary D-Pyr, found only in bone and dentine, provides an index of bone resorption whereas the evaluation of total Pyr does not allow to distinguish among the degradation of bone, synovium and joint and between free and glycosylated-Pyr.

The aim of the present study was to develop and validate an HPLC-fluorescence method for the measurement of Pyr and D-Pyr in free, glycosylated and total forms in healthy women and in children urine. Women were selected to compare our results with those reported by others in adult healthy groups. Children were enrolled as, being in growing age and having an extremely high remodelling rate, their physiological levels of urine collagen crosslinks in this stage of the life appeared of particular interest to be compared with those of an omogeneous group of pre-menopauseal women. Moreover, studies on children were performed in order to estimate GluGal-Pyr and Gal-Pyr amounts as these glycosylated pyridinolines have never been measured until now.

The specificity of the method was assured by a synthesized superior homologue of D-Pyr, here proposed as internal standard (IS) for the first time, added to the biological samples before any pre-analytical step. Moreover, pure synthesized Pyr, D-Pyr, Gal-Pyr and GluGal-Pyr were used as primary calibrators for the correct HPLC identification of each analyte by their corresponding retention times and for their accurate quantification by specific calibration curves.

To validate the method for the measurement of all the crosslinks of interest, various analytical performance parameters (linearity, recovery, within and between-day repeatabilities, reproducibility, limit of detection, limit of quantitation) have been considered and here reported.

The results demonstrate, for the validated method, good linearity, recovery, precision, limit of detection and limit of quantitation for all compounds.

The measurement of consisting amounts of GluGal-Pyr in both women and children urine and few but detectable Gal-Pyr amounts only in children confirm the importance for free and glycosylated crosslinks quantification. In fact, only the evaluation of free glycosylated and total crosslinks excretion can provide more information on different collagen catabolism, since total crosslinks amounts give no information on the intermediate products (pyridinoline glycosides) generated by various collagen degradations.

INTRODUCTION

Bone formation and matrix components

Bone is a living and dynamic tissue formed by cells (osteoclasts and osteoblasts) surrounded by an extracellular calcified amorphous matrix containing fibers and glycoproteins. The calcifying mineral compound [hydroxyapatite; $Ca_{10}(PO_4)_6(OH)_2$] confers hardness and mechanic resistance to bone, whereas fibers affect the flexibility. Bone undergoes two opposing processes determining the bone density, i.e bone formation (by osteoblasts) and bone resorption (by osteoclasts).

Osteoblasts, under the control of osteoclasts, synthesize in the endoplasmic reticulum both fibers and glycoproteins of the amorphous matrix, but the final matrix chemical structure is completed out of cells (1). Alkaline phosphatase, an enzyme able to split the phosphoric bounds of pyrophosphate, a physiological inhibitor of bone formation, is active in osteoblasts with specific membrane's receptors for estrogens. On the contrary, an acidic phosphatase enzyme, able to destroy the bone calcified matrix, is present in osteoclasts with specific membrane's receptors for parathyroid hormone.

During youth, the osteoblasts activity is higher than the osteoclasts one, during the middle-age the two processes are in equilibrium, whereas in the old-age the bone resorption is greater than bone formation. About 90% of total adult bone mass is accrued by age 20, and a significant proportion of this is achieved only during puberty (2).

Collagen and elastine are the components of the fibrous bone extracellular matrix. Collagen, the most abundant protein in the human body, constitutes approximately 30% of the body proteins, with up to 40% in skin and 50% in bone. Tropocollagen, the structural unit of collagen, is formed by three similar or different α-polypeptide chains linked by hydrogen bounds and with amino- and carboxyl- terminal groups. After their synthesis in the endoplasmic reticulum, the three tropocollagen subunits associate to build a right-handed triple helix. However, before this step, the nascent collagen polypeptide undergoes several post-translational modifications, comprising the enzymatic hydroxylation of proline and lysine residues by hydroxylases. Collagen is characterized by domains representing repeats of the triplet Gly-X-Y, where proline and lysine are often found at positions X and Y; approximately 100 proline and 10 lysine residues are present in every chain (3,4).

Hydroxyproline and hydroxylysine contribute to the stability of the collagen triple helix, where hydroxylysine is essential for the crosslinking of collagen molecules, ensuring the

strength of collagen fibrils. Hydroxylysine and hydroxyproline can be further modified by reaction with the specific β(1-*O*) galactosyl and α(1-2) glucosyl transferase, enzymes forming the glycosylated derivatives galactose-hydroxylysine and glucose-galactose hydroxylysine (5). In these two glycosylated derivatives, glucose and galactose are linked by β-glycosidic bonds to the hydroxyl groups of hydroxylysine residues. After the hydroxylation reactions, three pro-alpha chains fold together and procollagen molecules are excreted by the cells into extracellular space where the two non-helical extensions, called N- and C-terminal procollagen peptides are cleaved off by two specific peptidases (6).

The importance of post-translational modifications of collagen is reflected in diseases caused by defective collagen modifying enzymes giving a deglycosylated collagen form. Mutations of the lysyl hydrolase lead to connective tissue disorders (7-9).

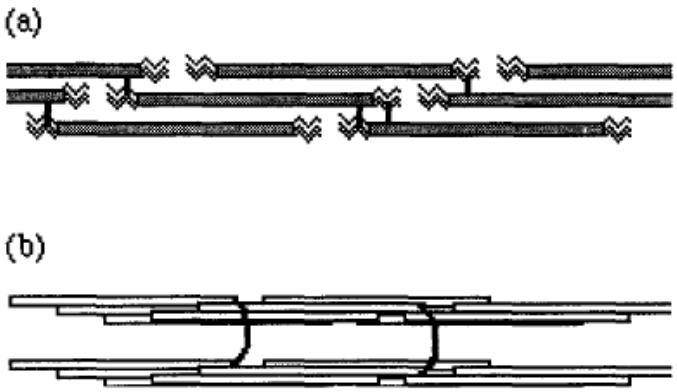
Collagen crosslinks

After the release from bone cells in the extracellular matrix, collagen molecules are stabilized by crosslinking aminoacids generating different bounds inside and among collagen molecules (10).

The intramolecular crosslinks, in which two α chains within the same molecule may be covalently linked, are mainly bifunctional. They are present in newly formed collagen and are also called reduced crosslinks as they may be analitically investigated by mild chemical reduction utilizing borohydride. The integrity and the physical-chemical properties of bone are depending on these bounds between adjacent collagen fibrils. For fibrous collagen, these bonds are conferring a very high tensile strength.

The intermolecular crosslinks, involving the formation of covalent bridges between chains in different molecules, are mainly trifunctional. They are present in mature tissue and cannot be reduced (they are referred as non-reducible mature crosslinks). These covalent crosslinks of collagen molecules begin with the COOH-terminal non α-helical region and this step may occur before intramolecular crosslinking formation (11) (Fig.1).

Fig.1 Schematic rapresentation of the location of immature and mature enzymatic crosslinks



(a) Immature, bivalent crosslinks within microfibrils (b) Mature microfibrils illustrating the interfibrillar linkage of collagen molecules in parallel allignment

To date, about 29 types of collagen have been identified and described, which are encoded by at least 44 genes (5,9) and are characterized by different α-chains bounded together. Depending on the collagen type, the three α chains can be either identical or the molecule can contain two or even three different α chains. Type I collagen is formed by two chains α1 and one chain α2, whereas Type II collagen is formed by three chains α1. Type I is ubiquitously distributed and forms the major protein in skin, bone, tendon, ligament, sclera, cornea, blood vessels but some collagens have a districted tissue distribution: for example, as Type II, IX and XI found almost exclusively in cartilage and Type X, found only in hypertrophic and mineralizing cartilage. (Tab. 1)

