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High-performance liquid chromatographic method for the determination of prolyl peptides in urine

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ABSTRACT

A rapid and accurate method is described for the determination of prolyl peptides in urine, with specific reference to the dipeptide prolylhydroxyproline, and free hydroxyproline and proline. Free amino acids and peptides were isolated from urine on cation-exchange minicolumns, and free imino acids and prolyl-N-terminal peptides were selectively derivatized with 4-chloro-7-nitrobenzofurazan, after reaction of amino acids and N-terminal aminoacyl peptides with *o*-phthalaldehyde. The highly fluorescent adducts of imino acids and prolyl peptides were separated on a Spherisorb ODS 2 column by isocratic elution for 12 min using as mobile phase 17.5 m*M* aqueous trifluoroacetic acid solution containing 12.5% acetonitrile (eluent A), followed by gradient elution from eluent A to 40% of 17.5 m*M* aqueous trifluoroacetic acid solution containing 80% acetonitrile in 20 min. Analytes of interest, in particular the dipeptide prolylhydroxyproline, can be easily quantified by fluorimetric detection (ε_{ex} = 470 nm, ε_{em} = 530 nm) without interference from primary amino-containing compounds.

INTRODUCTION

Total urinary hydroxyproline (Hyp) consists of 1–3% free Hyp and more than 95% of a peptide-bound form; the prolylhydroxyproline (Pro–Hyp) dipeptide represents about 60% of all Hyp peptides excreted [1,2]. The urinary oligo- and dipeptides containing hydroxyproline are a reflection of collagen metabolism [3,4]. A strongly increased excretion of dipeptides containing imino acids is observed in individuals suffering from diseases concerning collagen metabolism [5–7]. Thus, the determination of this dipeptide fraction in urine can be regarded as suitable for clinical biochemical studies of disorders of collagen metabolism.

The determination of urinary peptides together with free amino acids has been accomplished by ion-exchange chromatography on columns packed with sulphonated polystyrene resins [8]. The subsequent improvement of this technique has allowed the separation and identification of a large number of peptides of different molecular sizes, some of which contained Hyp [9–11].

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More recently, attention has been turned to the study of the fractionation of urinary dipeptides by gas chromatography [12–15]. Although very efficient, this method is tedious and time consuming and therefore unsuitable for routine determinations. High-performance liquid chromatography (HPLC) is simpler, but few data are available in the literature [16,17].

In this paper, we report an HPLC method for the selective determination of propyl peptides in urine, together with free imino acids.

EXPERIMENTAL

Materials

Hyp, proline (Pro), prolylleucine (Pro–Leu), prolylglycine (Pro–Gly), Pro–Hyp, prolylproline (Pro–Pro), 4-chloro-7-nitrobenzofurazan (NBD-Cl) and *o*-phthalaldehyde (OPA) were obtained from Sigma (St. Louis, MO, U.S.A.), analytical-reagent-grade trifluororacetic acid (TFA) and boric acid from Merck (Darmstadt, F.R.G.), methanol and acetonitrile (HPLC grade) from Inalco (Milan, Italy) and Dowex 50W-X8 (H^+) (100–200 mesh) from Bio-Rad Labs. (Richmond, CA, U.S.A.). Water was demineralized and glass-distilled.

A 25-m*M* NBD-Cl solution was prepared in methanol. OPA reagent was prepared at a 150 m*M* concentration in methanol.

The standard solution, prepared in 0.01 *M* hydrochloric acid, was a 2.5 m*M* mixture of each of Hyp, Pro, Pro–Hyp, Pro–Pro, Pro–Leu and Pro–Gly. This solution was stored at 4°C and freshly prepared every 2 weeks. The propyl peptides used as standards are the main dipeptides present in normal or pathological urine.

Sample preparation

Urine samples (24-h) were obtained from healthy subjects. The urine was kept at 4°C during collection. A 10-ml aliquot of the 24-h urine sample was adjusted to pH 2.0 with 6 *M* hydrochloric acid; a 5-ml aliquot of this sample was poured into a minicolumn (1 × 0.8 cm I.D.) of Dowex 50W-X8, previously washed with 5 ml of 0.01 *M* hydrochloric acid. After adsorption of the urine sample, the column was washed with 5 ml of water to remove salts, acids and neutral compounds. The adsorbed fraction of amino acids and peptides was then eluted with 5 ml of 2 *M* ammonia solution. A Rotavapor was used to remove the solvent from the collected fraction at 50°C. The dry residue was dissolved in 2.5 ml of 0.2 *M* borate buffer (pH 9.0) and derivatized according to the method described previously [18]. A 0.2-ml aliquot of this solution was poured into a screw-capped glass tube (5 × 0.5 cm I.D.) and 50 μl of OPA reagent were added. After 3 min at room temperature, 100 μl of NBD-Cl solution were added; the derivatization was carried out at 60°C for 15 min and the reaction was stopped by adding to the mixture 0.65 ml of eluent A. A 50-μl aliquot of the solution was injected into the chromatograph.

