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Simultaneous free and glycosylated pyridinium crosslink determination in urine: Validation of an HPLC-fluorescence method using a deoxypyridinoline homologue as internal standard

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ABSTRACT

Pyridinoline (Pyr), deoxypyridinoline (D-Pyr), galactosyl-pyridinoline (Gal-Pyr) and glucosyl–galactosyl pyridinoline (GluGal-Pyr) are enzymatic mature pyridinium crosslinks. Generally, only total Pyr and D-Pyr urinary amounts (free + bound forms) are evaluated by HPLC as indices of bone resorption. This report describes the validation of an HPLC-fluorescence method for the simultaneous evaluation of free Pyr and D-Pyr, together with GluGal-Pyr and Gal-Pyr, in urine of healthy women ($n = 20$, aged 27–41) and girls ($n = 20$, aged 5–10). The use of an unnatural D-Pyr homologue, here proposed for the first time as internal standard, and of pure Pyr, D-Pyr, GluGal-Pyr and Gal-Pyr synthesized to be used as primary calibrators, guarantees method specificity and correct crosslink quantification. Urine, spiked with IS, was solid-phase extracted prior to HPLC analysis. Total Pyr and D-Pyr amounts were also evaluated after urine hydrolysis. The HPLC method was validated for selectivity, sensitivity, linearity, precision, accuracy, recovery and stability for all measured crosslinks. Both free and total Pyr and D-Pyr as well as GluGal-Pyr and Gal-Pyr amounts were significantly higher in girls than in women ($p < 0.0001$), indicating an increased collagen turnover rather than only bone turnover. Gal-Pyr, for the first time evaluated in girls, was under its lower quantification limit (<LLOQ, <21.20 pmol/mL) in women. The measurement of free and glycosylated pyridinium crosslinks might provide more information on the degradation of various types of collagen than only that of total Pyr and D-Pyr. Moreover, this validated method could be a useful non-invasive technique for studying pathological conditions characterized by modified glycosylation enzyme activity and for more clinical investigation on bone fragility.

1. Introduction

Enzymatic mature pyridinium crosslinks, mainly derived from the degradation of bone collagen resorption, are released into circulation and excreted in urine either as free pyridinoline (Pyr) and deoxypyridinoline (D-Pyr), or bound to peptides or sugars, as galactosyl pyridinoline (Gal-Pyr) and glucosyl–galactosyl pyridinoline (GluGal-Pyr) [1–4].

D-Pyr, mainly present in bone and dentine, is considered a specific biochemical marker of bone resorption, useful in the prediction of fracture risk associated with osteoporosis or bone cancer, as well as for growth assessment in children with growth hormone deficiency [1,5,6]. This specific role, on the other hand, cannot be attributed to Pyr, due to its wider tissue distribution in bone, cartilage and synovium [7–9]. Very little information is available on glycosylated pyridinolines. Gal-Pyr has been chemically identified in cartilage, bone and urine, but never quantified [10,11]. GluGal-Pyr, first identified in the urine HPLC chromatogram [1], has been reported to be higher in healthy children than in healthy adults [12]. It has been proposed to consider GluGal-Pyr as a specific marker of the synovial metabolic state and as a possible predictor of joint tissue degradation [11], owing to its increased levels in synovium and synovial tissues of patients with osteoarthritis or synovial diseases [9,13–15]. A correlation between GluGal-Pyr amounts and radiographic progression in early rheumatoid arthritis has also been reported [9,16].

With the exception of a few authors [2,12,17–19], free Pyr and D-Pyr amounts have not been quantified, as the commonly used procedures submit urine to a prolonged acid hydrolysis (12–24 h at 110 °C), which cleaves both amidic and glycosidic bonds, allowing only the evaluation of total Pyr and D-Pyr (free + bound forms)

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[5,20,21]. Moreover, most of the existing analytical methods suffer from the lack of adequate internal standards and of pure synthetic reference standards to be used as primary calibrators, in particular for the evaluation of the glycosylated pyridinolines, not commercially available. These are necessary requirements to avoid errors occurring during pre-analytical steps (solid-phase purification and/or hydrolysis) and allow a correct quantification of all crosslinks. Inaccurate estimation can also be minimized through standardization of all pre-analytical and analytical steps or by the use of validated methods, allowing a clearer interpretation and comparison of the data reported from different laboratories [20,22,23].

In an attempt to find compounds suitable as internal standards, it has been proposed to use propylated or acetylated pyridinolines for an accurate pyridinium crosslink evaluation [7,17,20,24,25]. However, due to the fact that they are hydrolyzed under acidic conditions, they cannot be used for total Pyr and D-Pyr evaluation, but only for their free amounts. Recently, an internal standard, unknown to the authors from the chemical point of view, as unspecified by the factory (proprietary information), was used for total crosslink evaluation by an automated HPLC assay [21].

The aim of this study is to set up and validate a sensitive and selective HPLC-fluorescence method for the simultaneous quantification of free Pyr and D-Pyr, Gal-Pyr and GluGal-Pyr in the urine of healthy women and girls. The specificity of the method is guaranteed by the use of the synthesized unnatural D-Pyr superior homologue, with one longer carbon lysine side chain linked to the heterocyclic nitrogen [26] and proposed here as internal standard (IS). In addition, pure synthesized standards of Pyr, D-Pyr, Gal-Pyr and GluGal-Pyr were used as primary calibrators for both correct identification in biological samples and quantification through the use of calibration curves [27–30]. Pyr and D-Pyr total amounts were also evaluated after acidic hydrolysis of the urine.

