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SIMPLIFIED PROCEDURE FOR THE ANALYSIS OF 3- AND 4-HYDROXYPROLINE

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SUMMARY

A column chromatographic analysis for 3-hydroxyproline (3-Hyp), 4-hydroxyproline (4-Hyp), and γ-carboxyglutamic acid (Gla) is described. The analyses of urine and plasma were performed with a JLC-6AH amino acid analyzer. A 0.15 *M* sodium citrate buffer, pH 2.1, was used for elution. Urinary Gla, 3-Hyp, and 4-Hyp were among the seventeen peaks eluted before aspartic acid. Hyp, Gla, glutamine, and asparagine in plasma were separated by elution with 0.2 *M* sodium citrate buffer, pH 3.25, containing 10% methanol. This single-column procedure achieves the sequential separation and quantitation of Gla, 3-Hyp, and 4-Hyp in urine as well as plasma, and is applicable to the diagnosis of collagen metabolism disorders.

INTRODUCTION

Collagens from skin, tendon, bone, and vascular tissue contain large amounts of 4-hydroxyproline (4-Hyp), while basement membrane collagen from the kidney contains 3-hydroxyproline (3-Hyp) [1]. About 90% of Hyp derived from the catabolism of collagens is degraded to carbon dioxide and water and only 5—10% of Hyp is excreted in urine. The urinary Hyp fraction contains 5—10% free Hyp and 90—95% peptide-bound Hyp [2]. Under normal conditions, free 4-Hyp is usually not present in detectable amounts in urine and serum samples except for those from prematures, neonates, and infants. Collagen turnover can be estimated from the quantitation of either free 4-Hyp or total 4-Hyp in urine. In Paget's disease of the bone, in progressive systemic sclerosis, and in cancer metastatic to the bone, an increased degradation of collagen is observed. Since elevated urinary levels of free 4-Hyp and peptide-bound Hyp are found in the foregoing disorders, the assay of free and/or bound 4-Hyp is useful in the diagnosis of collagen disorders. In each α-chain of skin collagen, a single residue of 3-Hyp is presend per 90—120 residues of

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4-Hyp. As many as 10—15 residues of 3-Hyp, however, are present in every α-chain derived from renal basement membrane collagen. Although free 3-Hyp is not excreted in detectable amounts in normal urine, 3-Hyp-containing peptide is present in normal urine [3]. The ratio of 3-Hyp to 4-Hyp increases with increased degradation of basement membrane collagen, as occurs in acute nephritis.

3-Hyp and 4-Hyp in urine can be separated from other amino acids by the ion-exchange chromatographic procedures developed by Moore and Stein [4], Spackman et al. [5], Piez and Morris [6], and Hamilton [7]. All of these methods require several hours of analysis. Other methods of 4-Hyp determination require prior oxidation and decarboxylation followed by color reaction with Ehrlich reagent [8—12]. Szymanovicz et al. [13] have described a method for separating 3- and 4-Hyp on an amino acid analyzer which requires a preliminary separation of the urine sample on an ion-exchange column. This paper describes a procedure for the rapid analysis for free Hyp in urine and in protein hydrolysate using a single column.

MATERIALS AND METHODS

Twenty-four hour urine samples were collected from ten healthy adult individuals and two patients with scleroderma. Each 24-h urine sample was collected in a plastic bottle, stored in an ice bath during collection, and kept frozen at —20°C before analysis. Blood samples, obtained by venipuncture, were collected in a vacutainer tube with or without anticoagulation. Plasma or serum was separated from blood cells by centrifugation within 1 h of collection. Samples showing even a slight hemolysis were discarded.

Standard amino acid mixture, 4-Hyp, and chemicals and reagents used in amino acid analysis, were obtained from Pierce (Rockford, IL, U.S.A.). Taurine, cysteic acid, and calf skin collagen were purchased from Sigma (St. Louis, MO, U.S.A.). Burro aortic collagen was purified as previously described [14]. γ-Carboxyglutamic acid (Gla) was purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.). A sample of 3-Hyp was kindly provided by Drs. A. Szymanovicz and J.P. Borel, Laboratoire de Biochimie Medicale, Reims, France.

Aliquots (5 ml) of a urine sample were adjusted to pH 2.0 with concentrated hydrochloric acid, then stirred for 10 min and the precipitate removed by centrifugation.

