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Note

Analysis of total hydroxyproline in urine by high-performance liquid chromatography and pre-column derivatization

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In man, endogenous 4-hydroxyproline is confined almost exclusively to collagen, where it constitutes about 14% of the total amino acid content[1,2]. More than half the body's collagen may be found in bone, with the remainder being mainly in the skin[3]. The hydroxyproline-containing products of collagen degradation cannot be reutilized, and are either catabolized or excreted; about 85–90% of the amino acids is oxidized, the remainder appearing in the urine[2,3]. Newly formed collagen is preferentially degraded[4]. Hydroxyproline is liberated during the intracellular maturation of collagen. Furthermore, degradation of the N-terminal peptides which contain 4-hydroxyproline in type I procollagen is probably also a source of urinary hydroxyproline. Therefore, increased collagen synthesis as well as breakdown will result in elevated hydroxyproline levels. In fasting individuals, urinary hydroxyproline may therefore be used as a specific index of collagen metabolism[3]. Increased urinary levels have been shown to be associated particularly with the accelerated growth phase at puberty, as well as with various diseases affecting bone, Paget's disease being the most notable example[2,3]. There has recently been renewed interest in urinary hydroxyproline as a marker for bony secondary cancer[5]. In particular, a number of workers have reported on its usefulness in the diagnosis and management of metastatic carcinoma of the prostate[6].

Over 90% of the hydroxyproline excreted in urine is peptide-bound[3]. Quantitation of the total urinary hydroxyproline therefore involves acid hydrolysis of the hydroxyproline-containing peptides. The conventional method of analysis by Ehrlich's reaction after chloramine T oxidation can be carried out manually or with the use of an automated continuous flow analyzer[7–9]. However, the concentration of free urinary hydroxyproline is usually too low to be measured accurately. Recently, high-performance liquid chromatographic (HPLC) analysis of hydroxyproline in fibroblast cultures[10], urine[11] and serum[12] has been reported. These methods are not suitable for the analysis of large numbers of samples.

Our objective was to develop a reliable, optimized and more rapid HPLC method for urinary hydroxyproline analysis. The method of sample treatment, which involved hydrolysis of urinary peptides and chemical derivatization, had to be reliable and reproducible.

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EXPERIMENTAL

Materials

Acetonitrile and acetone (both of chromatography grade), L-4-hydroxyproline, N-methyltaurine, 4'-dimethylaminoazobenzene-4-sulphonyl chloride (dabsyl chloride), alanine, arginine, glycine, glutamine, proline and other amino acids were obtained from E Merck (Darmstadt, F.R.G.). Quantitative urine-control sets were from Bio-Rad (Anaheim, CA, U.S.A.). The Dowex 50W-X8 (H^+) resin (100–200 mesh) was from Fluka (Buchs, Switzerland). All other chemicals were of AnalaR grade from E. Merck or BDH (Poole, U.K.).

Apparatus

An automated liquid chromatographic system was used (Waters Assoc., Milford, MA, U.S.A.). It consisted of a Model 6000A solvent-delivery system, a Model 481 UV–VIS absorbance detector, a Model 730 data module and a column temperature-control accessory. Hibar pre-packed RP-18 columns (125 mm × 4 mm) were from E. Merck. A Model 1040A diode-array detection system (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used to determine the optimum wavelength for dabsylated hydroxyproline. Water purification was performed with a Milli-Q Water Polishing System (Millipore, Bedford, MA, U.S.A.).

Buffer preparation

Phosphate–citric acid buffer (pH 5.0) comprised 0.2 *M* disodium monohydrogenphosphate–0.1 *M* citric acid (10.3:9.7, v/v). Acetate–citrate–citric acid buffer (pH 6.0) was prepared by dissolving 57.1 g of sodium acetate trihydrate, 58.7 g of trisodium citrate dihydrate and 5.5 g of citric acid in 800 ml of water. Carbonate–bicarbonate buffer (pH 9.2) comprised sodium carbonate–sodium hydrogencarbonate (14:86, v/v).