2. Experimental

2.1. Materials and reagents

Methanol and acetonitrile (HPLC-grade), hydrochloric acid (37%), acetic acid and heptafluorobutyric acid (HFBA) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Microgranular cellulose powder (CC31 type) was bought from Whatman, Inc. (NJ, USA). Empty SPE columns (12 mL) with 20-μm frits came from Maisch GmbH (Ammerbuch, Germany).

2.2. Apparatus and chromatographic conditions

HPLC analyses were performed using a Perkin Elmer system, equipped with an LC pump 200 system, an LC 240 fluorescence detector with a xenon lamp and an NCI 900 series interface (Perkin Elmer, U.K.). Data were acquired and processed using the Totalchrom Navigator software (Perkin Elmer, U.K.). The best crosslink chromatographic separation was obtained injecting 20 μL of sample onto the analytical reversed-phase (RP) HPLC ReproSil-Pur Basic C18 column (200 mm × 3 mm, 5 μm) provided with a ReproSil-Pur Basic C18 guard column (10 mm × 3 mm, 5 μm) (Maisch GmbH, Ammerbuch, Germany). Separation was carried out in isocratic assay using an aqueous HFBA solution (0.015 mol/L):acetonitrile (88:12, v/v) as mobile phase at 0.6 mL/min flow rate. Eluting peaks were detected by fluorescence with 295 nm excitation and 400 nm emission. The retention times (min) were: GluGal-Pyr (14.32), Gal-Pyr (15.63), Pyr (18.40), D-Pyr (21.40), and IS (27.90). Total runtime for each analysis was 35 min. Peak identification was based on the retention time of each corresponding pure synthesized primary calibrator.

2.3. Primary calibrators and internal standard

The synthesized Pyr, D-Pyr, GluGal-Pyr, Gal-Pyr and IS (Fig. 1) were purified by a preparative RP HPLC on a μBondpak C18 column (100 mm × 40 mm, 10 μm) (Waters Corporation, MA, USA), using an aqueous trifluoroacetic acid (0.015 mol/L):acetonitrile (90:10, v/v) as mobile phase at 20.0 mL/min flow rate. The chemical structure and the purity (≥99%) of each compound were confirmed by elemental analyses, mass spectrometry, nuclear magnetic resonance and HPLC analysis (Section 2.2) [26–30]. Molar absorption coefficients (ε = L mol^{-1} cm^{-1}) of the compounds in HCl (0.1 mol/L) at 295 nm (λ_{max}) were: 6520 (Pyr), 6480 (D-Pyr), 6400 (GluGal-Pyr, Gal-Pyr) and 6490 (IS).

2.4. Standard stock and working solutions

Standard stock solutions, prepared by dissolving weighed pure standards in HCl (0.1 mol/L), were aliquoted and stored at −40 °C. The final concentrations, confirmed by reading absorbance values, were: D-Pyr (9.50×10^{-5} mol/L), Pyr (1.20×10^{-4} mol/L), GluGal-Pyr (1.06×10^{-4} mol/L), Gal-Pyr (2.04×10^{-4} mol/L) and IS (1.20×10^{-4} mol/L).

Working standard solutions were prepared by diluting standard stock solutions with aqueous HFBA solution (0.015 mol/L), aliquoted and stored at −40 °C. 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).

2.5. Subjects and urine collection

Twenty healthy female volunteers (n = 20, aged 33.9 ± 7.1) and twenty girls (n = 10, aged 5.8 ± 0.8, n = 10, aged 9.6 ± 0.5), all in good health, were included in the study. Informed consents were obtained from the subjects themselves or their parents. None of the subjects had any history of metabolic bone diseases and premenopausal women were not receiving any medication affecting calcium absorption and metabolism.

First-morning urine samples were used rather than 24 h samples, as their collection is easier, especially when children are involved. The urine collected was aliquoted and stored at −40 °C until analyzed.

Urine samples from healthy women (n = 7, aged 35.6 ± 7.6) were pooled, aliquoted to avoid multiple thawing, stored at −40 °C and used for urine calibration curve preparation, study of hydrolysis reaction and quality control (QC) samples preparation.

2.6. Hydrolysis reaction

In order to search for the best hydrolysis conditions for Gal-Pyr and GluGal-Pyr, and confirm the chemical stability of the selected internal standard, water samples (1 mL) spiked with Pyr and D-Pyr (210 pmol/mL) or GluGal-Pyr and Gal-Pyr (210 pmol/mL) and with IS (210 pmol/mL) were hydrolyzed in HCl (6 mol/L, final concentration) for 2, 4, 6, 10 or 15 h at 110 °C, and then submitted to the complete work-up (Section 2.7). Urines (1 mL) spiked with IS (210 pmol/mL) were submitted to the same hydrolysis reaction time.

