Total Urinary Hydroxyproline Determined with Rapid and Simple High-Performance Liquid Chromatography

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A precolumn derivatization method was optimized for rapid and specific analysis of total urinary hydroxyproline by HPLC. After an overnight hydrolysis, urine samples were dried and reconstituted with the internal standard cysteic acid (in sodium hydrogen carbonate, pH 9.3) were derivatized with N,N-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA) at 100 °C for 20 min. The FDNDEA-hydroxyproline adduct was separated on an Ultrasphere ODS column with a mobile phase of acetate buffer (containing triethylamine, 6 mM/L, pH 4.3) and acetonitrile (80/20, by vol), and was detected at 360 nm. A single run took 18 min with a hydroxyproline retention time of 7.3 min. The assay showed a linear response to hydroxyproline concentrations from 5 to 100 mg/L with a detection limit of 0.8 ng injected, corresponding to 2 mg/L in urine. Mean (SD) analytical recovery was 94.2 (13)% and 104 (9)% at 10 and 50 mg/L, respectively. Within-run and between-run CVs (n = 10) were 3.74% and 4.33%, respectively, for 25 mg/L. Results for samples (n = 50) analyzed by HPLC (y) vs ion-exchange chromatography with postcolumn ninhydrin reaction (x) correlated well: y = 0.98x + 1.02 (r = 0.985, Sxy = 3.13). In another comparison, involving 173 samples, a colorimetric procedure (Hypronosticon®) gave slightly higher values than the HPLC method (y): y = 0.83x + 2.21 (r = 0.937, Sxy = 4.6).

Additional Keyphrases: chromatography, reversed-phase, bone protein, amino acids

In humans, most of the body collagen is in bone, which represents the major reservoir of this protein (1). About 14% of the total amino acids content of collagen is 4-hydroxyproline (2), derived from hydroxylation of proline residues after protein is synthesized (3). Because the hydroxyproline released from collagen degradation is not reused, but only catabolized and excreted, the amounts of free hydroxyproline and hydroxyproline-containing peptides in urine are strictly related to collagen metabolism (4, 5) and to bone-related diseases (2, 6). Quantification of urinary 4-hydroxyproline allows diagnosis and monitoring of a variety of diseases, such as osteoporosis, Paget disease (2), bone secondary cancers (7), and hereditary disorders (8).

Early colorimetric methods for urinary hydroxyproline determination involved oxidation of hydroxyproline followed by colorimetric reaction with Ehrlich's reagent (9). Because many urinary components can interfere with these assays, different modifications of the original method were proposed (10–13), including a preliminary purification of urine hydrolysates before analysis (14) with an ion-exchange sulfonic column. Recently several high-performance liquid-chromatographic (HPLC) methods have been developed. These assays combine the good resolution power of HPLC with the sensitivity and specificity of various derivatizing agents, e.g., dabsyl chloride (15–18), phenylisothiocyanate (19–21), dansyl chloride (22), 4-chloro-7-nitrobenzofurazan (23–25), and 9-fluorenylethyl chloroformate (26–28). Most of these derivatization methods present problems with quantification and require an initial cleanup step with p-phthalaldehyde, which removes interfering peaks by selectively reacting with primary amino acids (26, 28).

Our purpose was to obtain a sensitive, specific, and rapid HPLC method for determination of total urinary 4-hydroxyproline. We recently suggested the use of N,N-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA) as a precolumn derivatizing agent that allows determination of primary and secondary amino acids (29). In addition we tested the reliability of this procedure for quantifying amino acids in serum (30). Here we present a modification of the previous HPLC method, designed to quantify hydroxyproline in hydrolyzed urine samples. In this method, the hydroxyproline peak is not affected by interfering compounds, so the preliminary purification step with p-phthalaldehyde is avoided.

Materials and Methods

Chemicals. 4-Hydroxy-L-proline and L-cysteic acid were from Sigma Chemical Co. (St. Louis, MO). FDNDEA and triethylamine were purchased from Fluka (Buchs, Switzerland). Sodium hydrogen carbonate, acetic acid, and of the inulin plasma single-shot clearance. Contrib Nephrol 1990;81:220–8.

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and all other chemicals, including HPLC-grade acetonitrile, were from Merck (Darmstadt, F.R.G.). All salts and reagents were AR grade. Water was always doubly distilled, and the buffers were filtered through a 0.45-μm pore-size filter (Millipore, Bedford, MA) before HPLC analysis.

Reagents and solutions. A stock solution of hydroxyproline (1 g/L) was prepared in HCl (10 mmol/L). Dilutions (to 5, 10, 25, 50, and 100 mg/L) were prepared in water and stored at −20 °C until use. A fresh solution of sodium hydrogen carbonate (2 mol/L, pH 9.3) was prepared every week. Cysteic acid (1 g/L) was dissolved in water, stored at −20 °C, and diluted 25-fold (to 40 mg/L) with the sodium hydrogen carbonate solution immediately before sample preparation. FDNDEA (5 g/L) was prepared weekly in acetonitrile and stored at 4 °C in the dark.