Protein was removed from plasma samples by adding four volumes of 1% picric acid solution. The precipitate was removed by centrifugation, and the supernatant either analyzed directly or diluted with four volumes of 0.2 *M* sodium citrate buffer, pH 2.2. A 0.8-ml aliquot of the deproteinized sample was applied to the analyzer for the quantitation of free amino acids. Usually only two of the three dilutions (1-, 5- or 25-fold) of a sample were analyzed. Triplicated analyses were performed on each dilution and means ± S.D. were calculated. The precision for free Hyp assay is 0.4 nmole.

Protein hydrolysis

Collagen was hydrolyzed with 6 *N* hydrochloric acid for 20 h at 110°C

in a sealed evacuated pyrex tube. After hydrolysis, the samples were dried in vacuo and then dissolved in 0.20 *M* sodium citrate buffer, pH 2.2. The humin formed was separated from the solution by centrifugation. A 0.8-ml aliquot of the hydrolysate was used for analysis. Proteins and peptides containing γ-carboxyglutamyl residues were hydrolyzed with 2 *N* potassium hydroxide. The hydrolysate was titrated to pH 2.0 with 2 *N* hydrochloric acid, and diluted to appropriate concentrations for analysis.

Chromatography on the amino acid analyzer

Amino acid analysis was performed on JLC 6AH autoanalyzer equipped with a high-sensitivity cuvette (10 mm light path) and a single-column elution system. Two single columns (25 × 0.8 cm) packed with JEOL LCR-2 resin, a spherical resin of sulfonated polystyrene (7% cross-linkage, 10—12 μm in size). Free Gla and Hyp in urine were separated from other amino acids by a one-step elution with 0.15 *M* sodium citrate buffer, pH 2.1 (slow elution program) at the flow-rate of 0.51 ml/min. Column temperature was adjusted to 55°C and at 62 min after elution the temperature was raised to 62°C. Column regeneration was done by eluting with 0.2 *M* sodium hydroxide for 10 min, then equilibrated with a pH 2.1 buffer for 30 min.

In the fast elution program, a sample was first eluted with a 0.2 *M* sodium citrate buffer, pH 3.25, containing 10% (v/v) methanol, followed by a 0.2 *M* sodium citrate buffer, pH 4.25, and finally with a 0.35 *M* sodium citrate buffer containing 0.1 *M* sodium borate adjusted to pH 9.6 with sodium hydroxide [15]. The column was regenerated by elution with 0.2 *M* sodium hydroxide for 5 min, followed by equilibration with pH 3.25 buffer for 25 min. The amino acids were monitored by absorbance at 440 and 570 nm after reaction with ninhydrin. The recorder sensitivity was set at 0.155 a.u.f.s. In the slow elution program, a 0.15 *M* sodium citrate buffer, pH 2.1, was used for elution until the appearance of 4-Hyp, a pH 4.25 buffer was followed to elute neutral and acidic amino acids. Finally a pH 9.6 buffer was applied to complete the elution of basic amino acids. If only 3-Hyp or 4-Hyp is to be quantitated, the elution time for each sample can be shortened by regenerating the column as soon as the Hyp is eluted.

RESULTS

The fast elution program with a pH 3.25 buffer containing 10% (v/v) methanol accelerated the elution of Hyp and/or Gla from other amino acids in protein hydrolysate and plasma. The elution order of a standard amino acid mixture is: cysteic acid (21 min), Gla (35 min), 3-Hyp (39 min), 4-Hyp (42 min), and aspartic acid (Asp) (45 min) (Fig. 1). The elution pattern of an aortic collagen hydrolysate is shown in Fig. 2. The 4-Hyp shows a distinctive peak, while 3-Hyp appears only as a shoulder before the 4-Hyp peak.

During the analysis of free amino acids in urine, most amino acids in diluted urine (25-fold) eluted within the full scale expansion of 0.155 absorbance except for glutamine (Gln) (eluted with threonine), glycine (Gly), and histidine (Fig. 3, left). The first three peaks eluted with a pH 3.25 buffer were composed of at least sixteen components, as determined by elution with a