2.7. Free and total crosslink extraction from urine samples

The solid-phase extraction (SPE) procedure was carried out according to Spacek et al. [31] with some modifications. Cellulose (800 mg), suspended in a solution of aqueous acetic acid in 1-butanol (8 mL butanolic solution; 1-butanol:water:acetic acid, 4:1:1, v/v/v), was loaded onto a column and allowed to settle. It was then washed with the same butanolic solution (2 mL) and the

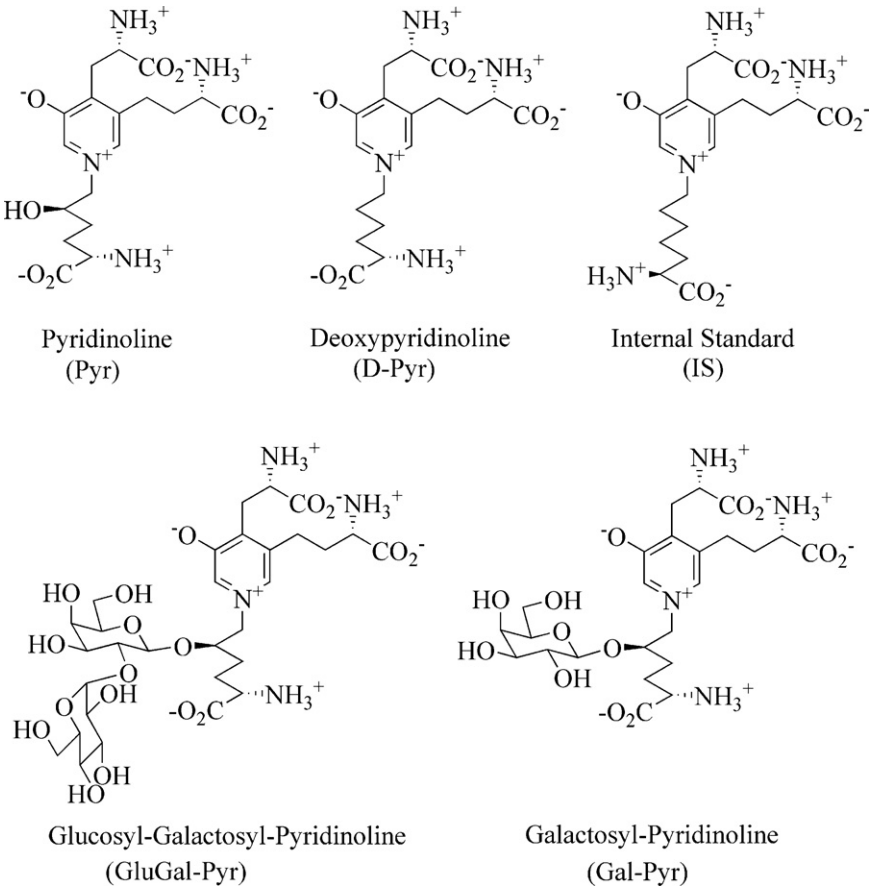

Fig. 1. Chemical structures of pyridinium crosslinks and internal standard.

bed top protected by a filter disk. For free and glycosylated crosslink extraction, urine (1 mL), spiked with IS (210 pmol/mL), was mixed with an acidic solution of methanol, HCl (37%), acetic acid and 1-butanol (7 mL, 1:1:1:4, v/v/v/v), and loaded onto the cellulose-packed column. The column was then washed with the butanolic solution (10 mL × 2) to eliminate all interfering fluorophores, while the crosslinks were eluted with distilled water (4 mL). The aqueous phase obtained was then centrifuged, the upper butanolic layer removed, water phase dried, the residue dissolved in aqueous HFBA solution (0.1 mL, 0.015 mol/L) and injected (20 μL) into the HPLC system for analysis.

For total crosslink extraction, urine (1 mL) added with IS (210 pmol/mL) was first hydrolyzed for 6 h at 110 °C in a sealed tube, with an equal volume of HCl (37%). The sample was then centrifuged, precipitated proteins were separated, and the supernatant was loaded onto the cellulose column and submitted to SPE procedure as for free crosslink extraction.

2.8. Method validation

Method validation was partially carried out according to the FDA and emeA guidelines [32,33] drawn up for toxicokinetic and pharmacokinetic studies on non-endogenous compounds (drugs, xenobiotics), usually added to the authentic biological matrix for method validation. However, the determination of endogenous analytes, such as the pyridinium crosslinks formed during physiological bone resorption, has so far been hampered by the absence of official validation guidelines [34]. Moreover, to overcome the lack of analyte-free matrix, one approach is the use of a surrogate analyte-free matrix (water, buffer) for the preparation of calibration standards and quality control samples at the lower limit of quantification [34]. As pyridinium crosslinks show high solubility in water, the latter was selected as the surrogate analyte-free matrix. The method reported was validated for linearity, selectivity, sensitivity, precision, accuracy, recovery and stability.

2.8.1. Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) is the lowest amount of analyte which can be quantified reliably, with 20% precision and 80–120% accuracy. Moreover, the analyte response at the LLOQ should be at least five times the blank response. The LLOQ was evaluated in water samples with the addition of primary calibrators at 10.5, 16, 21 and 52 pmol/mL (five determinations).

2.8.2. Calibration curves preparation for linearity

Three different calibration curves were prepared consisting each of a blank sample (without IS), a zero sample (with IS) and six to seven non-zero samples covering the expected concentration range, including LLOQ value.

A seven-point calibration curve of pure standards (Curve 1) was prepared with increasing concentrations of Pyr, D-Pyr, GluGal-Pyr and Gal-Pyr working standard solutions over a concentration range of 10.5–840 pmol/mL (i.e., 10.5–21–52–105–210–420–840 pmol/mL) and a constant amount of IS (210 pmol/mL). Samples were dried and redissolved in aqueous HFBA solution (0.1 mL, 0.015 mol/L). SPE procedure was omitted and samples were directly injected into the HPLC system (20 μL).