Reversed-phase chromatography

HPLC was performed by using an apparatus consisting of two Jasco 880-PU pumps (JASCO, Tokyo, Japan) and a Shimadzu (Kyoto, Japan) FC 530 spectrofluorimeter, equipped with a xenon lamp and a 12-μl quartz flow cell. The NBD derivatives were detected by setting the monochromators at 470 nm for excitation and

at 530 nm for emission. The detector was connected to a Model 3390 A integrator (Hewlett-Packard, San Diego, CA, U.S.A.). A Model 910 injection valve (Negretti, Southampton, U.K.), whose loop had been replaced with a Guard-Pak C_{18} module (Waters Assoc., Milford, MA, U.S.A.), was used as a precolumn. The separation was performed on a 15 × 0.4 cm I.D. Spherisorb ODS 2 (5 μm) column.

Chromatographic analysis was carried out by isocratic elution followed by binary gradient elution as described below. Eluent A was 17.5 m*M* aqueous TFA solution containing 12.5% acetonitrile and eluent B was 17.5 m*M* aqueous TFA solution containing 80% acetonitrile. Both mobile phases were briefly degassed under reduced pressure. Prior to analysis, the column was equilibrated with eluent A at room temperature for 20 min at a flow-rate of 1 ml/min. After sample injection, elution was first carried out under isocratic conditions with eluent A for 12 min and then by gradient elution from eluent A to 40% of eluent B in 20 min. After the analysis, the column was washed with eluent B for 5 min and then the initial conditions were re-established by passing eluent A through the analytical column for 20 min.

The concentration of the analytes was determined from peak areas in comparison with those of known standards.

A calibration graph was obtained from working standard solutions containing amounts ranging from 10 to 100 nmol/ml of analytes in 0.2 *M* borate buffer (pH 9.0); 0.2-ml aliquots of these solutions were derivatized according to the procedure described above.

RESULTS AND DISCUSSION

HPLC with precolumn derivatization

The chromatogram shown in Fig. 1 represents a typical separation of standard Hyp, Pro, Pro–Hyp, Pro–Gly, Pro–Leu and Pro–Pro (1 nmol of each). The chromatographic system allows the baseline separation of these analytes; a non-interfering peak, due to the side-product 7-nitro-4-benzofurazanol (NBD-OH), is present in the chromatogram. The optimum elution conditions were chosen after several trials, performed with different eluents with pH from 7 to 3 and with different percentages of organic modifier. The elution conditions chosen, *i.e.*, the TFA–acetonitrile system established by an isocratic step coupled with a gradient elution step, allow the baseline separation of all the components and particularly of the pair Pro–Hyp and Pro–Gly, which have similar retention times.

The reliability of the chromatographic analyses was checked from 0.01 to 1 nmol of the analytes injected in the column; the linearity of the detector response to analyte concentration was excellent for all the compounds. Calibration graphs for Hyp, Pro–Hyp, Pro, Pro–Gly, Pro–Leu and Pro–Pro showed linearity over the concentration range from 0.01 to 1 nmol. Correlation coefficients between peak areas and amounts of analytes, ranging from 0.997 to 0.999, were obtained by linear regression analysis. The precision was determined with five aliquots of a working solution, at the same concentration (1 nmol) of each standard. The within-assay relative standard deviations (R.S.D.) for Hyp (3.1%), Pro (2.8%), Pro–Hyp (3.3%), Pro–Gly (2.6%), Pro–Leu (2.5%) and Pro–Pro (3.2%) indicate good reproducibility. The between-assay R.S.D.s were less than 6.1%. Recovery experiments ($n = 5$), performed by using the same aliquot (1 ml) of a working standard solution (5 nmol/ml of 0.01 *M*