Tab. 1 Types of collagen and associated disorders

Type	Notes (location)	Gene(s)	Disorders
I	This is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. It is found in tendons, skin, artery walls, the endomysium of myofibrils, fibrocartilage, and the organic part of bones and teeth.	COL1A1 COL1A2	osteogenesis imperfecta, Ehlers-Danlos Syndrome, Infantile cortical hyperostosis aka Caffey's disease
II	Hyaline cartilage, makes up 50% of all cartilage protein. Vitreous humour of the eye.	COL2A1	Collagenopathy, types II and XI
III	This is the collagen of granulation tissue, and is produced quickly by young fibroblasts before the tougher type I collagen is synthesized. Reticular fiber. Also found in artery walls, skin, intestines and the uterus	COL3A1	Ehlers-Danlos Syndrome
IV	basal lamina; eye lens. Also serves as part of the filtration system in capillaries and the glomeruli of nephron in the kidney.	COL4A1 COL4A2 COL4A3 COL4A4 COL4A5 COL4A6	Alport syndrome, Goodpasture's syndrome
V	most interstitial tissue, assoc. with type I, associated with placenta	COL6A1 COL6A2 COL6A3	Ehlers-Danlos syndrome (Classical)
VI	most interstitial tissue, assoc. with type I	COL6A1 COL6A2 COL6A3	Ulrich myopathy and Bethlem myopathy
VII	forms anchoring fibrils in dermal epidermal junctions	COL7A1	epidermolysis bullosa dystrophica
VIII	some endothelial cells	COL8A1 COL8A2	Posterior polymorphous corneal dystrophy 2
IX	FACIT collagen*, cartilage, assoc. with type II and XI fibrils	COL9A1 COL9A2 COL9A3	EDM2 and EDM3
X	hypertrophic and mineralizing cartilage	COL10A1	Schmid metaphyseal dysplasia
XI	Cartilage	COL11A1 COL11A2	Collagenopathy, types II and XI

* FACIT collagen: Fibril Associated Collagens with Interrupted Triple helices.

Physiological and pathological bone resorption

Bone mass may be influenced by several factors that can be grouped into factors that cannot be modified, such as gender, age, genetics and ethnicity, and those that can be modified, as lifestyle including physical activity levels, smoking, alcohol consumption and diet (12). Under physiological conditions, extracellular matrix production balances the breakdown, thereby ensuring the continuous renewal of this critical tissue component. When the bone formation cannot keep up with the degradation processes, a loss of the structural integrity of bone and/or cartilage emerges as a net result (10). From this unbalance, the skeletal fragility can result due to: (a) a failure to produce a skeleton of optimal mass and strength during growth; (b) an excessive bone resorption resulting in decreased bone mass and microarchitectural deterioration of the skeleton; and (c) an inadequate formation response to increased resorption during bone remodelling. The high rates of resorption are not always associated with bone loss, for example, during the pubertal growth.

Drastic bone and/or cartilage resorptions have been observed and studied in pathological conditions such as osteoporosis, osteoarthritis, rheumatoid arthritis, osteosarcoma and joint desease (13-17). The first three diseases are shortly described below.

Osteoporosis is a global health problem that will take on increasing significance as people live longer and the world's population continues to increase. Moreover, it is also a socio-economic problem because long-term therapy is needed to prevent fractures and chronic disability. Osteoporosis is defined as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture particularly common in the spine, hip, and distal forearm (14).

Rheumatoid arthritis and osteoarthritis are severe chronic debilitating diseases with articular cartilage destruction, loss of periferal joint function and consequent pain, impaired mobility and disability. Rheumatoid arthritis is an autoimmune disease with chronic inflammation of the joints characterized by an influx of inflammatory cells like monocytes, macrophages and fibroblasts into the synovial fluid with subsequent release of pro-inflammatory agents and induction of cartilage degradation through activation of different proteases (16,17). Osteoarthritis, a prevalent age-related disease with a strong genetic component, is characterized by progressive destruction of articular cartilage.

Markers of bone turnover for clinical investigations.

Clinical information on bone health and/or skeletal diseases and the monitoring of the response to pharmacological treatments are achieved measuring specific markers of bone turnover in serum or in urine samples. Usually, these markers are the following:

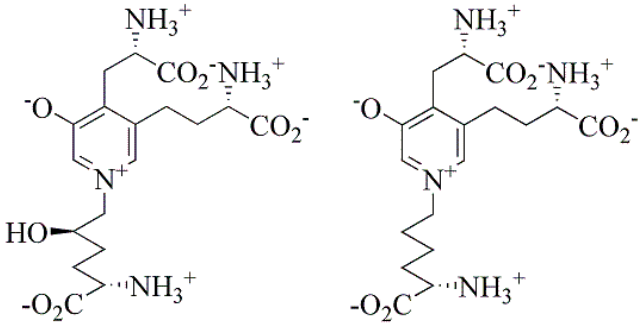
- Bone formation markers:
    1) Serum alkaline phosphatase: active enzyme in osteoblasts, ables to split the phosphoric bounds of pyrophosphate, a physiological inhibitor of bone formation. This enzyme originates from various tissues, such as liver, bone, intestine and kidney. Its enzymatic activity is measured by radioimmunological or immunoassay methods with monoclonal antibodies.
    2) Serum osteocalcin: non collagenic small protein (49 amino acids) synthesized by osteoblasts and incorporated into the extracellular matrix. It is known as bone Gla-protein because it contains three vitamin-K-dependent γ-carboxyglutamic acid (Gla) residues which serve as calcium binding sites and may be involved in bone mineralization.
    3) Serum procollagen type 1 propeptides: procollagen type 1 contains N- and C-terminal extensions, (called P1NP and P1CP, respectively), which are removed by specific proteases during the extracellular conversion of procollagen to collagen. During bone resorption, these short fragments, still crosslinked through Pyr and D-Pyr and called telopeptides, are cleaved by osteoclasts and enter the circulation. Radioimmunological or immunoassay methods are used for their evaluation.

- Bone resorption markers:
    1) Acidic phosphatase: active enzyme in osteoclasts ables to destroy the bone calcified matrix.
    2) Measurement of urine calcium concentration.
    3) Urinary hydroxyproline: this aminoacid represents 13% of the total collagen aminoacids and is released in serum and urine in free and bound forms.
    4) Pyridinium crosslinks: pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) form covalent crosslink bonds among adjacent collagen chains stabilizing the extracellular matrix.

Markers for bone resorption: pyridinium crosslinks

The pyridinium crosslinks, known as pyridinoline (Pyr: hydroxylysyl-pyridinoline) and deoxy- pyridinoline (D-Pyr: lysyl-pyridinoline) (Fig.2), are the main non-reducible fluorescent trifunctional crosslinks in mature skeletal tissues.

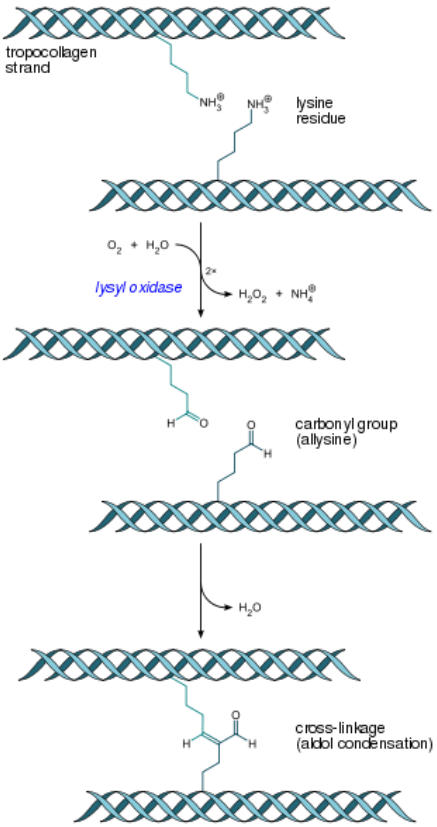
Fig.2 Pyr and D-Pyr structural formulas



Pyridinoline is formed by the reaction of two hydroxyallysines with an hydroxylysine residue, while deoxypyridinoline by the reaction of two hydroxyallysines and a lysine residue.

Several non-enzymatic mechanisms have been proposed for Pyr and D-Pyr biosynthesis; a reaction between two hydroxyallysine and one hydroxylysine residue or an aldol condensation of hydroxyallysine with a divalent crosslink followed by an intramolecular condensation (10,18) (Fig.3).