A six-point urine calibration curve (Curve 2) was prepared by spiking urine samples pooled from healthy women (1 mL) with increasing amounts of pure standards (21–52–105–210–420–840 pmol/mL) and with a fixed IS amount

(210 pmol/mL). The sample spiked with 10.5 pmol/mL of primary calibrators was omitted in contrast to Curve 1 as this amount is not high enough to result in a concentration that can be reliably distinguished from the endogenous concentration of the pyridinium crosslinks. Each urine calibration sample was subjected to the SPE procedure as reported for free crosslink extraction (Section 2.7).

A six-point water calibration curve (Curve 3) was prepared by spiking water samples (1 mL) at the same IS concentrations and working standard solutions as reported for Curve 2. Samples were then subjected to the same SPE procedure as Curve 2 samples.

The three calibration curves were prepared in triplicate; each sample was dried, redissolved in aqueous HFBA solution and injected twice.

Least-squares regression analysis was performed for linearity evaluation, by plotting the peak area ratios of each analyte to that of IS (A/A_{IS}) (Y-axis) against the known added concentrations of each primary calibrator (pmol/mL) (X-axis). Blank and zero samples were not taken into consideration to assess calibration curve parameters. The linearity of the three types of calibration curves was evaluated from the slope, y-intercept, correlation coefficient and standard deviation of residuals. The concentration of each analyte in real women and girls' urine samples was calculated as A/A_{IS} divided by the mean slope value of the urine calibration curve (Curve 2).

2.8.3. Selectivity

The selectivity of the method was assessed by evaluating potential interference deriving from endogenous urine components or metabolites at the retention time corresponding to the four pyridinium crosslinks and the internal standard. This was done by comparing the slopes obtained from urine and water calibration curves (Curves 2 and 3), and the HPLC chromatograms of six independent women urine samples.

2.8.4. Preparation of quality control samples

Due to the presence of endogenous crosslink amounts in urine, quality control samples were prepared according to van de Merbel [34].

Quality control samples, with the addition of IS, were independently prepared, either from working standard solutions of the pure primary calibrators directly injected or from pooled urine and water samples (1 mL), at five levels of primary calibrator concentrations as follows: unspiked urine (zero sample), water with 21 pmol/mL (LLOQ), urine with 21 pmol/mL (low QC), 210 pmol/mL (medium QC) or 840 pmol/mL (high QC). The QC samples were aliquoted and stored at −40 °C until analyzed.

2.8.5. Precision and accuracy

The precision and accuracy of the HPLC method were assessed for within- and between-day assays by analyzing the QC samples (Section 2.8.4) seven times on the same day and on five different days in 2 replicates. The precision, reported as the percentage coefficient of variation (%CV), is calculated by means of the formula: SD/(mean measured concentration) × 100, where SD is the standard deviation of the response. Accuracy is expressed as the percentage of the mean measured concentration obtained from both the LLOQ water sample and the urine QC samples divided by the nominal spiked concentration.

2.8.6. Extraction recovery

In order to evaluate the recovery of each analyte from a urine sample, the peak area ratios (A/A_{IS}) obtained from spiked urine QC samples, at low, medium and high concentration levels, were compared with the mean A/A_{IS} measured from the primary calibrators QC samples at the same concentrations and directly injected, representing 100% recovery [32]. Analyte recovery percentage was calculated by subtracting the mean y-intercept value of urine calibration curve (Curve 2) from each A/A_{IS} value measured in the urine QC samples. The result was divided by the mean A/A_{IS} value obtained for the primary calibrators QC samples directly injected and multiplied by 100.

2.8.7. Stability

The pyridinium crosslink standard stock solutions were aliquoted and stored at −40 °C for up to 3 years. The long-term stability was assessed by analyzing working standard solutions of all the pyridinium crosslinks at 52 and 840 pmol/mL concentrations prepared by diluting the stock solutions stored for 1, 6 months, 1, 3 years, and by comparing the concentrations measured with those obtained before the storage period. The stability of the working standard solutions was evaluated by comparing working standard solutions at 52 and 840 pmol/mL stored at −40 °C for 1 year with fresh ones prepared on the day of the analysis. The long-term storage stability of endogenous crosslinks in pooled women urine samples was also assessed. The stability is expressed as percentage recovery.

3. Results and discussion

3.1. Crosslink extraction, HPLC-fluorescence detection and selectivity

The high polarity and solubility in water of the free and glycosylated collagen crosslinks require their preventive solid-phase extraction from biological matrices and the use of ion-pairing agents for their HPLC separation [7,18,23,31]. Cleaner extracts and higher crosslinks recovery were obtained by mixing urine samples with an acidic solution containing methanol [31]. Better HPLC resolution and reduced run time analysis were observed when HFBA was added as an ion-pairing agent to the aqueous HPLC mobile phase instead of pentafluoropropionic acid.