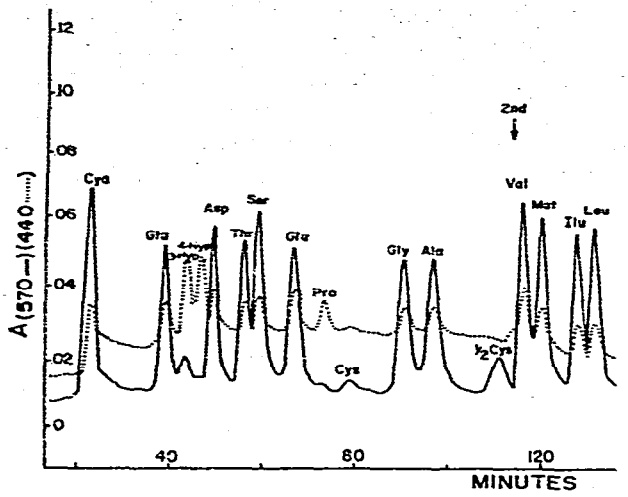

Fig. 1. The elution pattern of a mixture of standard amino acid (6 n*M* each), including cysteic acid (Cya), Gla (10 n*M*), 3-Hyp and 4-Hyp (20 n*M* each) on a column of LCR-2 resin (25 × 0.8 cm) of JLC 6AH amino acid analyzer. The first buffer contains 0.2 *M* sodium citrate, pH 3.25, with 10% (v/v) methanol. The second buffer contains 0.2 *M* sodium citrate at pH 4.25. The third buffer contains 0.1 *N* sodium borate and 0.35 *N* sodium citrate at pH 9.60. The equilibration with the first buffer for 40 min is performed before applying the sample. The arrows denote positions of buffer change of second buffer. Peaks eluted after leucine are not shown.

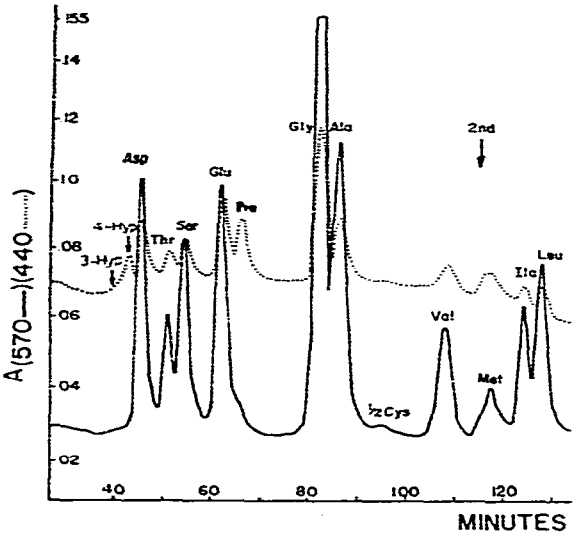

Fig. 2. The elution pattern of a burro aortic collagen hydrolysate. The elution procedure is exactly the same as that described in Fig. 1 except that the equilibration with the first buffer for only 20 min is performed before applying the sample. Peaks eluted after leucine are not shown.