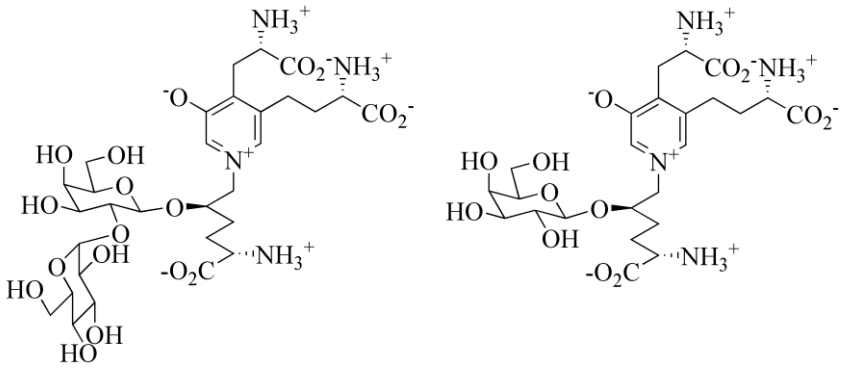
Fig.3 Intramolecular aldol condensation



As a consequence of bone resorption, both Pyr and D-Pyr are released into the circulation as small peptides that can be further degradated into free crosslinks in kidney and excreted in urine in both free (~ 40%) and peptides or sugars bound forms (~ 60%) (14,19,20-22). They are widely accepted as specific markers of skeletal collagen degradation (22-27), as they are not reused for new collagen synthesis or metabolized in the liver. Pyridinoline, the most abundant, has been found in bone, cartilage, tendon, and some other connective tissues, but not in skin (19). On the contrary D-Pyr is almost exclusively present in bone and dentine where its concentration is about one fourth of that of Pyr (19).

Some studies involved as degradation markers also two Pyr analogues deriving from post-translational modification of collagen: glucose-galactose Pyr (GluGal-Pyr) and the galactose Pyr (Gal-Pyr) (18,21,22,27-32) (Fig.4).

Fig.4. Glucose-galactose-Pyr and galactose-Pyr chemical structures



As reported in Table 2, both Pyr and D-Pyr have been found in bone (Type I collagen), Pyr in cartilage (Type II collagen), Pyr and GluGal-Pyr in synovium (Type III collagen) (29,33), whereas Gal-Pyr mainly in bone (31). GluGal-Pyr is considered an important predictor of joint tissue degradation being found associated with pain and physical function and increased in urine of patients with osteoarthritis (19) and in synovial tissue (31), but is absent from bone tissue (31,32). In patients with early rheumatoid arthritis, a 70-100% increase of GluGal-Pyr in urine was observed respect to healthy controls (32). As concern Gal-Pyr, its presence in synovium and bone has only been observed in HPLC chromatograms, but its concentration has never been measured (32).

Tab.2 Pyridinium crosslinks markers of bone, cartilage and synovium turnover

Tissue (Collagen)	Degradation Compounds
Bone (Type I)	Pyr + D-Pyr + Gal-Pyr
Cartilage (Type II)	Pyr
Synovium (Type III)	Pyr + GluGal-Pyr

The molar ratio of Pyr to D-Pyr in urine is very similar to that found in bone (19,20) indicating that both Pyr and D-Pyr are derived mainly from bone. However, D-Pyr would be a more appropriate bone marker respect to Pyr for patients with cartilage disease (e.g. rheumatoid arthritis) who have a significant increase in Pyr from non-bone tissue. Some variables such as age, gender, ethnicity and body mass index may influence background levels of these biomarkers. Moreover, levels of the bone turnover markers are influenced by a marked circadian variation (34), whereas cartilage markers show little diurnal variability (13).

Among compounds forming crosslink bounds, there are also hydroxyproline and hydroxylysine that, as Pyr and D-Pyr, are excreted in urine after bone resorption as free or glycosylated (linked with glucose or glucose-galactose). However, hydroxyproline cannot be considered as a specific marker of collagen breakdown since it derives from both the degradation of newly synthesized collagens originated from different tissues other than bone and the diet. Moreover, most of the free hydroxyproline amount is further metabolized to other products; thus only a fraction of the totally produced hydroxyproline is recovered in urine (4,35). Regarding hydroxylysine, studies reported its higher concentration in skin than in bone, therefore this compound is thought to reflect skin collagen metabolism rather than bone metabolism (6,35,36). However, hydroxylysine and its glucose-galactose conjugated compound are commonly measured in children and adolescents urine to have clinical information on their skeletal health (35-38).

Analytical methods for pyridinium crosslinks measurement

Usually, the analytical procedures submit urine to an overnight acid hydrolysis, allowing the evaluation of total Pyr and D-Pyr (15,28,34,41,42), whereas only few authors quantified free urinary pyridinolines amounts (16,27,39,40).

Currently, high-performance liquid chromatography (HPLC) coupled to a fluorescence detector is a valid analytical method allowing the separation of Pyr and D-Pyr for quantitative determinations. This measurement takes advantage of fluorescent properties of these compounds but is a time-consuming method requiring extensive sample pre-treatment and purification steps before the HPLC analysis. Thus, for routine use, simple and less expensive immunoassays have been developed to measure urinary Pyr and D-Pyr. Commercially available methods include enzyme immune assays (EIA) specific for free

Pyr and D-Pyr, and radioimmuno assays (RIA) for total or free D-Pyr (25,39,41,43,44). D-Pyr is also measured by chemiluminescence immunoassay.

Requirements necessary for quantitative measurements of biological analytes: importance of the internal standard and of pure primary calibrators

The specificity of the existing analytical methods for the crosslinks evaluation is often compromised by the lack of an adequate internal standard and of pure synthesized primary calibrators, in particular for the glycosylated compounds. The internal standard is necessary to avoid errors occurring during all the pre-analytical steps (hydrolysis, solid-phase purification), whereas the primary calibrators are necessary to improve the analytes quantification process.

Thus, the variability of literature results on urine crosslinks amounts, depending also from physiological factors (age, gender) (26,34), renders the results from different analytical laboratories difficult to be compared (25,26,41).

Generally, an ideal internal standard has to be an analog of the target compound exibiting physico-chemical properties virtually identical and must be added in known amounts to the biological sample before any pre-analytical step. Moreover, it should be clearly resolved from the analyte that has to be measured, recovered from any extraction procedure in the same fraction as the analyte, stable under the assay conditions employed and absent in the biological matrix. Thus, being the partition coefficient for the standard and the analyte the same, any potential error caused by an inefficient and/or variable procedure is avoided and the ratio of metabolite to internal standard is unmodified during all the analytical procedures. Therefore, the use of an adequate internal standard guarantees good precision, specificity and reproducibility for the method, since it reduces problems of extraction recovery and biological matrix effects.

The need of an internal standard for pyridinium crosslinks measurement has been underlined by many authors (22,25-27,39). Moreover, as no reference standards of the compounds of interest are available, every laboratory performing crosslinks measurements use different compounds or primary calibrators, such as Pyr or D-Pyr, often isolated and prepared by each laboratory from various sources, including animal or human.

In attempt to search for a compounds useful as internal standard for crosslinks measurement, isodesmosine, propylated or acetylated pyridinoline and pyridoxine have been proposed as internal standards for crosslinks measurement (16,22,26,27,39,45). Isodesmosine, an elastine crosslink, is not suitable as an internal standard being

endogenously present in urine and recovered in the same extraction fraction as the crosslinks (46). Propylated and acetylated pyridinolines themselves hydrolyzed under acidic conditions, cannot be used for Pyr and D-Pyr total amounts evaluation, but eventually only for their free forms (16,45). Pyridoxine (Vitamine B6) is not suitable having a different chromatographic behaviour than the crosslinks. Pyridoxine has been also used as an external calibrator, added to biological samples after the hydrolysis reaction (24,27). Recently, an internal standard unknown from a chemical point of view to the authors as not specified by the factory (proprietary information), was used for total crosslinks evaluation by an automated HPLC method (42).

AIM OF THE STUDY

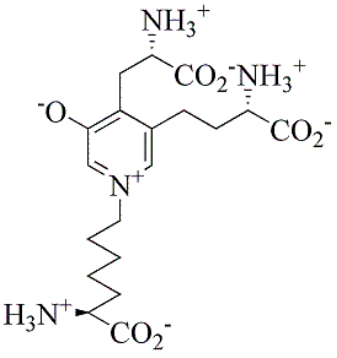
In spite of extensive literature on the crosslinks evaluation, some major points have still not been solved:

1) the choice of an adequate internal standard for the quantitative crosslinks measurement, required by many researchers working in this issue;
2) the pure authentic reference standards, obtained by synthesis, to be used as primary calibrators necessary to improve the crosslinks quantification process;
3) the evaluation of crosslinks in their total (free + bound forms) and/or free forms.

In fact, the assessment of total urinary D-Pyr, found only in bone and dentine (19), provides an index of bone resorption, whereas the evaluation of total Pyr does not allow to distinguish between bone, synovium and joint degradation and between free and glycosylated-Pyr.