Typical isocratic ion-pair reversed-phase HPLC chromatograms of the free and glycosylated crosslinks are reported in Fig. 2 (panels A–C). The first one, corresponding to a mixture of the pure synthesized primary calibrators added with IS (210 pmol/mL) at 1:1 ratio, shows a satisfactory resolution of Pyr, D-Pyr and the IS. GluGal-Pyr and Gal-Pyr show a small overlapping area of their peaks. This incomplete separation can only be observed when high concentrations of the two glycosylated pyridinolines calibrators are present (panel A), whereas at physiological levels (panels B and C) the amount of Gal-Pyr is very low and good resolution can be observed between these two compounds. However, even when peak overlapping occurs, the contribution of each peak to the total area of the other could be measured with an acceptable level of accuracy by the software using the drop method [35].

Moreover, by comparing the chromatograms (Fig. 2) obtained injecting an extract of the pooled women urine with the addition of (panel B) or lacking in IS (panel C), the absence of endogenous urine components at the retention time of the internal standard was observed, thereby proving method selectivity for the internal standard proposed. Good selectivity was also confirmed by the absence of interference at the retention time of the analytes and of internal standard when injecting six independent women's urine samples.

3.2. Method validation

3.2.1. Linearity of calibration curves and lower limit of quantification (LLOQ)

Fluorescent-HPLC responses were linear for Pyr, D-Pyr, Gal-Pyr and GluGal-Pyr up to 1680 pmol/mL (336 pmol injected). The highest crosslink concentration used for all the calibration curves was 840 pmol/mL (168 pmol injected), which is higher than the

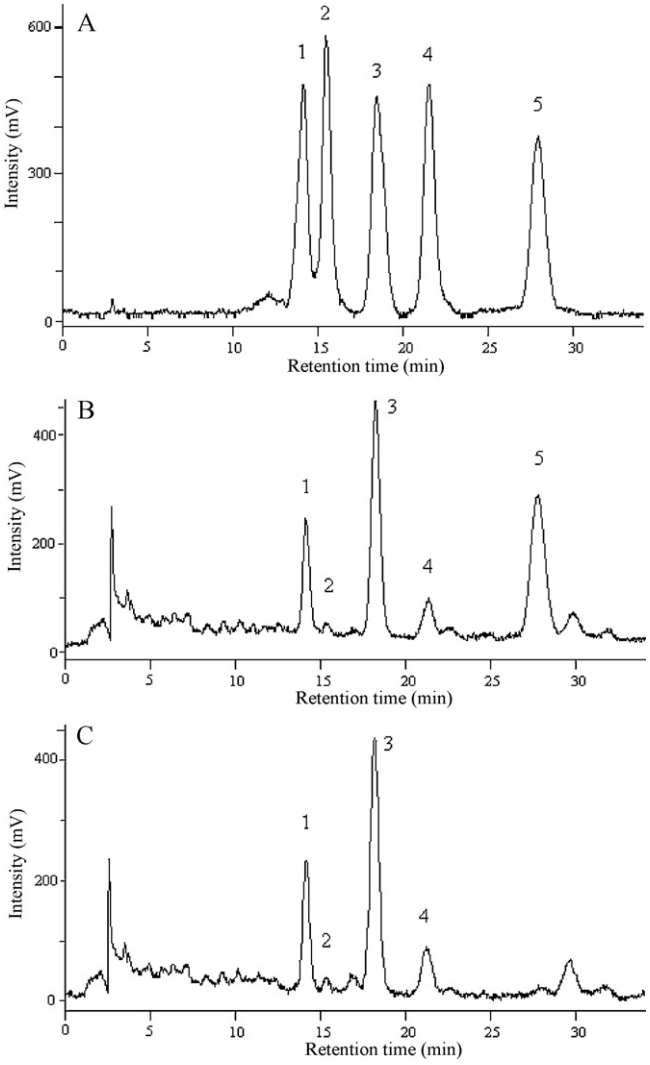

Fig. 2. HPLC-fluorescence chromatograms obtained using a ReproSil-Pur Basic C18 column (200 mm × 3 mm, 5 μm) with an aqueous HFBA solution (0.015 mol/L):acetonitrile (88:12, v/v) as mobile phase: pure synthesized primary calibrators mixed with IS (ratio 1:1) (panel A), extracts obtained from the non-hydrolyzed pooled women urine with the addition of (panel B) or lacking in IS (panel C). Peaks: GluGal-Pyr (1), Gal-Pyr (2), Pyr (3), D-Pyr (4), and IS (5). Free and glycosylated pyridinoline concentrations obtained from the pooled women urine sample (mean ± SD, $n = 6$) (panel B) were: GluGal-Pyr (92.94 ± 3.2 pmol/mL), Gal-Pyr (<LLOQ, <21.20 pmol/mL), Pyr (215.88 ± 7.3 pmol/mL), D-Pyr (36.68 ± 2.7 pmol/mL).

total Pyr and D-Pyr concentrations from hydrolyzed pooled women urine samples. The lower limit of quantification (LLOQ) for all the crosslinks was established at 21.20 pmol/mL.

Mean values for linear regression parameters of the three calibration curves are shown in Table 1. The slopes obtained from the calibration curves prepared in urine and water samples (Curves 2 and 3, respectively) indicate a similar extraction yield and the lack of both biological matrix effects and interference from endogenous urine components and good selectivity in the determination of the four pyridinium crosslinks. The mean correlation coefficient (r) of the three different calibration curves was better than 0.997 for all the pyridinium crosslinks. The low Gal-Pyr intercept on Y-axis (Curve 2) indicates the absence and/or undetectable amount of this compound in healthy women's urine.