Resin-catalysed hydrolysis

To a 0.5-ml urine sample in a 5-ml test-tube were added 1.5 ml phosphate–citric acid buffer (pH 5.0) and 200 mg Dowex 50W-X8 (H^+) resin. The mixture was gently shaken for 10 min and then centrifuged. The supernatant was discarded, and 6 ml water were added. The mixture was centrifuged at 1000 *g*, and the supernatant was again discarded. The tube containing the resin was screw-capped and left overnight (16 h) at 120°C. On the next day, hydroxyproline and other amino acid residues were eluted with 1 ml of acetate–citrate–citric acid buffer (pH 6.0).

Derivatization

An aliquot of N-methyltaurine (40 μl of a 10 m*M* solution) as internal standard was added to the eluate. To a 50-μl portion of the mixture, transferred to a 4-ml glass vial, were added 450 μl carbonate–bicarbonate buffer (pH 9.2). A 50-μl aliquot of this solution was then mixed with 100 μl dabsyl chloride solution (4 m*M* in acetone). The vial was screw-capped and incubated at 70°C for 10 min with constant shaking. The solvent was removed by a stream of nitrogen at 40°C in a fume-cupboard. The dry residue was reconstituted in 500 μl mobile phase, and 10 μl were injected into the chromatographic system.

Chromatography

We used an isocratic mobile phase of 30% (v/v) acetonitrile in 12 m*M* sodium acetate (pH 4.14). Its aqueous and organic components had been filtered through a 0.45-μm nitrocellulose membrane and a 0.45-μm fluorocarbon membrane (Millipore, Bedford, MA, U.S.A.) respectively. Degassing was carried out by sonication and sparging with helium before use. A reversed-phase Hibar C_{18} column (particle size 5 μm, 125 mm × 4 mm I.D.) was used. The column temperature was 40°C and the flow-rate of the mobile phase was 0.7 ml/min. The eluate was monitored at 471 nm and 0.005 absorption units full scale (a.u.f.s.).

After establishment of the standard calibration curves and confirmation of retention times and recovery rates, the Data Module of the HPLC system was programmed to calculate chromatographic results. The samples were normally set up in injection vials just before the end of a working day and analysis was performed unattended during the night. Print-outs of chromatograms with retention times and calculated results were available the next morning.

Creatinine measurement

Urinary creatinine was determined by the Jaffe reaction in an Astra 8 autoanalyser (Beckman, Palo Alto, CA, U.S.A.).

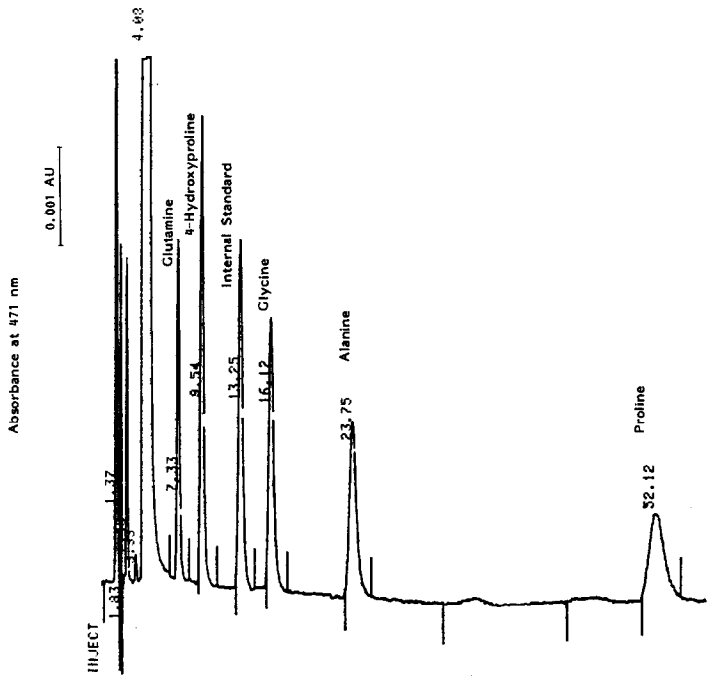

Fig. 1. Chromatographic separation of a standard mixture of dabsylated amino acid residues. This is a direct print-out from the Data Module.