In view of the above, the aim of the present study was to develop and validate an HPLC-fluorescence method for the quantification of both Pyr and D-Pyr in their free and total forms, together with Gal-Pyr and GluGal-Pyr in urine of healthy women and children. The specificity of the method was allowed by the use of a synthesized superior homologue of D-Pyr, having one carbon longer lysine side chain at the eterocyclic nitrogen, here proposed as internal standard (IS) for the first time (Fig. 5). The internal standard is added to the biological samples before any pre-analytical steps. Pure synthesized Pyr, D-Pyr, Gal-Pyr and GluGal-Pyr were used as primary calibrators for both the correct HPLC identification and quantification of each analyte by their corresponding calibration curves.

Fig.5. Chemical structure of the internal standard.



Internal Standard
(IS)

MATERIALS AND METHODS

Chemicals and Reagents

Methanol and acetonitrile (HPLC-grade), hydrochloric acid (37%), acetic acid, heptafluorobutyric acid (HFBA) and pentafluoropropionic acid (PFPA) were purchased from Sigma-Aldrich-Chemie GmbH (Steinheim, Germany). Microgranular cellulose powder (CC31 type) was from Whatman Inc (NJ, USA). Empty SPE columns (12 mL) with 20-μm frits and ReproSil-Pur Basic RP C18 column (200 x 3 mm, 5 μm) were from Maisch GmbH (Ammerbuch, Germany). RP Nucleosil 100 C18 column (200 x 2.1 mm, 5 μm) was from Teknokroma (S. Coop. C. Ltda, Barcelona). Both analytical columns were protected by a guard column ReproSil-Pur Basic C18 (10x3 mm, 5 μm) from Maisch GMBH (Ammerbuch, Germany). SPE vacuum manifold was from J.T. Baker (NJ; USA).

Primary calibrators and Internal standard

Pyr, D-Pyr, GluGal-Pyr, Gal-Pyr and IS were synthesized, purified and chemically characterized by Nuclear Magnetic Resonance (NMR) and Mass Spectrometry as previously reported (47-53) by Prof. Mario Anastasia and Prof. Pietro Allevi (Dipartimento di Chimica, Biochimica e Biotecnologie per la Medicina, Facoltà di Medicina e Chirurgia, Università degli Studi di Milano). For their chemical structures see Fig.2, 4 and 5.

The molar absorption coefficients (ε= L mol^{-1} cm^{-1}) in HCl 0.1 mol/L at 295 nm (λ_{max}) were: 6520 (Pyr), 6480 (D-Pyr), 6400 (Glugal-Pyr and Gal-Pyr) and 6490 (IS).

Instrument and HPLC analysis

The HPLC system included a LC pump 200 system (Perkin Elmer, U.K.) equipped with a Perkin Elmer LC 240 fluorescence detector with xenon lamp (Perkin Elmer, U.K.). Data were integrated using Turbo Chrom Navigator connected with an interface NCI 900 series (Perkin Elmer, U.K.).

Two HPLC columns were tested: 1) Nucleosil 100 RP C18 and 2) ReproSil-Pur Basic C18. Various mobile phases added with ion-pairing agents as pentafluoropropionic acid (PFPA) or heptafluorobutyric acid (HFBA), were compared (22,23,39). Firstly, we tested an aqueous PFPA solution (0.009 mol/L) as mobile phase at 0.4, 0.5, 0.6 mL/min flow rate

but no adequate separation of all the peaks was reached. No better results were obtained using as mobile phase an aqueous PFPA solution (0.009 mol/L) in H_2O:ACN (90:10, 95:5, 98:2) as GluGal-Pyr, Gal-Pyr and Pyr peaks were overlapped or IS and D-Pyr peaks not well resolved or too long retention time were observed. Even the use of aqueous PFPA solution (0.009 mol/L) in H_2O:MeOH (97:3) at 0.2 or 0.6 mL/min as flow rate did not improve the peaks resolution.

The best HPLC crosslinks resolution was obtained in isocratic assay using ReproSil-Pur Basic C18 column with aqueous HFBA solution (0.015 mol/L): acetonitrile (88:12) as mobile phase at 0.6 mL/min flow rate with the fluorescence detector set at λ_{ex} 295 nm and λ_{em} 400 nm. The total runtime for each analysis was 35 min.

The identification of all peaks was made by the absolute retention times of the corresponding pure synthesized crosslinks. Every 3-4 samples injected, the column was washed with acetonitrile and reconditioned with the mobile phase. Each analyte was quantified using its calibration curve.

Standard and Working Solutions

Standard stock solutions were prepared by dissolving weighed pure standards amounts in 0.1 mol/L HCl, aliquoted and stored at –20°C. The final concentrations, confirmed reading the absorbance values were: D-Pyr ($9.50x10^{-5}$ mol/L), Pyr ($1.20x10^{-4}$ mol/L), GluGal-Pyr ($1.06x10^{-4}$ mol/L), Gal-Pyr ($2.04x10^{-4}$ mol/L) and IS ($1.20x10^{-4}$ mol/L).

Working standard solutions were prepared by diluting the standard stock solutions with aqueous HFBA solution (0.015 mol/L), aliquoted and stored at -20°C until use. The final working solutions concentrations were: D-Pyr and Pyr (26.60 μmol/L), GluGal-Pyr (26.62 μmol/L), Gal-Pyr (26.75 μmol/L) and IS (26.67 μmol/L).

Subjects and urine collection

Twenty healthy women volunteers (n=20, 33.9±7.1 years) and twenty girls (n=10; 5.8±0.8 years, n=10; 9.6±0.5 years), all in good general health, were included in this study.

Informed consent was obtained from the subjects themselves or parents, respectively. All subjects had no history of metabolic bone diseases and all pre-menopausal women were receiving no medications affecting calcium absorption and metabolism.

Fasting first-time urine was collected in the morning, aliquoted and stored at -40°C until analysis. Urines from healthy women (n=7; 35.6±7.6 years) were pooled, aliquoted, stored at -40°C and used for urine calibration curves preparation and for studying the hydrolysis reaction.

Free and glycosylated crosslinks extraction from urine

Different reported conditions for urine crosslinks purification by solid phase extraction (SPE) were compared (23,39). The following variables were tested:

-Cellulose amount for SPE column: 100, 400, 800 mg

-Washing solution to elute urine interfering compounds :

1) acetonitrile: acetic acid: water (4:1:1; v/v/v), without or with tetrahydrofuran (1 mL) (39). Different elution volumes of this mixture were used (10, 20, 30, 40 mL).
2) butanol: acetic acid: water (4:1:1; v/v/v), without or with methanol (0.5 or 1 ml) (23). Different elution volumes of this mixture were used (10, 20, 30, 40 mL).

The best results were obtained according to Space et al. (23) with some modifications. The SPE procedure was performed under vacuum (SPE vacuum manifold). Cellulose-packed column was prepared as follows: cellulose (800 mg), suspended in a solution of aqueous acetic acid in 1-butanol (8 mL; 1-butanol, water, acetic acid; 4:1:1; v/v/v; named butanolic solution), was loaded onto a column and allowed to settle. Then, it was washed with the same butanolic solution (2 mL) and the bed top protected by a filter disk. For free crosslinks extraction, urine (1mL), spiked with IS (210 pmol/mL), was mixed with an acidic solution (7 mL) of methanol, HCl (37%), acetic acid and 1-butanol (1:1:1:4; v/v/v/v) and loaded onto the cellulose-packed column. Then the column was eluted with butanolic solution (10 mL x 2) to eliminate all interfering fluorophores whereas the crosslinks were eluted with distilled water (4 mL). The obtained aqueous phase was centrifuged, the upper butanolic layer removed, water phase dried, the residue redissolved with aqueous HFBA solution (0.1 mL; 0.015 mol/L) and injected (20 µL) for HPLC analysis.

Method validation

Various analytical performance parameters need to be considered for the validation of the proposed method thus, three different calibration curves were prepared using pure synthesized primary calibrators and IS.