Apparatus. The analysis was carried out with a System Gold (Beckman Instruments, Fullerton, CA) liquid-chromatography system comprising two pumps (Model 126), a diode-array detector (Model 168), an auto sampler (Model 507), and a PS/2 50 IBM computer for the control of the whole apparatus. The analysis was performed with a 150 × 4.6 mm, 5-μm-particle size reversed-phase C18 column (UltraspHERE ODS; Beckman) and a 30 × 4.6 mm (5 μm) precolumn of Spheri 5 ODS (Brownlee, Santa Clara, CA).

Chromatographic conditions. Separation of hydroxyproline from other urinary components was carried out at room temperature and isocratically with a mixture (80/20, by vol) of acetate buffer (3 mL of glacial acetic acid, 6 mL of triethylamine, and 1 L of water, adjusted to pH 4.3 with HCl, 5 mol/L) and acetonitrile. The flow rate was 1.3 mL/min and the ultraviolet detector was set at 360 nm. After elution of the hydroxyproline peak, the acetonitrile proportion in the mobile phase was increased to 90% in 1 min and the column was washed for 5 min. Re-equilibration to the initial 80/20 volume proportion lasted 2 min, and the next sample was injected after another 3 min.

Sample treatment. Urine specimens were kept refrigerated at 4 °C during the 24-h collection period and then were frozen at −20 °C until hydrolysis. After thawing, specimens were stirred and centrifuged to remove any sediment, and 50 μL was transferred into screw-capped borosilicate-glass tubes with 50 μL of water and 100 μL of concentrated HCl. These samples were hydrolyzed for 16 h at 110 °C so that any bound hydroxyproline would also be detected (17). The hydrolysates were evaporated with a Speed Vac Concentrator centrifuge (Savant, Hicksville, NY) under reduced pressure, reconstituted with 200 μL of acetonitrile, and dried again. The residue was redissolved with 250 μL of cysteic acid solution (pH 9.3). After thorough mixing, 50 μL of the resulting solution was added to 50 μL of FDNDEA solution, derivatized at 100 °C for 20 min, and dried. The DNDDEA adducts were dissolved in 500 μL of the HPLC eluent, with mild heating if necessary, until complete dissolution was achieved, and 20 μL was automatically injected onto the HPLC.

Analytical variables. To evaluate the precision of the method, we calculated within-run coefficients of variation (CVs) for two urine samples at different concentrations that were hydrolyzed and then assayed repeatedly (n = 10) on the same day. To calculate the between-run CV, we analyzed 10 aliquots of a urine sample that were frozen and hydrolyzed on 10 subsequent days. Analytical recovery was evaluated by analyzing five urine samples to which different amounts of authentic hydroxyproline standard (10 and 50 mg/L) were added before hydrolysis.

Quantification. The concentration of the analyte was determined by using calibration curves for aqueous hydroxyproline standards. Aliquots (50 μL) of the standard solutions with increasing concentrations (5, 10, 25, 50, and 100 mg/L) of hydroxyproline were diluted with water (50 μL) and processed according to the derivatization procedure described above. The ratio between the derivatized hydroxyproline peak area and the derivatized internal standard area (γ) was plotted vs the concentration of the standard solutions (x). The standard curves were analyzed by linear-regression analysis to determine linearity. Calibration curves in urine were also prepared by adding 50 μL of the hydroxyproline standard solutions to 50 μL of pooled urine and, after hydrolysis, proceeding as described above.

Comparison procedure. To evaluate the reliability of our procedure, we hydrolyzed 50 urine samples obtained from the hospital laboratory and quantified their hydroxyproline content by using a High Performance Amino Acids Analyzer (Model 6300; Beckman) set at 440 and 570 nm. In addition, we tested 173 urine samples with both the HPLC and a routine spectrophotometric assay (9, 10) (Hypronosticon®; Organon Teknika, Rockville, MD). Except for age and sex, no information regarding health, disease, or diet restriction of the patients from whom the samples were obtained was available. To estimate the normal reference interval, we collected the 24-h urine specimens from 20 apparently healthy volunteers, ages 20–40 years (seven men and 13 women), working in our department. No diet restriction was imposed in the four days before the collection.

Results

Derivatization dependence on pH. The effect of pH on the derivatization reaction was investigated to determine the conditions for maximizing the amount of the DNDEA-hydroxyproline adduct. Four aliquots of hydroxyproline solution (17 mg/L) were hydrolyzed, desiccated, and resuspended with the internal standard solution at different pH values (range 9.2–9.9). As shown in Figure 1, the best reaction yields, in terms of peak areas, were obtained at a pH of 9.3.