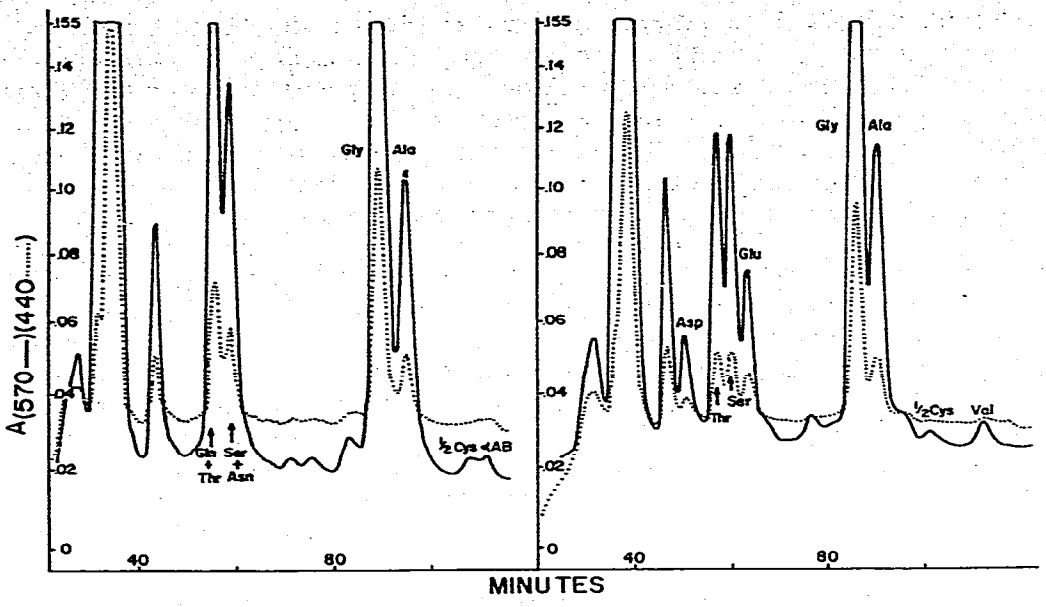

Fig. 3. Resolution of acidic and neutral amino acids in 0.8 ml of 25-fold diluted urine (left) and the same urine sample after *E. coli* asparaginase treatment (right). The appearance of Asp and Glu after asparaginase digestion is observed. Gla, 3-Hyp and 4-Hyp are obscured by the peaks eluted before Asp. The column is eluted with 0.2 *M* sodium citrate buffer, pH 3.25, containing 10% methanol.

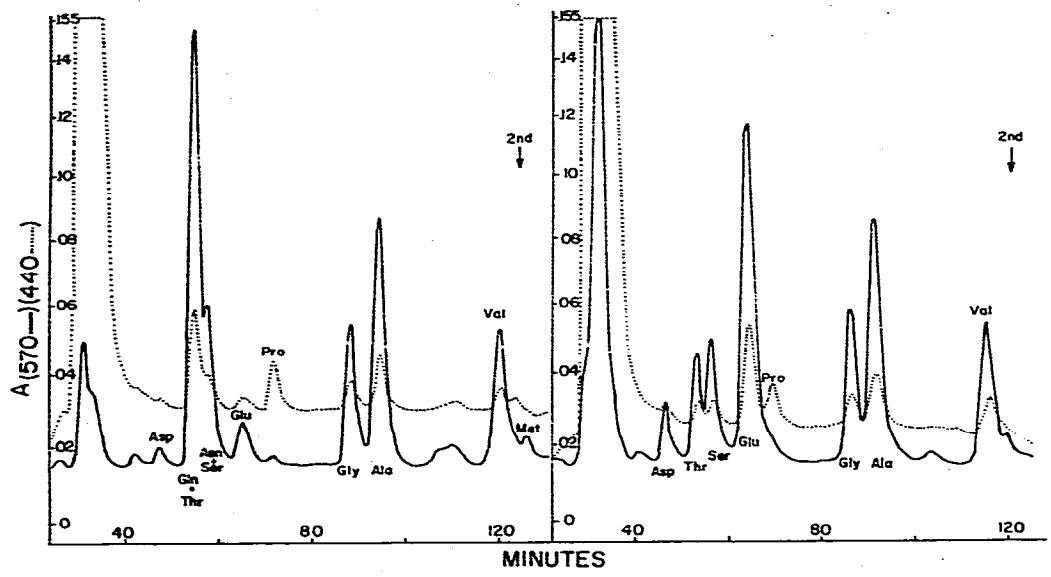

Fig. 4. Separation of acidic and neutral amino acids from 0.8 ml of a 25-fold diluted deproteinized serum sample. Left, without asparaginase treatment; right, after asparaginase treatment. The elution condition is the same as that described in Fig. 3.

pH 2.10 buffer. Thus, 3- and 4-Hyp were not resolved from other components.

For the quantitation of plasma amino acids, the sample was deproteinized by picric acid. A 0.8-ml aliquot of diluted (25-fold) and deproteinized sample with picric acid content intact was analyzed on a single column system. The result is shown in Fig. 4 (left). Most amino acids exhibited absorbance lower than 0.155. Since glutamine (Gln) did not separate from threonine (Thr), nor did asparagine (Asn) separate from serine (Ser), the quantitation of Thr, Ser, Asn, and Gln could not be achieved by this program. Although picric acid elutes at the void volume of the column with a strong absorbance at 440 nm and may interfere with the quantitation of taurine, urea, and phosphorylated amino acids, it eluted well ahead of 3- and 4-Hyp and thus did not affect the quantitation of these two amino acids. Incubation of the serum and urine sample with *Escherichia coli* asparaginase completely converted Asn and Gln into Asp and Glu (1 unit enzyme per 2 ml serum or 4 ml urine). The quantitation of Thr, Ser, Asn, and Gln was accomplished by this simple treatment (Fig. 3, right and Fig. 4, right). Deamidation of Asn and Gln in urine with asparaginase treatment was slower and took overnight incubation at 37°C.