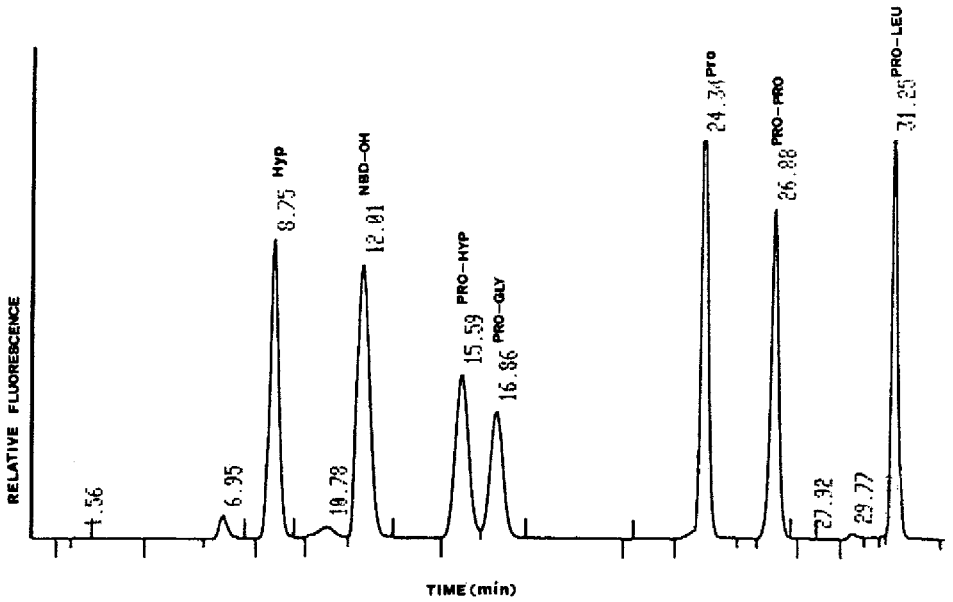

Fig. 1. Chromatogram of standard Hyp, Pro–Hyp, Pro–Gly, Pro, Pro–Pro and Pro–Leu, derivatized according to the procedure described in the text. Analyte peaks correspond to 1 nmol of each compound. Chromatographic conditions: 15 × 0.4 cm I.D. Spherisorb ODS 2 (5 μm) column; elution at a flow-rate of 1 ml/min isocratically for 12 min with 17.5 m*M* aqueous TFA solution containing 12.5% acetonitrile, then a 20-min linear gradient from this eluent to 40% of 17.5 m*M* aqueous TFA solution containing 80% acetonitrile was used. Attenuation detector sensitivity: ×8.

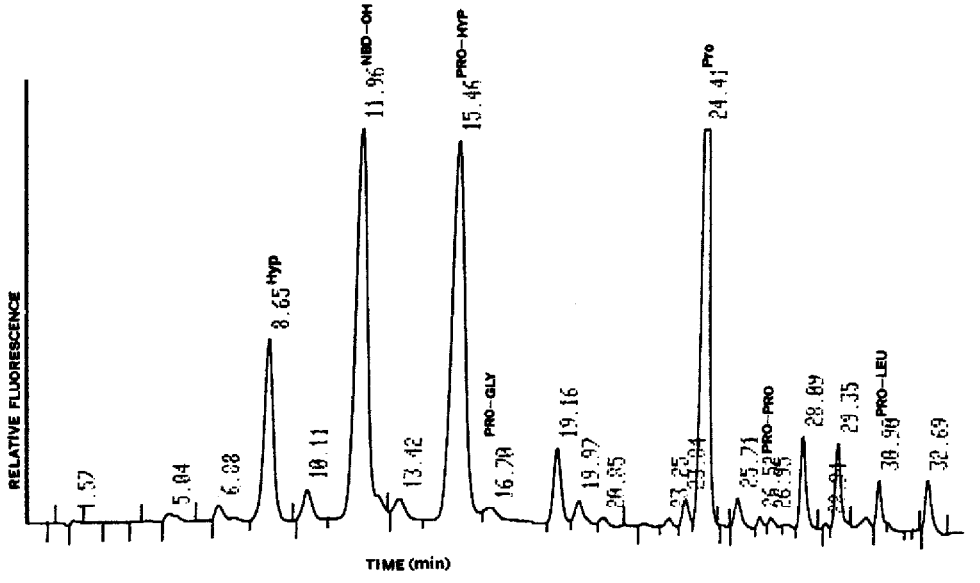

Fig. 2. Representative chromatogram of a urine sample, treated and derivatized as described in the text. Chromatographic conditions as in Fig. 1. Attenuation detector sensitivity: ×6.

hydrochloric acid) which had been treated according to the complete procedure used for the biological sample, gave recoveries above 90% for all the compounds, and the maximum R.S.D. was 8%.

Finally, the detection limit, referred to the amount of standards injected into the analytical column, was calculated to be 10 pmol for all of the compounds.

Urine analysis

Fig. 2 shows the chromatogram of a urine sample with selective separation of free amino acids and peptides having Pro as the N-terminal amino acid. Pro–Hyp and the free imino acids can be easily determined without interference from compounds containing amino groups. In this study, quantification was performed for free imino acids, Pro–Hyp and Pro–Gly, the most abundant collagen-related metabolites. The amounts (μmol/g creatinine) of free Hyp, Pro, Pro–Hyp and Pro–Gly, determined in urine of normal adult subjects, were (mean $\pm$ S.D., $n = 7$) 6.2 $\pm$ 2.3; 62.5 $\pm$ 12; 36.7 $\pm$ 8 and 1.6 $\pm$ 0.7, respectively.

As can be seen in Fig. 2, several other peaks are present in minute amounts compared with imino acids, Pro–Hyp and Pro–Gly. Two of these peaks were tentatively identified as indicated in Fig. 2, on the basis of their retention times and by standard coelution experiments.

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