Calibration curves:

- Pure standard calibration curves (Curves 1): samples were prepared by adding increasing amounts of Pyr, D-Pyr, GluGal-Pyr, Gal-Pyr working standard solutions (0-10.5-21-52-105-210-420-840 pmol/mL) and a constant IS amount (210 pmol/mL) to obtain a ratio (pmol analyte/pmol IS) corresponding to 0-0.05-0.1-0.25-0.5-1.0-2.0-4.0. Samples were dried and redissolved with aqueous HFBA solution (0.1 mL; 0.015 mol/L). The SPE procedures were omitted and samples were directly injected for HPLC analyses.
- Urine calibration curves (Curves 2): pooled urine samples from healthy women (1 mL) were spiked with increasing amounts of pure working standard solutions (0-52-105-210-420-840 pmol/mL) and with a constant IS amount (210 pmol/mL) to obtain a ratio (pmol compound/pmol IS) corresponding to 0-0.25-0.5-1.0-2.0-4.0. Due to the presence of Pyr, D-Pyr and GluGal-Pyr in endogenous amounts in urine, samples at 0.05 and 0.1 ratio (pmol compound/pmol IS) were omitted in contrast to Curve 1. Each urine calibration sample was subjected to the full SPE procedure as we reported for free and glycosylated crosslinks extraction.
- Water calibration curves (Curves 3): water samples (1 ml) were added with IS and working standard solutions as reported for Curve 2 and then subjected to the full SPE procedure.

The three calibration curves were prepared in triplicate, each sample was dried, the residue redissolved in aqueous HFBA solution (0.1 mL; 0.015 mol/L) and injected twice (20 μL) as reported for Curve 1 samples. The maximum calibration curve concentration was higher than 70-120% of the urine basal value of all the analytes measured, as required for analytical method performance (55,56). These curves were usefull to determine, for all the analytes, the linearity, recovery, precision, detection limit (LOD) and quantification limit (LOQ) of the assay.

-Linearity of the method

For linearity calculation, least-square regression analysis was performed by plotting the peak area ratio (A/A_{IS}) of each analyte to that of the IS (Y-axis) against the ratio of known added amounts of each analyte to that of IS (pmol compound/pmol IS) (X-axis).
The concentrations of each analyte in urine were calculated using the parameters (slope and intercept) of the corresponding calibration curve. The used regression line was: $Y=aX + b$ (a: slope, b: intercept on Y axis).
The comparison among the slopes obtained from Curve 2 and 3 was used to exclude any matrix effect for all the measured compounds.

-Recovery (accuracy)

To test the recovery of each compound from the biological matrix, peak area ratio (A/A_{IS}) of urine samples (Curve 2), at low and high concentrations of analytes corresponding to a ratio of 0.25 (52 pmol compound/210 pmol IS) and 2.0 (420 pmol compound/210 pmol IS), were compared with Curve 1 samples at the same ratio containing the analytes at the theoretical maximum amount representing 100% recovery as not submitted to the SPE procedure, but directly injected.
The percentage of analytes recovery for both low and high concentrations was calculated as the mean A/A_{IS} value in urine added with primary calibrators subtracted by the mean A/A_{IS} basal urine value and divided by the mean A/A_{IS} of the known added concentrations of the primary calibrators directly injected and multiplied by 100.

-Precision of the method

Within-day and between-day precisions of the method were assessed by analysing Curve 1 samples seven times on the same day and once daily in seven consecutive days, respectively. The precision of the method is reported as percentage of coefficient of variation (CV %), calculated at two concentrations of all the analytes corresponding to a ratio of 0.25 and 2.0 as those used for recovery. The CV% was calculated according to the formula: SD/mean values x 100, where SD is the standard deviation of the response.

-Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) for each analyte, expressed as a concentration (pmol/mL), obtained by injecting Curve 1 sample at 0.1 ratio (21 pmol compound/210 pmol IS) once daily in seven different days, were estimated based on the standard deviation of the response and the slope of calibration Curve 1. The formulas 3.3 (SD/S) and 10 (SD/S) were used for LOD and LOQ, respectively, where SD is the standard deviation of the response and S is the slope of the analyte corresponding calibration curve.

Acid hydrolysis conditions for total Pyr and D-Pyr evaluation in urine

To find the best conditions for the hydrolysis reaction, the following variables were tested:

- HCl final concentration: 6 mol/L and 3 mol/L
- Hydrolysis time: 2, 4, 6, 8, 12 and 15 hours

The hydrolysis reactions, for water and urine pool samples (1 mL), were carried out using sealed Pyrex ampoules in an heating block system at 110°C.

1) Water sample hydrolysis was performed to evaluate and confirm the chemical stability of both internal standard and primary calibrators to the drastic used conditions and to evidence the desappearance in time of the glycosylated crosslinks due to their complete hydrolysis.

Water samples (1 mL) were prepared by adding Pyr and D-Pyr, both at 210 pmol/mL, or GluGal-Pyr and Gal-Pyr, both at 210 pmol/mL, and IS (210 pmol/mL) (ratio 1:1:1 for crosslinks and IS).

Water samples were hydrolyzed in the presence of two different HCl concentrations obtained by adding HCl 37% (1 mL) to water (1 mL) (HCl final concentration: 6 mol/L) or HCl (6 mol/L) to water (1 mL) (HCl final concentration: 3 mol/L).

Water samples were hydrolyzed for 2, 4, 6, 10, 15 h and then submitted to the complete work-up.

2) Pooled urine samples (1 mL), added with IS (210 pmol/mL), were hydrolyzed under the different indicated conditions. At the end of the hydrolysis reaction, urine samples were centrifuged and the precipitated proteins separated from supernatant. The obtained supernatant was loaded onto SPE column and submitted to the complete work-up as reported for free crosslinks extraction and for HPLC analysis.

The chemical stability of the IS to the hydrolysis conditions, was also tested by adding the IS to urine samples (n=3) both before or immediately after the hydrolysis reaction done before the protein separation by centrifugation.

RESULTS AND DISCUSSION

Free and glycosylated crosslinks extraction from urine and their HPLC-fluorescence evaluation

Due to the extreme polarity of Pyr, D-Pyr and glycosylated crosslinks, several conditions for their HPLC separation and their solid-phase extraction from biological matrices have been reported in literature (22,23,39).

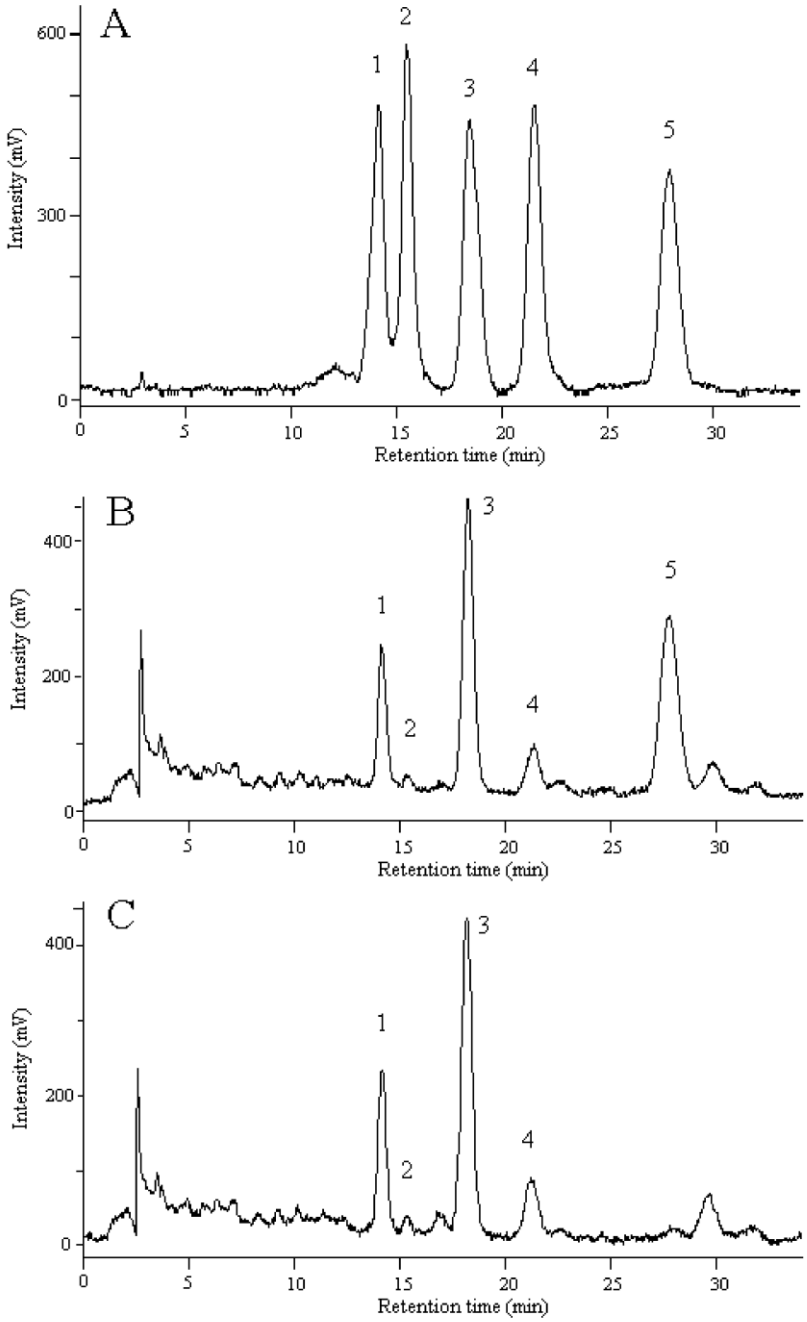
Our preliminary studies, carried out using working primary calibrators standard solutions, were performed to achieve the best HPLC crosslinks separation. Two HPLC columns were tested: Nucleosil 100 RP C18 and Reprosil-Pur Basic C18, together with various mobile phases (H_2O:ACN and H_2O:MeOH) added with two ion-pairing agents, pentafluoropropionic acid (PFPA) or heptafluorobutyric acid (HFBA), necessary to retain the positively charged crosslinks on HPLC column. The hydrophobicity of the ion-pairing agent and the composition of mobile phase (pH, organic solvent) condition the retention times and the resolution of the pyridinium crosslinks from each other and from interfering fluorophores present in the biological matrix.