3.2.2. Precision, accuracy and extraction recovery

Within- and between-day precision and accuracy of the method were assessed by determining QC samples at five different concentration levels (Tables 2 and 3). The use of the internal standard allows all the pyridinium crosslinks to be determined with a good analytical precision and accuracy. The values of all the compounds are within an acceptable criterion as suggested by the guidelines [32–34]. The acceptable criterion for Gal-Pyr LLOQ level, zero sample and low QC level is ≤20% as its physiological level in women urine is under the LLOQ value.

The extraction recoveries of all the crosslinks from the urine QC samples (Table 2) are higher than 77%, 91% and 93% at low, medium and high concentration levels, indicating also good accuracy of the analytical method developed.

3.2.3. Stability

The standard stock solutions are stable at −40 °C for at least three years, the percentage recovery ranging from 95 to 103% for all crosslinks at 52 and 840 pmol/mL. During this period, they were defrosted and thawed at room temperature several times to prepare new working standard solutions. Their stability was confirmed for at least one year, with recovery percentage ranging from 96 to 101% for all crosslinks at the two concentration levels. Aliquoted pooled women urine samples were kept at −40 °C for different storage periods (0, 3 and 6 weeks) with no variation of the endogenous crosslink concentrations compared to those measured in fresh urine (recovery percentage from 97 to 101% for Pyr, D-Pyr and GluGal-Pyr). The recovery percentage for Gal-Pyr was not evaluated as its endogenous concentration in women urine is under the LLOQ (<21.20 pmol/mL). The crosslinks stability in urine has also been previously reported [7].

3.3. Hydrolysis conditions for total Pyr and D-Pyr evaluation

Pure Gal-Pyr and GluGal-Pyr in water samples were fully hydrolyzed after 2 h at 110 °C, in agreement with others [7]. The chemical stability of pure Pyr, D-Pyr and IS was confirmed by their complete recovery from water samples after all tested hydrolysis reaction times (data not shown). When pooled women urine samples were hydrolyzed, a time-dependent increase in Pyr and D-Pyr amounts was observed up to 6 h [7]. However, fluorophore compounds, less polar than GluGal-Pyr, appeared in the HPLC chromatogram after more than 10 h. Since interference was absent when pure standards of the pyridinolines were hydrolyzed under similar conditions, we exclude the possibility that they should derive from pyridinolines decomposition. The pyridinolines' chemical stability under hydrolysis has already been proved [7]. They might derive from other five-ring membered collagen crosslinks which could be destroyed during acidic hydrolysis as reported by Eyre et al. [36]. Therefore, we decided to hydrolyze urine for 6 h to avoid the formation of these interfering compounds obtaining a satisfactory analysis of all the crosslinks, even if their final total amounts in urine resulted 10% lower than after a longer reaction time. The chemical stability of IS in urine was also confirmed by the same total Pyr and D-Pyr quantities measured when IS was added before or immediately after 6 h of hydrolysis.

3.4. Free, glycosylated and total crosslink evaluation in urine

We tested the feasibility of the method proposed evaluating free, glycosylated and total crosslinks in healthy women and girls' urine. Girls, in growing age with a high bone remodeling rate, were selected as their physiological levels of urinary Gal-Pyr and GluGal-Pyr, have never been quantified, and free Pyr and D-Pyr appeared particularly interesting, when compared with those of pre-menopausal women. We also measured total Pyr and D-Pyr urinary amounts in the selected groups in order to compare our results with data from current literature [5,11,16–19,21].

Table 1
Statistical evaluation of pyridinium crosslinks linearity response from the calibration curves of pure primary calibrators directly injected, spiked urine and water samples.

	Pyr	D-Pyr	Gal-Pyr	GluGal-Pyr
Curve 1				
Slope	0.0043 (0.0001)	0.0044 (0.0002)	0.0042 (0.0001)	0.0042 (0.0001)
Intercept	0.012 (0.008)	0.030 (0.023)	0.022 (0.017)	0.019 (0.013)
r[a]	0.999	0.999	0.998	0.998
Sy/x[b]	0.023	0.028	0.020	0.057
Curve 2				
Slope	0.0042 (0.0001)	0.0044 (0.0001)	0.0039 (0.0001)	0.0039 (0.0001)
Intercept	0.978 (0.034)	0.159 (0.015)	0.023 (0.016)	0.324 (0.021)
r[a]	0.997	0.998	0.997	0.997
Sy/x[b]	0.052	0.047	0.079	0.065
Curve 3				
Slope	0.0042 (0.0001)	0.0043 (0.0001)	0.0039 (0.0001)	0.0040 (0.0001)
Intercept	0.033 (0.008)	0.052 (0.013)	0.027 (0.014)	0.022 (0.013)
r[a]	0.999	0.999	0.998	0.998
Sy/x[b]	0.019	0.036	0.022	0.033

Calibration curve for pure primary calibrator mixtures directly injected in HPLC (Curve 1) (range 10.5–840 pmol/mL). Calibration curves obtained from extracts of pooled women urine samples (Curve 2) and water samples (Curve 3), submitted to SPE before HPLC-fluorescence analysis (range 21–840 pmol/mL). Values are reported as mean (SD). Each calibration curve was prepared in triplicate and each sample twice injected.

[a] Correlation coefficients.

[b] Standard deviation of residuals.