RESULTS

4-Hydroxyproline in a mixture of standards was separated from the other major amino acid residues of urinary hydroxyproline-containing peptides (Fig. 1). Under the chromatographic conditions described, the proline peak was eluted at about 50 min. The use of our isocratic mobile phase system was satisfactory for the analysis of 4-hydroxyproline in urine samples from normal subjects (Fig. 2) and from patients (Fig. 3). The whole chromatographic analysis required about 75 min. An absorption maximum at 471 nm was obtained for the hydroxyproline during its chromatographic elution.

Analytical data were obtained from the analysis of hydroxyproline in quantitative urine-control sets (Bio-Rad) at low (50 μM) and high (200 μM) levels. The intra-assay coefficient of variation (C.V.) was 6.8% (n = 12) and inter-assay C.V. was 12% (n = 5). Linearity ranged from 10 to 100 pmol at 0.01 a.u.f.s. and from 2 to 30 pmol at 0.005 a.u.f.s. Therefore the use of different absorption scales in the detector enabled us to obtain an extended range from 2 to 100 pmol (corresponding to 5–250 μM hydroxyproline in the starting solution). The use of either peak height or peak-height ratio was satisfactory. The absolute recovery of hydroxyproline in the assay was 72% and the recovery rate relative to N-methyltaurine (internal standard) was 97%. We have also assessed the analysis of urine specimens by determining the

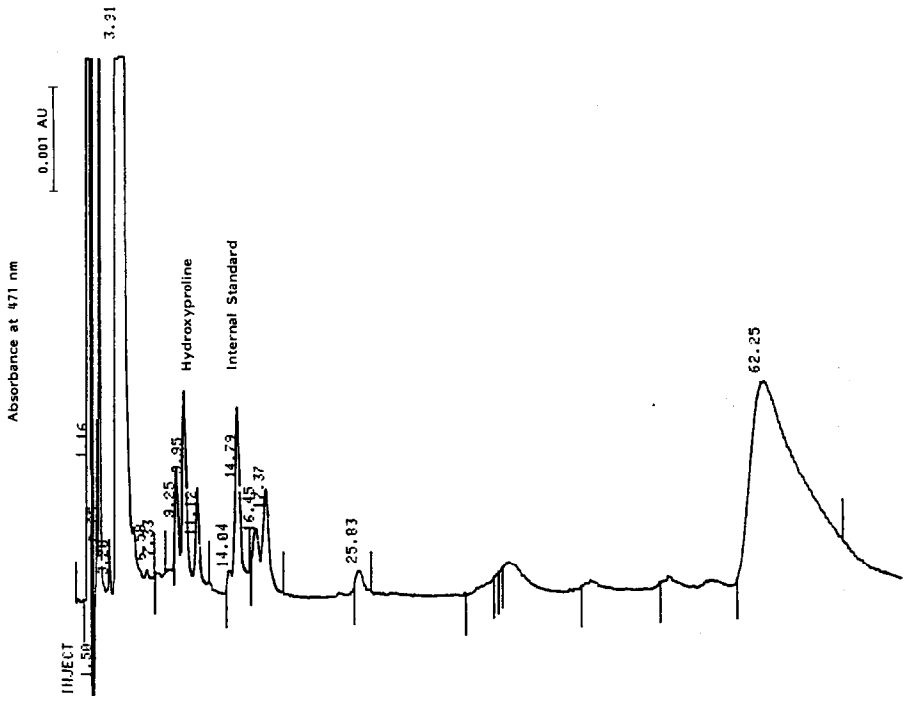

Fig. 2. A typical chromatogram of total hydroxyproline analysis of an urine sample from a fasting normal subject. Chart speed: 0.3 cm/min; flow-rate 0.9 ml/min. Conditions for resin-catalysed hydrolysis, derivatization and chromatography are as in the text.