The best HPLC peaks resolution and run time of the analysis were obtained in isocratic condition using the Reprosil-Pur Basic C18 column with aqueous HFBA solution (0.015 mol/L):acetonitrile (88:12; v:v) as mobile phase at 0.6 mL/min flow rate (λ_{ex} 295 nm and λ_{em} 400 nm).

Studies were also performed for urine crosslinks purification by solid phase extraction (SPE) comparing different reported conditions (23,39). Usually, most of fluorescent undesirable compounds is extracted from urine on a cellulose column by a butanolic solution whereas crosslinks are removed by water. However, being butanol miscible in water, there is the possibility of an incomplete butanolic phase separation from water, causing the presence of impurities in the crosslinks extract. Thus, the addition of methanol (23) or tetrahydrofuran (39) has been proposed. Our results showed cleaner extracts and higher crosslinks recovery by the addition of methanol to the acidic solution used to load the urine samples on the SPE column.

The typical isocratic ion-paired reversed-phase HPLC chromatogram for free and glycolylated crosslinks separation in the best tested conditions is reported in Fig.5. The chromatogram of a pure synthesized primary calibrators mixture added with IS (pannel A) shows good resolution of all the compounds as distinct peaks. By comparing the chromatograms obtained injecting an extract of the pooled women urine added with IS (pannel B) or without IS (pannel C), the absence in urine (pannel C) of a peak at the retention time of that of the selected IS suggests its possible use for this purpose. Moreover, it is possible to note the absence of a peak at the retention time of the pure synthesized Gal-Pyr in women urines (pannels B and C).

Fig.5 Reversed-phase HPLC chromatograms



(A) pure synthesized primary calibrators mixed at ratio 1:1 with IS (210 pmol/mL) and directly injected. (B) pooled women urine added with IS (210 pmol/mL) before SPE extraction or (C) without the addition of IS.

Peak 1, GluGal-Pyr; peak 2, Gal-Pyr; peak 3, Pyr; peak 4, D-Pyr; peak 5, IS. The concentrations in pooled women urine were: GluGal- Pyr (91 pmol/mL), Gal- Pyr (n.q.; <LOQ), Pyr (233 pmol/mL) and D-Pyr (43 pmol/mL).

Performance of the assay

The performance of the assay was tested in samples not submitted to the hydrolysis reaction.

- Linearity

Fluorescent-HPLC responses were linear for Pyr, D-Pyr, Gal-Pyr, GluGal-Pyr up to 1680 pmol/mL (336 pmol injected) corresponding to a ratio of 8 (pmol compound/pmol IS) higher than ratio of 4 (170 pmol injected), here reported for the calibration curves, being the last ratio over the pooled urine concentration.

Mean values for linear regression parameters of the three calibration curves are presented in Table 3.

The good linearity of the assay is confirmed by the coefficients of correlation (R^2) > 0.99 for all the tested compounds for the three types of calibration curves. The biological matrix does not interfere on the determination of all the analytes, as evidenced by the similar slopes in all the calibration curves. The low Gal-Pyr intercept indicates the absence of this compound in pooled women urine, whereas that of GluGal-Pyr, even if 3-fold lower than that of Pyr, suggests a contribution of this glycosylated-Pyr to the total Pyr amounts.

Table 3. Linearity data: Calibration curves.

	Pyr	D-Pyr	Gal-Pyr	GluGal-Pyr
CURVE 1				
Slope	0.897 (0.016)	0.926 (0.040)	0.867 (0.028)	0.889 (0.014)
Intercept	0.012 (0.008)	0.030 (0.023)	0.022 (0.017)	0.019 (0.013)
R^{2} [a]	0.999	0.999	0.998	0.998
Sy/x [b]	0.023	0.099	0.020	0.057
CURVE 2				
Slope	0.887 (0.033)	0.928 (0.014)	0.816 (0.012)	0.805 (0.024)
Intercept	0.981 (0.046)	0.159 (0.015)	0.027 (0.014)	0.323 (0.022)
R^{2} [a]	0.997	0.998	0.997	0.997
Sy/x [b]	0.069	0.052	0.086	0.073
CURVE 3				
Slope	0.882 (0.014)	0.914 (0.009)	0.820 (0.017)	0.833 (0.013)
Intercept	0.027 (0.016)	0.044 (0.024)	0.023 (0.015)	0.024 (0.020)
R^{2} [a]	0.999	0.999	0.998	0.998
Sy/x [b]	0.022	0.033	0.017	0.040

CURVE 1: pure standards mixture samples were directly injected in HPLC-fluorescence. CURVE 2: pooled women urine samples and CURVE 3: water samples were submitted to SPE before HPLC-fluorescence analysis.

Values are reported as mean (±SD). [a] Correlation coefficients; [b] Standard deviation of residuals. Each calibration curve was prepared in triplicate as indicated in Materials and Methods and each sample twice injected.

Analytical characteristics of the method

- Precision

The use of the internal standard allows to determine all the compounds of interest with a good analytical precision (Table 4). For all compounds, the percentage of coefficients of variation (CV%) are lower than 4.86 % and 5.11%, for both within- and between-day analysis, respectively, at the lower (52 pmol/mL) and the higher (420 pmol/mL) tested concentrations.

Table. 4. Analytical characteristics of the method: precision of the assay.

	within-day (n=7) %CV		between-day (n=7) %CV	
compounds	ratio 0.25 [a]	ratio 2.0 [a]	ratio 0.25 [a]	ratio 2.0 [a]
Pyr	4.86 (0.29)	3.07 (1.62)	2.52 (0.49)	2.79 (2.30)
D-Pyr	4.86 (0.29)	2.94 (1.62)	3.71 (0.22)	3.96 (2.15)
Gal-Pyr	4.57 (0.27)	1.03 (0.64)	3.68 (0.23)	3.91 (2.40)
GluGal-Pyr	4.35 (0.26)	1.56 (0.93)	5.11 (0.31)	3.09 (1.79)

[a] Pooled urine samples were spiked with all primary calibrators at 52 or 420 pmol/mL and IS (210 pmol/mL) to obtain the ratio 0.25 or 2.0 (calibrator/IS), respectively (samples from Curve 2).

Results are reported as mean values %CV (SD). The %CV was calculated according the formula: SD/mean values x 100, where SD is the standard deviation of the response.

- Recovery, LOD and LOQ

The results reported in Table 5 indicate a recovery higher than 80% for all the studied compounds, at both the lower and higher amounts injected. The lowest determined limit of quantification (LOQ) for Pyr shows the possibility of its evaluation in urine at the concentration of 10.85 pmol/mL, D-Pyr at 22.81 pmol/mL, Gal-Pyr 28.25 pmol/mL and GluGal-Pyr at 23.43 pmol/mL.

Table. 5. Analytical characteristics of the method: recovery, LOD and LOQ.