The results, reported in Table 4, show a significant increase in Pyr, D-Pyr (both free and total) and GluGal-Pyr in girls compared with women. Gal-Pyr, under its lower quantification limit (21.20 pmol/mL, 3.15 nmol/mmol creatinine) or absent in women, was quantifiable in girls with values significantly lower in the 5–6 year-old girls than in the 9–10 year-old ones. The Pyr and D-Pyr concentrations in girls' urine higher than in women were expected, considering the high rate of skeletal growth and the rapid bone turnover during childhood. Similarly, by comparing the fractions of free with total forms of crosslinks, the significantly higher percentage of both free Pyr/total Pyr and free D-Pyr/total D-Pyr in girls than in women might reflect an increased collagen turnover rather than only bone turnover.

The free Pyr and D-Pyr evaluated, as well as the total amount of pyridinolines in our selected population, are within the wide range of values reported by some authors [5,17,19–21], but higher than

Table 2
Within-day assay: precision, accuracy and extraction recovery for pyridinium crosslinks.

Within-day assay (n = 7)[a]						
Analyte	Nominal spiked concentration (pmol/mL)	Mean measured concentration (SD) (pmol/mL)	Precision %CV	Accuracy (%)	Extraction recovery (%)	
					Mean (SD)	%CV
Pyridinoline						
Zero sample[b]	0.00	215.88 (4.97)	2.13			
LLOQ water[c]	21.33	23.80 (1.23)	5.18	111.6		
QC low[d]	21.33	21.33 (3.73)	14.49	100.0	84.8 (14.6)	17.2
QC medium	213.30	220.85 (17.29)	6.40	103.5	96.0 (7.5)	7.8
QC high	853.35	855.79 (17.62)	1.89	100.3	98.9 (2.0)	2.0
D-pyridinoline						
Zero sample[b]	0.00	36.81 (1.74)	4.72			
LLOQ water[c]	21.30	21.51 (1.62)	7.51	101.0		
QC low[d]	21.30	21.03 (2.57)	12.21	98.7	77.3 (8.7)	11.2
QC medium	212.80	225.69 (15.65)	6.93	106.1	100.7 (6.4)	6.3
QC high	840.55	851.29 (17.01)	2.00	101.3	101.3 (1.8)	1.8
GluGal-Pyr						
Zero sample[b]	0.00	92.32 (1.88)	2.04			
LLOQ water[c]	21.30	25.57 (2.38)	9.30	120.0		
QC low[d]	21.30	20.99 (2.71)	12.92	98.5	79.5 (10.3)	12.9
QC medium	212.90	211.56 (12.29)	5.81	99.4	90.9 (5.3)	5.8
QC high	840.90	842.22 (14.75)	1.75	100.2	93.5 (1.6)	1.7
Gal-Pyr						
Zero sample[b]	0.00	n.q.	n.q.	n.q.	n.q.	n.q.
LLOQ water[c]	21.20	25.11 (2.32)	9.26	118.4		
QC low[d]	21.20	23.20 (4.15)	16.35	109.4	93.2 (15.2)	16.4
QC medium	212.15	217.31 (13.11)	5.88	102.4	96.0 (5.6)	5.9
QC high	838.05	848.02 (15.27)	1.69	101.2	94.6 (1.6)	1.7

Within-day assay was performed by analyzing each QC sample seven times on the same day. Precision is calculated as percent coefficient of variation (%CV). Accuracy is expressed as the percentage of the mean measured concentration value to the nominal value. Extraction recovery (%) was calculated by subtracting the mean y-intercept value of urine calibration curve from each A/A_{IS} value measured in the urine QC samples. The obtained result was divided by the mean A/A_{IS} obtained from the corresponding primary calibrators QC samples directly injected and multiplied by 100. n.q., not quantifiable (<LLOQ, <21.20 pmol/mL).

[a] Number of individual determinations.

[b] Zero sample: urine spiked only with IS.

[c] Water sample spiked with LLOQ.

[d] QC samples were prepared by spiking urine samples with known nominal analyte concentration; the endogenous analyte concentration was subtracted from the mean measured concentration.

Table 3
Between-day assay: precision and accuracy for pyridinium crosslinks.

Between-day assay ($n = 10$)[a]				
Analyte	Nominal spiked concentration (pmol/mL)	Mean measured concentration (SD) (pmol/mL)	Precision %CV	Accuracy (%)
Pyridinoline				
Zero sample[b]	0.00	216.52 (6.45)	2.98	
LLOQ water[c]	21.33	23.93 (1.46)	6.09	112.2
QC low[d]	21.33	22.14 (2.70)	14.17	103.8
QC medium	213.30	220.21 (19.04)	7.40	103.2
QC high	853.35	859.80 (26.24)	3.14	100.8
D-pyridinoline				
Zero sample[b]	0.00	36.58 (2.94)	8.03	
LLOQ water[c]	21.30	21.32 (3.05)	14.32	100.1
QC low[d]	21.30	22.68 (2.83)	12.50	106.5
QC medium	212.80	230.27 (15.98)	6.94	108.2
QC high	840.55	852.18 (18.89)	2.22	101.4
GluGal-Pyr				
Zero sample[b]	0.00	91.37 (3.33)	3.65	
LLOQ water[c]	21.30	25.55 (4.74)	18.56	119.9
QC low[d]	21.30	21.92 (2.97)	13.54	102.9
QC medium	212.90	211.86 (12.90)	6.09	99.5
QC high	840.90	842.99 (15.16)	1.80	100.2
Gal-Pyr				
Zero sample[b]	0.00	n.q.	n.q.	n.q.
LLOQ water[c]	21.20	25.11 (3.57)	14.21	118.5
QC low[d]	21.20	24.93 (4.06)	16.30	117.6
QC medium	212.15	209.97 (19.22)	9.15	99.0
QC high	838.05	849.55 (16.29)	1.92	101.4

Between-day assay was performed by analyzing each QC sample two times on five different days. Precision is calculated as percent coefficient of variation (%CV). Accuracy is expressed as the percentage of the mean measured concentration value to the nominal spiked value. n.q., not quantifiable (<LLOQ, <21.20 pmol/mL).