Chromatographic separation of DNDEA-hydroxyproline. Figure 2A shows the separation of DNDEA derivatives of standard hydroxyproline (25 mg/L) and of cysteic acid used as internal standard. The retention times of cysteic acid and hydroxyproline are 4.2 and 7.3 min, respectively, with hydroxyproline being the first amino
acid to elute from the column. Figure 2B and C show typical chromatographic profiles of derivatized urine samples containing 25.1 and 14.2 mg of hydroxyproline per liter. The organic solvent concentration in the mobile phase was chosen to allow rapid analysis and efficient resolution of the hydroxyproline peak from other urinary components, especially from histidine, the amino acid eluting immediately after hydroxyproline. No peaks were detected at the retention time of cysteic acid in urine specimens that were derivatized without this component.

Analytical variables. Regression analysis of the calibration curves gave linear responses in the tested calibration range (5–100 mg/L). Slopes (±SE) of three aqueous standard curves prepared and analyzed on three different days were 0.0091 (0.0002), 0.0087 (0.0002), and 0.0091 (0.0001), with a day-to-day CV of 2.6%. Coefficients of correlation (r) were always > 0.999 with an SE of the estimate of 0.019, 0.013, and 0.016, respectively. The intercepts on the y-axis did not differ significantly from zero. Similar results were obtained for standard curves constructed by using hydroxyproline in urine.

The detection limit of the assay was 0.8 ng of hydroxyproline injected onto the column, at a signal-to-noise ratio of 2; this corresponds to a urinary concentration of 2 mg/L. The absolute analytical recovery, calculated with the pure DNDEA derivative of hydroxyproline, was 94.2 (SD 13%) and 104 (SD 9%) (n = 5) at 10 and 50 mg/L, respectively. The results of within-run and between-run precision studies are summarized in Table 1.

Comparison methods. The normal range for total urinary hydroxyproline calculated with our HPLC method for 20 healthy subjects was 22.3 (SD 6.2) mg/L. No significant sex-related difference was found, as already reported by other authors (26). Fifty urine samples collected without conscious bias from those submitted to the analysis laboratory were analyzed in triplicate by HPLC, by the routine colorimetric assay, and by the amino acid analyzer, generally considered the standard method for amino acid determination. Regression analysis of the HPLC (y) and the amino acid analyzer (x) results gave a linear fit with the following equation: \( y = 0.98 (±0.03)x + 1.02 (±0.88) (±SE), r = 0.985, S_{xy} = 3.13 \). This slope was not significantly different from 1 and the intercept was not different from 0. Statistical analysis of the data detected four outliers that were rejected (31) after additional evaluations. An analysis performed on a plot of the results of the colorimetric assay vs those from the analyzer gave a slope of 0.87 (±0.07) and an intercept of 4.69±2.6), (r = 0.878, S_{xy} = 9.39, with three outliers). When the DNDEA and the colorimetric methods were compared for 50 urine samples, correlation was poor: \( y = 0.76 (±0.06)x + 4.67 (±2.4), r = 0.860, S_{xy} = 9.01 \). Increasing the number of samples examined with these two methods (to 173) showed a better correlation: \( y = 0.83 (±0.03)x + 2.21 (±0.65), r = 0.937, S_{xy} = 4.69 \). This slope was significantly different from 1 and the intercept was significantly different from 0. Although the mean (SD) value by HPLC did not differ statistically from the colorimetric value (22.4 ± 16.2 vs 24.2 ± 17.8), these results indicate a general overestimation by the colorimetric assay with respect to the HPLC assay.

We also compared the results obtained from the HPLC analysis of the 173 urine samples both with and

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**Table 1. Precision of the Method**

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<tr>
<th>Sample</th>
<th>Hydroxyproline, mg/L</th>
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<tr>
<td></td>
<td>Mean</td>
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<tr>
<td>Within assay</td>
<td>26.2</td>
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<tr>
<td>Between assay</td>
<td>98.9</td>
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<td>24.7</td>
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n = 10 each.
without the use of the internal standard cysteic acid. Although no significant differences were found between the two methods of quantification (22.4 ± 16.1 vs 20.7 ± 14.7), we chose to use the more reliable internal-standard method to determine sample concentration.

Discussion

The aim of our study was to develop a practical and reliable method for quantifying hydroxyproline in a complex and heterogeneous biological sample such as urine. Because hydroxyproline is one of the first-eluting amino acids when FDNDEA is used to derivatize amino acids in serum (29, 30), we thought that this reagent might also work well with urine samples.