	Recovery %		LOD (pmol/mL)	LOQ (pmol/mL)
compounds	ratio 0.25 [a]	ratio 2.0 [a]	ratio 0.1 [b]	ratio 0.1 [b]
Pyr	95.1 (4.3)	97.1 (4.2)	3.58	10.85
D-Pyr	85.9 (5.6)	91.9 (3.5)	7.53	22.81
Gal-Pyr	81.8 (5.2)	94.4 (5.2)	9.35	28.25
GluGal-Pyr	88.5 (8.4)	95.7 (1.3)	7.73	23.43

[a] Pooled women urine samples were spiked with all primary calibrators at 52 or 420 pmol/mL and IS (210 pmol/mL) to obtain the ratio 0.25 or 2.0 (calibrator/IS), respectively (samples from Curve 2).
Results are reported as mean recovery % (SD).
[b] Samples (Curve1) at 0.1 ratio (21 pmol/210 pmol IS). Each sample was injected once daily in 7 different days.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated by 3.3(SD/S) and 10(SD/S) formula, respectively. SD: standard deviation of amounts (pmol). S: slope (mean) of calibration Curve 1.

Hydrolysis conditions for total Pyr and D-Pyr evaluation

Total urinary crosslinks are routinely assayed after overnight hydrolysis in the presence of HCl 6 mol/L (final concentration) at 110°C. However, these drastic conditions could affect the chemical structure of the pyridinium crosslinks and of our selected internal standard. In fact, stereoisomers or epimers formation under hydrolysis has been described using aminoacids (11,18).

To search for the best hydrolysis conditions for Gal-Pyr and GluGal-Pyr, and to confirm the chemical stability of Pyr, D-Pyr and of the selected internal standard, different reaction times (2, 4, 6, 10, 15 h) were first tested in water samples.

According to others (22), we found that after two hours at 110°C, pure Gal-Pyr and GluGal-Pyr, added to water samples, were totally hydrolyzed. After all the selected reaction times, also the chemical stability of pure Pyr, D-Pyr and IS in water samples, was confirmed by the recovery of Pyr/IS and D-Pyr/IS at the same ratio as that added to the water samples before the hydrolysis reaction.

When pooled women urine were hydrolyzed in the presence of HCl 3 mol/L, the obtained Pyr and D-Pyr amounts were about the 90% of those formed in the presence of HCl 6 mol/L.

Moreover, when pooled women urine were hydrolyzed in the presence of HCl 6 mol/L for longer time, Pyr and D-Pyr amounts increased, but after 10 and 15 h, fluorophores compounds, less polar than GluGal-Pyr, appeared in the HPLC chromatogram (but not in that of children).

Thus, we decided to perform the hydrolysis in the presence of HCl 6 mol/L for 6 h, to avoid the formation of these interfering compounds that could compromise the HPLC resolution of the compounds of interest. This was a good compromise that allowed a satisfactory analysis of Pyr and D-Pyr, even if the final crosslinks amount in urine resulted 10% lower than that determined after longer reaction time.

At these selected hydrolysis conditions, the chemical IS stability was confirmed by the same total Pyr and D-Pyr amounts measured in pooled women urine when IS was added before or immediately after the hydrolysis reaction.

Total Pyr and D-Pyr amounts in pooled healthy women urine (n=7) account for 477.11±8.6 and 91.03±6.3 pmol/mL, respectively (mean±SD of six injections).

Free, glycosylated and total crosslinks evaluation in healthy women and children urine

We tested the feasibility of the studied method evaluating free, glycosylated and total crosslinks in urine of healthy women and girls.

To minimize the biologic variability, due to age and gender, only women (30-40 years-old) were selected to compare our results with those reported by others in adult healthy groups.

Girls were enrolled as, being in growing age and having a bone remodelling rate extremely high, their physiological levels of urine collagen crosslinks in this stage of the life appeared of particular interest to be compared with those of an omogeneous group of pre-menopausal women. Moreover, girls were selected as, except some authors (27,42,54), D-Pyr, hydroxylysine (Hyl), galactose-Hyl and glucose-galactose-Hyl, are usually measured as bone and collagen turnover markers in children (35-38) and GluGal-Pyr amounts in children are not available. In particular, GluGal-Pyr has been identified, but not measured, by Roth et al. (27) in adult controls and children urines, suggesting the possibility for GluGal-Pyr to be formed not only by synovium, as reported by Gineyts et al. (32), but also by other tissues.

Due to the crosslinks variable dilution in urine, it is common practice to adjust urinary values according to a reference parameter such as urine creatinine amounts. However, as reported (26,55), many endogenous (age, gender, renal insufficiency, hydration state, changing in muscle mass, circadian rhythm) and exogenous factors (life style, dietary intake) migh affect creatinine concentration together with the day-time for urine collection (morning or 24 h urine) and with the analytical methods for creatinine measurement (26,34,39,55). Thus, creatinine correction is far from satisfactory as its excretion over 24 h can vary not only between individuals but also within individuals.

Even if the 24 h urine offers the advantage of allowing a mean of daily urine excretion, its collection is complex expecially when children are involved, and first-morning urine are often used for crosslinks measurements.

As proposed by some authors (34), we reported our crosslinks results both as molar concentrations (pmol/mL urine), and corrected by urine creatinine level (nmol/mmol creatinine), measured in clinical laboratory, to compare our values with those reported by others (16,27,28,32,39).

The results reported in Table 3, show a significant increase in Pyr, D-Pyr (both free and total) and GluGal-Pyr in girls respect to women. Gal-Pyr was under its lowest quantification limit (LOQ≥28.25 pmol/mL) or absent in women, while it was quantifiable in girls with values significantly lower in 5-6 year-old girls respect to the 9-10 year-old

ones. The observed higher Pyr and D-Pyr concentrations in girls urine respect to women were expected, considering the high skeletal growth velocity and the rapid bone turnover during childhood growth. Similarly, by comparing the fractions of free to total crosslinks forms, the significantly higher percentages of both free Pyr/total Pyr and free D-Pyr/total D-Pyr in girls than in women might reflect both an higher availability of free Pyr and D-Pyr to form crosslinks in children and an increased collagen turnover rather than bone turnover alone.

Table 6. Free, glycosylated and total crosslinks amounts in healthy women and girls urine.

Compounds	Women (n=20) (33.9±7.1 y)	Girls (n=10) (5.8±0.8 y)	(n=10) (9.6±0.5 y)
f Pyr (pmol/mL)	231.3* (45.5) [172-282]	989.7 (166.7) [799-1151]	1026.0 (73.9) [962-1107]
f Pyr/Crea (nmol/mmolCr)	33.8* (6.6) [24-39]	111.8 (19.9) [90-131]	118.0 (8.8) [108-127]
f D-Pyr (pmol/mL)	41.7* (9.3) [36-55]	180.3 (24.5) [159-200]	214.0 (41.4) [171-263]
f D-Pyr/Crea (nmol/mmolCr)	6.1* (1.5) [4.8-7.6]	20.4 (3.2) [17-23]	24.7 (3.9) [21-30]
f Pyr/f D-Pyr	5.7 (1.0) [4.5-6.5]	5.5 (0.6) [5.0-6.3]	4.9# (0.8) [4.2-5.9]
t Pyr (pmol/mL)	468.5* (121.8) [307-612]	1394.5 (261.2) [1140-1743]	1497.4 (155.7) [1347-1663]
t Pyr/Crea (nmol/mmolCr)	77.7* (7.9)	156.0 (29.4)	171.9 (20.6)
t D-Pyr (pmol/mL)	84.5* (23.9) [50-112]	275.3 (55.2) [210-333]	239.9 (55.5) [277-340]
t D-Pyr/Crea (nmol/mmolCr)	12.4* (3.9) [7-16]	30.7 (5.9) [23-37]	37.7 (5.5) [33-46]
t Pyr/t D-Pyr	5.7 (1.0) [4.3-6.7]	5.1 (0.6) [4.4-5.6]	4.6 (0.5) [4.1-5.2]
% f Pyr/t Pyr	50.38* (1.0)	71.9 (11.9)	68.5 (4.4)
% f D-Pyr/t D-Pyr	51.3° (12.9)	67.1 (13.2)	65.1 (5.0)
GluGal-Pyr (pmol/mL)	90.3* (15.0) [76-105]	226.9 (27.4) [201-249]	196.3 (44.1) [153-246]
GluGal-Pyr/Crea (nmol/mmolCr)	13.2* (2.0) [11-16]	25.6 (3.5) [22-28]	22.2 (4.0) [19-27]
Gal-Pyr (pmol/mL)	n.q.	41.1 (8.2) [33-50]	53.0 (11.5) [42-65]
Gal-Pyr/Crea (nmol/mmolCr)	n.q.	4.6 (0.9) [3.7-5.6]	6.1 (1.4) [4.7-7.5]

Results are reported as mean (±SD) and as median [10th-90th] percentile range.

f: free; t: total; Crea: creatinine; n.q.: not quantifiable (≥LOQ).