[a] Number of individual determinations.
[b] Zero sample: urine spiked only with IS.
[c] Water sample spiked with LLOQ.
[d] QC samples were prepared by spiking urine samples with known nominal analyte concentration; the endogenous analyte concentration was subtracted from the mean measured concentration.

others' [11]. This discrepancy between our data and that of previous studies could be attributed to the lack of an internal standard for accurate quantification of all the crosslinks. Moreover, inter-method comparison is rather difficult as no validated method is available for the determination of free and glycosylated crosslinks as most existing HPLC methods measure only total pyridinoline and deoxypyridinoline amounts, without an internal standard for their quantification.

The presence of GluGal-Pyr in healthy women's urine and higher amounts in adolescents' urine with no apparent cartilage problems confirms the observations previously reported by some authors [1,12]. They identified GluGal-Pyr in the HPLC chromatograms of adult controls and of 11 year-old adolescent urine samples, hypothesizing that GluGal-Pyr formed not only in synovium, but also in other tissues. Therefore, GluGal-Pyr might play a role not only as a specific marker for synovium degradation [11,14]. Moreover, considering that GluGal-hydroxylysine and Gal-hydroxylysine, two glycosylated amino acids involved in immature enzymatic collagen crosslinks formation and related to the mature glycosylated pyridinolines, are used as biochemical markers for children bone health studies [6,37,38], GluGal-Pyr and Gal-Pyr assessment might be useful to strengthen our understanding of bone formation and resorption during childhood and the change from immature to mature crosslinks.

Recently, the biological importance has been reported of collagen glycosylation on the stability of the triple-helical structure and on the mechanical properties of bone [39,40]. Furthermore, the evaluation of GluGal-Pyr and Gal-Pyr could be useful for the study of pathological conditions characterized by the modified activity of galactosyl- and glucosyl-transferases, the enzymes involved in collagen glycosylation.

Table 4
Urinary excretion amounts of free, glycosylated and total pyridinium crosslinks evaluated in healthy women and girls.

	Women	Girls	
	($n = 20$) (33.9 ± 7.1 years)	($n = 10$) (5.8 ± 0.8 years)	($n = 10$) (9.6 ± 0.5 years)
f Pyr (nmol/mmolCr)	33.8* (6.6)	111.8 (20.0)	118.7 (8.8)
f D-Pyr (nmol/mmolCr)	6.1* (1.5)	20.4 (3.2)	24.7 (3.9)
f Pyr/f D-Pyr	5.7 (1.0)	5.5 (0.6)	4.9# (0.8)
t Pyr (nmol/mmolCr)	68.0* (16.2)	156.0 (29.4)	171.9 (20.6)
t D-Pyr (nmol/mmolCr)	12.4* (4.0)	30.7 (5.9)	37.7 (5.5)
t Pyr/t D-Pyr	5.7 (1.0)	5.1 (0.6)	4.6 (0.5)
f Pyr/t Pyr (%)	50.4* (5.2)	71.9 (12.0)	68.5 (4.8)
f D-Pyr/t D-Pyr (%)	51.3** (13.0)	67.2 (13.2)	65.2 (5.0)
GluGal-Pyr (nmol/mmolCr)	13.2* (2.0)	25.6 (3.5)	22.2 (4.0)
Gal-Pyr (nmol/mmolCr)	n.q.	4.6 (0.9)	6.1 (1.4)

Results are reported as mean (SD). f, free; t, total; Cr, creatinine; n.q., not quantifiable (<LLOQ, <3.15 nmol/mmol creatinine).

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

4. Conclusions

This study highlights the set-up and the validation of the first HPLC-fluorescence method for the simultaneous quantification of free and glycosylated urinary pyridinium crosslinks, which uses a superior unnatural D-Pyr homologue as internal standard. The specificity of the method is guaranteed by the use of both the internal standard and the pure synthesized primary calibrators. Our method shows remarkable selectivity, sensitivity, linearity, precision, accuracy, extraction recovery and stability for all compounds.

The measurement of consistent quantities of GluGal-Pyr in urine from both women and girls, along with low but detectable Gal-Pyr amounts only in girls, confirms the importance of free crosslink quantification. As a matter of 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 us to distinguish the degradation of bone from that of synovium or joints, and the free from the glycosylated-Pyr.

Therefore, the evaluation of free, glycosylated and total pyridinolines in urine may provide more information on bone and collagen catabolism, particularly in pathological conditions, and it may also be useful for monitoring disease progression and response to medical treatment. Furthermore, due to recent assumptions on the biological importance of collagen glycosylation on bone structure stability and mechanical properties, the non-invasive analytical method herein reported could be of great interest for the study of pathological conditions characterized by the modified activity of the enzymes involved in the collagen glycosylation (i.e., galactosyl- and glucosyl-transferases).

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