DNDEA derivatives dried under reduced pressure are quite stable for several weeks if stored at 4 °C. Once dissolved in the HPLC eluent, they may be kept in daylight and at room temperature for a week without appreciable degradation (30). The chromatographic analysis is performed in the isocratic mode, and both the internal standard and the hydroxyproline are very well resolved from the other urinary components. One chromatographic run takes 18 min from injection to injection, including washing and reconditioning the column. This short time is quite advantageous when compared with ion-exchange analysis, in which the hydroxyproline peak elutes at 12 min and a long conditioning time is required. With time (after ~250 analyses), the separation of hydroxyproline from adjacent peaks slowly deteriorates, so the initial acetonitrile percentage may have to be adjusted.

The method provides an acceptable analytical precision and a very good sensitivity, comparable with that of other HPLC methods with fluorescence detection (16). Most of the commonly used HPLC methods for hydroxyproline determination involve purifying urine samples by reactions of primary amino acids with o-phthalaldehyde (26, 28, 32); a great advantage of the present method is the avoidance of this time-consuming step. Moreover, our procedure requires fewer sample manipulations than do other methods (20, 22, 24, 25); hence the procedure is more rapid.

The accuracy of the precolumn derivatization procedure, although tested with only a few samples, was proved by the better correlation of the ion-exchange postlabeling method with the HPLC method (r = 0.985) than with the routine colorimetric procedure (r = 0.878).

The main drawback of the present method is that the derivatization yield is strictly related to the reaction pH. FDNDEA reacts quantitatively with amino groups in the presence of a very carefully controlled pH (>9.0). The most critical step of our procedure is, therefore, the drying of the hydrochloric acid, because a low pH adversely affects the recovery of DNDEA derivatives, especially that of cysteic acid.

In conclusion, this technique has a relatively low cost and requires a nondedicated instrument that, because of the stability of the derivatized products, may be coupled to an automated sampler. The good sensitivity, specificity, and reliability of the present method suggest that it merits consideration for determination of urinary 4-hydroxyproline in clinical laboratories.

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References


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D-Mannose as a Preservative of Glucose in Blood Samples

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We studied the changes in blood glucose concentration in blood samples collected in heparinized specimen tubes containing no other preservative, or containing NaF, D-mannose, or a combination of NaF and D-mannose. Blood concentration in samples taken into NaF decreased by 0.40 mmol/L in the first 2 h; thereafter, there was no change. In samples collected into mannose there was a small but significant decrease in blood glucose concentration with time. When samples containing mannose were analyzed immediately after collection, the concentration of glucose was higher than in later analyses, probably because of an exchange of intracellular glucose for extracellular mannose. When a combination of NaF and mannose was used, the blood glucose concentration was relatively stable but slightly higher than nonpreserved samples for the next 24 h. However, samples containing mannose were unsuitable for electrolyte analysis. We conclude that a combination of D-mannose and NaF may be a better preservative for blood glucose than either compound alone.

Additional Keyphrases: sample handling • enzymatic methods • electrolytes

Sodium fluoride (NaF), the most widely used anti-glycolytic agent for preserving glucose (J), has been reported to be the best available preservative (2). Nevertheless, even in the presence of NaF, glucose concentration decreases by 5–15% within the first 2–4 h after sample collection (3). D-Mannose has recently been suggested to be a useful anti-glycolytic agent to preserve glucose in blood samples (4–6) and reportedly acts faster than NaF (5). However, D-mannose is known to interfere with several methods for glucose analysis (7), causing positive interference in the glucose oxidase method and negative interference in the hexokinase method (8). Although Nakashima et al. (4) found that the rate of decrease in blood glucose in mannose-containing tubes was less than that in the presence of NaF, at 5 h after collection the blood glucose concentration was still decreasing. Because another reported advantage of D-mannose is that samples containing mannose can be used for electrolyte determination, we studied the changes in plasma glucose and electrolytes in blood samples collected in tubes containing NaF, mannose, or a combination of NaF and mannose.

Materials and Methods

A known amount of NaF and (or) D-mannose (Sigma Chemical Co., St. Louis, MO) dissolved in water was added to 1-mL screw-capped plain tubes and dried in an oven at 70 °C.

To study the effectiveness of D-mannose as a preservative for glucose, we obtained heparinized blood samples from 16 adults and aliquoted each sample into seven sets of tubes containing no other preservative (P); D-mannose, 3 g/L (M); NaF, 6 g/L (F); or a mixture of NaF (6 g/L) and D-mannose (3 g/L) (C). The contents of each tube were gently mixed. One set was centrifuged immediately and the remaining sets were left at room temperature (25 °C) and centrifuged after 2, 4, 6, 8, 12, and 24 h. The plasma was collected and stored at −70 °C for glucose analysis.

To study the short-term effect of D-mannose, we obtained heparinized blood samples from 17 adults and