*$p<0.0001$ vs girls; °$p<0.020$ vs girls; #$p<0.01$ vs women. The significant differences were evaluated by Student's t-test.

It is important to note that both GluGal-Pyr and Gal-Pyr have never been evaluated in children. GluGal-Pyr is considered by some authors (28,32) an useful marker of synovium degradation as its

increase was observed in subjects affected by rheumatoid arthritis. However, the present study shows that GluGal-Pyr is present in urine of healthy women and in higher amounts in children, all with no apparent cartilage problems. Our findings agree with results reported by Roth et al (27) who identified, but not measured, GluGal-Pyr in the HPLC chromatogram of adult controls and of eleven-old adolescent urines. Thus, as suggested by Roth (27), synovium cannot be the only tissue originating GluGal-Pyr and this glycosylated compound cannot be a specific marker only for synovium degradation.

Our results seem to evidence a role for both GluGal-Pyr and Gal-Pyr in bone and collagen formation during children growth. In fact, Gal-Pyr, which is under our detection limit or absent in women urine, is present in children, and GluGal-Pyr is higher in children than in women. Since the urinary excreted GluGal-Pyr in children urine is 6-fold higher respect to Gal-Pyr amounts, we might speculate that Gal-Pyr could be the substrate for the Pyr disaccharide formation.

Pyridinoline derives from hydroxylysine (Hyl) by chemical reactions and is transformed into its glycosylated forms by specific transferase. It has been reported that for the HyL crosslinks formation, the GluGal-Hyl is assembled in a stepwise manner through the action of specific galactosyl and glucosyl transferase and that the presence of galactose linked to hydroxylysine (Gal-Hyl) represents the good substrate to form the disaccharide units by glucosyl transferase (5,56). As GluGal-Pyr and Gal-Pyr derive from Pyr by reaction with specific transferase, there is the possibility for GluGal-Pyr to be formed through the preformed Gal-Pyr (57).

Hyl, GluGal-Hyl and Gal-Hyl, are often used as bone markers for children bone health studies (35-38); they form crosslinks in the newly generated collagen (10,36), whereas pyridinium forms crosslinks in the mature collagen. GluGal-Hyl and Gal-Hyl are excreted in urine of 6-7 years-old girls in the same concentrations (36), whereas our measured GluGal-Pyr is 6-fold higher respect to Gal-Pyr amounts. Moreover, it has been reported that GluGal-Hyl reflects skin collagen metabolism better than bone metabolism (6,36), whereas Gal-Hyl that of bone (38). Thus, the evaluation of both GluGal-Pyr and Gal-Pyr might increase the knowledge on bone formation and resorption during children growth together with hydroxilysine crosslinks.

The biological importance of collagen glycosylation on the stability of the triple-helical structure and on the mechanical properties of bone has been recently reported (57,58). Thus, the evaluation of GluGal-Pyr and Gal-Pyr could be useful also to study pathological conditions characterized by modified activity of galactosyl- and glucosyl-transferase, the enzyme involved in the collagen glycosylation.

When we compare our free, glycosylated and total crosslinks amounts, measured in woman and girls urine, with the wide range of values reported by others (Tab.7), it appears evident the very little data concerning both free and glycosylated crosslinks both in adult subjects and in children.

Our free D-Pyr amounts in women urines are in agreement with the results reported by some authors (36,38,54), but contrast with others (22,16,39). On one hand, our total crosslinks amount is in agreement with one obtained by some authors (15,41,42), but on the other hand it is higher respect to others (32) who carried out the hydrolysis reaction in the presence of NaOH instead of HCl. In fact, it has been reported that about less than 50% of the total crosslinks are recovered under basic conditions respect to those obtained after acidic conditions (22). Also for GluGal-Pyr, our values are 2-fold higher respect to those reported in controls urine by Garnero et al. (28,32), the only authors measuring this glycosylated pyridinium derivative.

These discrepancies might be attributable to control groups, often not well matched for age and gender, but more likely to the lack in their method of an adequate internal standard and of pure primary calibrators.

Table 7. Literature reference intervals for urinary crosslinks in adult and children urine.

Ref.	Sex	Age (years) ADULT	Pyr/Crea (nmol/mmol) Free	Pyr/Crea (nmol/mmol) Total	D-Pyr/Crea (nmol/mmol) Free	D-Pyr/Crea (nmol/mmol) Total	Pyr (nmol/L) Free	Pyr (nmol/L) Total	D-Pyr (nmol/L) Free	D-Pyr (nmol/L) Total	GluGal-Pyr/ Crea (nmol/mmol)	Gal-Pyr/ Crea (nmol/mmol)
[22]			14-63	14-181	4-6	5-106	----	----	----	----	----	----
[16]	(F)	24-50	10	45	2	9	----		----		----	----
[32]		30-40	----	28±4	----	6±1	----	----	----	----	4±1	----
[15]			----	231-365	----	40-60	----	----	----	----	----	----
[6]			----	----	----	----	----	115-150	----	45-70	----	----
[39]			----	----	----	----	51	222	19	53	----	----
[41]	(F)		----	49	----	7	----	----	----	----	----	----
[54]	(M+F)		16±6	22±12	6±5	8±6	----	----	----	----	----	----
[42]	(F)	30-39	----	44±12	----	9±2	----	----	----	----	----	----
Our results	(F)	**27-45**	**24-38**	**45-82**	**5-8**	**7-16**	**172-282**	**307-612**	**36-55**	**50-112**	**11-16**	**n.d**
		CHILDREN										
[38]		4-10	----	79-210	-----	20-66	----	----	----	----	----	----
		10-12	----	79-387	-----	25-104	----	----	----	----	----	----
[36]		8-11	----		----	14-23	----	----	----	----	----	----
[42]	(M+F)	6-10	116±30	189±72	28±10	63±26	----	----	----	----	----	----
[54]		5-8	----	343±207	----	77±50	----	----	----	----	----	----
		8-11	----	275±122	----	51±24	----	----	----	----	----	----
Our results	**(F)**	**5-6**	**90-131**	**128-193**	**17-23**	**23-37**	**799-1151**	**1140-1743**	**159-200**	**210-333**	**22-28**	**3.7-5.6**
		9-10	**108-127**	**148-195**	**21-30**	**33-46**	**962-1107**	**1347-1663**	**171-263**	**277-340**	**19-27**	**4.7-7.5**

CONCLUSION AND FUTURE RESEARCH

Herein we set-up and validate the first HPLC-fluorescence method for the simultaneous quantification of free and glycosylated crosslinks in urine, which uses a superior unnatural homologue of D-Pyr as internal standard. The specificity of the method is guaranteed by the use of the internal standard and of pure synthesized primary calibrators of Pyr, D-Pyr, Gal-Pyr and GluGal-Pyr. Our method demonstrates to have good linearity, recovery, precision, LOD and LOQ for all compounds.

The measurement of consisting amounts of GluGal-Pyr in both women and children urine and few but detectable Gal-Pyr amounts only in girls confirms the importance for free and glycosylated crosslinks quantification. In fact, whereas the assessment of total urinary D-Pyr, found only in bone and dentine, provides an index of bone resorption, the evaluation of total Pyr, found in bone, cartilage and synovium, does not allow to distinguish among the degradation of bone, synovium or joint, and between free and glycosylated-Pyr.

Thus, the evaluation of both free, glycosylated and total crosslinks excretion in urine might provide more information on bone and collagen catabolism, particularly in pathological conditions and in monitoring both the disease progression and the response to medical treatment.

Furthermore, since recently it has been suggested a biological importance of collagen glycosylation on the bone structure stability and on the mechanical properties, the analytical method herein reported could be useful also to study pathological conditions characterized by modified activity of the enzymes involved in the collagen glycosylation (i.e. galactosyl- and glucosyltransferase).

Thus, our results stimulate additional researches to clarify the origin and the meaning of these glycosylated collagen crosslinks particularly in children and their possible variation under pathological conditions.

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