An improved resolution of Hyp and Gla from other urinary peaks can be achieved by using the slow elution program with a pH 2.1 buffer. Altogether 16 to 17 peaks eluted before Asp from a urine sample of a scleroderma patient who possibly had kidney involvement (Fig. 5). The first three peaks eluted as incompletely separated peaks each of which probably contained more than one component. Among these peaks which co-eluted with standard references were cysteic acid, phosphoserine, and phosphothreonine (25 min); phosphoethanolamine and taurine (29 min); and urea (35 min). The remaining peaks were well separated from each other and included Gla (73 min), 3-Hyp (111 min), Δ^1-pyrroline 3-hydroxy-carboxylic acid (a metabolic product of 3-Hyp) (60 min), and 4-Hyp (184 min). Peaks eluted at 49, 80, 100, 125,

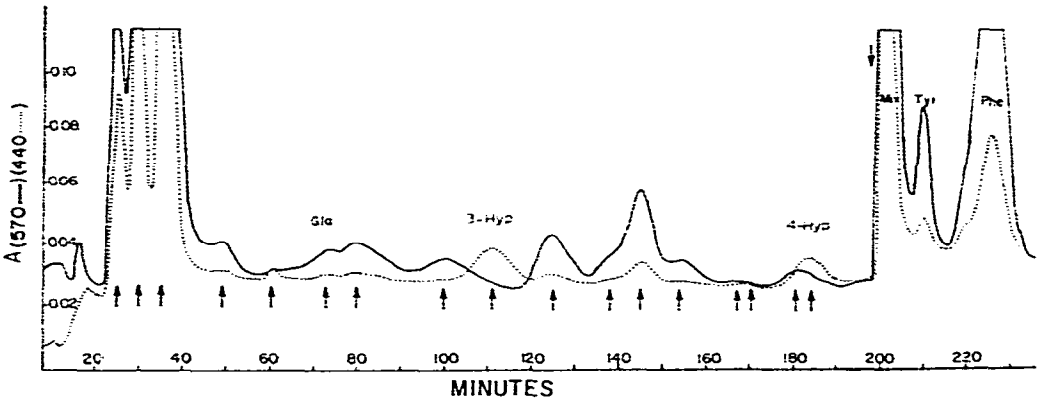

Fig. 5. Improved resolution of Gla, 3-Hyp and 4-Hyp in urine from other acidic amino acids. A 0.8-ml volume of a 5-fold diluted sample is applied to the column, the column is eluted with a 0.15 *M* sodium citrate buffer, pH 2.1, followed by a 0.2 *M* sodium citrate buffer, pH 4.25. The arrows indicate the position of peaks eluted.

138, 145, 154, 167, 170, and 181 min were not characterized. Although 3-Hyp was not present in the urine of normal individuals, a small quantity of a metabolite of 3-Hyp was sometimes peaked at 60 min. 3-Hyp and 4-Hyp and their metabolite could be easily identified because of much higher absorbance at 440 nm than at 570 nm. The presence of free 3-Hyp in the urine of this patient with scleroderma strongly suggests the renal involvement of the disease.

DISCUSSION

Many amino acid derivatives from the post-translational modification of protein are only partially re-utilized after the protein is degraded. Such modified amino acids and peptides are mostly excreted in the urine. The quantitation of modified amino acids and their peptides in urine can be used to estimate the turnover of a particular protein containing the unique amino acid derivative.

Although most of the hydroxyproline can be utilized for an energy source, the increased turnover of collagen results in an elevated level in the blood of hydroxyproline and its peptides. Thus, increased amounts of free and peptide-bound hydroxyproline were excreted in urine. The increased turnover of collagen could be detected by measuring the content of 3- and 4-Hyp and their peptides in urine. Since the content of 3-Hyp is higher in basement membrane collagen than in collagen from skin, tendons, and bone, the simultaneous quantitation of 3- and 4-Hyp and their peptides could furnish important information for the differential diagnosis of collagen disorders affecting various tissues [13]. The amount of 3-Hyp was increased from normal in the urine of patients suffering from acute nephritis, while 4-Hyp was increased in patients with scleroderma, Paget's disease of bone, or metastatic bone disease [2]. All free hydroxylated prolines found in the urine and serum can be sequentially quantitated by one-step chromatography on an amino acid analyzer without any preliminary purification of the sample. This procedure has the potential capability of detecting the deterioration of collagen in kidney and other tissues.

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