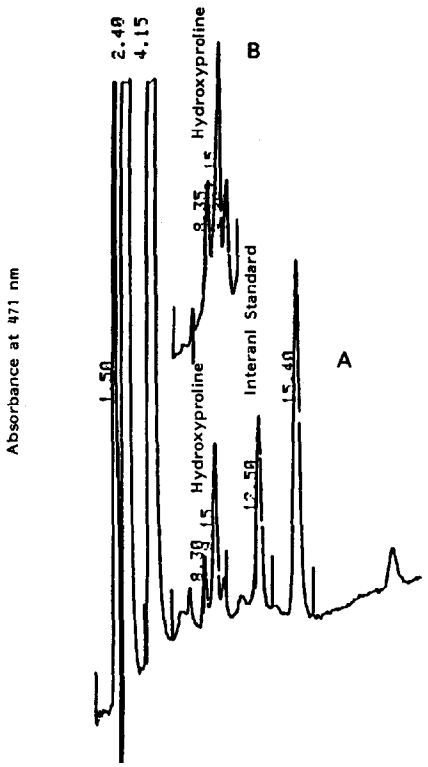

Fig. 3. Hydroxyproline (Hyp) analysis of (A) an urine sample from a patient with elevation in alkaline phosphatase from bone, (B) the same urine sample spiked with hydroxyproline (3 pmol). Only the earlier parts of the chromatograms are shown.

precision of the retention time of hydroxyproline relative to N-methyltaurine in the chromatogram. The intra-assay C.V. was 0.26% (n = 12) and the inter-assay C.V. was 3.6% (n = 9).

We assayed urine specimens from eight fasting healthy adults (ages 28–37) and from seven young female adolescents (ages 10–12). The results for the adult controls ranged from 8 to 49 μmol hydroxyproline per mmol creatinine, the mean and standard deviation (S.D.) being 26.4 ± 16.0 μmol. The values for the young adolescents ranged from 77 to 173 μmol hydroxyproline per mmol creatinine, the mean and S.D. being 120.9 ± 33.7 μmol.

DISCUSSION

In the past, HPLC of hydroxyproline in biological specimens has invariably involved gradient elution[10–12], although dabsylated amino acids have been separated by isocratic elution[13]. We found the use of the isocratic mobile phase system to be simple and reliable. A good separation of dabsylated hydroxyproline from the other amino acid residues was obtained. The chromatographic analysis can be completed in 40 min.

Incubation in 6 *M* hydrochloric acid overnight at a temperature above 105°C has been the common method for peptide hydrolysis. This is also frequently used for the hydrolysis of hydroxyproline-containing peptides[9,11]. The use of a sulphonated polystyrene cation-exchange resin to catalyse peptide hydrolysis has been shown to be effective[14]. Hodgkinson and Thompson[15] used a sulphonated cross-linked polystyrene–divinylbenzene resin for their peptide hydrolysis, and measured the released hydroxyproline by an automated colorimetric assay. We used the same cation exchanger and combined the resin-catalysed hydrolysis with HPLC analysis. More than 500 injections could be carried out with one C_{18} HPLC column. No interference from pigments or other substances[16] has been observed.

Initially we used a detection wavelength of 455 nm as used by others[11]. A wavelength of 425 nm had also been used for determination of dabsylated amino acids[13]. With the diode-array detector, the optimum wavelength of 471 nm is readily obtainable and contributed to improved sensitivity.

The analytical recovery of 72% is not very satisfactory. Major losses could have been incurred during the resin-catalysed hydrolysis procedure because it involves several steps of washing, incubation and elution. Nevertheless, in a separate experiment we detected no significant difference in hydroxyproline yield between acid hydrolysis (6 *M* hydrochloric acid, 120°C for 16 h) and the described resin-catalysed hydrolysis. The detection limit of 2 pmol in our method should enable free hydroxyproline to be detected in urine specimens. We are now making efforts to achieve a reliable and accurate quantitation of such low levels. The results of the measurement of total hydroxyproline in urine samples from healthy subjects were consistent with published values[17,18]. Higher levels of hydroxyproline excretion in adolescents[19,20] were also found.

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