Amino Acids (1999) 17: 315–322

Amino
Acids

Urine glycyl-L-proline increase and skin trophicity

Short Communication

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Accepted November 11, 1998

Summary. Glycyl-L-proline (gly-pro) is an end product of collagen metabolism that is further cleaved by prolidase (EC 3.4.13.9); the resulting proline molecules are recycled into collagen or other proteins. We postulated a relationship between defective gly-pro hydrolysis, increased collagen degradation and skin destruction. This relationship was tested using HPLC to measure the gly-pro in urine. 24 hour urine samples were collected from 27 old people (86 ± 6 years old), of whom 15 were suffering from skin pressure sores of the sacrum or calcaneus. The urine from patients with pressure sores contained significantly more gly-pro than the urine from the control. A cut-off at 7 μmol/mmol creatinine gave the test a positive predictive value of 70%. Collagen breakdown was also increased as indicated by the increase of hydroxyproline (hyp) in the urine. But this breakdown seemed to stop at the gly-pro step.

Keywords: Amino acids – Glycyl-L-proline – Urine – Trophicity – Collagen

Introduction

The dipeptide glycyl-L-proline (gly-pro) accounts for about one third of type I collagen. It is also an end product of collagen breakdown. Its production is the result of the collagen turnover, which occurs in as much as 3–5% of total skin collagen per day (Laurent, 1987). The gly-pro is further digested by prolidase (EC 3.4.13.9) and the proline molecules may be recycled into collagen or other proteins. As a result, there is normally little or no gly-pro in the urine. The recycling of proline is of great metabolic importance, not only for collagen synthesis, but for all protein synthesis, because this amino-acid plays a key role in protein folding. Proline recycling is affected by fasting, because collagen and muscle proteins then become the major sources of amino acids in the body during starvation (Berg and Kerr, 1992).

Interest was focused on gly-pro following the discovery of a rare inherited disease, prolidase deficiency (McCusik 26413). This disease is characterized

by iminodipeptiduria, especially gly-pro (Goodman et al., 1968). The iminodipeptiduria is associated with dermatological symptoms, such as refractory ulcers of the legs, purpuric, erythematous, papular or scaly lesions and photosensitivity. These symptoms can be related to the importance of prolidase in wound healing (Senboshi et al., 1996). Skin fibroblasts taken from prolidase deficiency patients and kept in tissue culture also show increased breakdown of newly synthesized collagen (Myara et al., 1983; Chamson et al., 1989). We have therefore postulated that there is a link between the defective gly-pro hydrolysis, the increase in collagen breakdown and skin trophicity. Our hypothesis is that the disturbed proline recycling stimulates the breakdown of proline-rich proteins, mainly collagen, which gives rise to the skin trophicity. This pathophysiology may concern, not only inherited prolidase deficiency, but also the trophic lesions that occur in old people. We have tested this relationship using HPLC method to measure gly-pro in the urine of elderly subjects suffering from pressure sores.

Materials and methods

Reagents

Thimerosal (mercury-[(O-carboxyphenyl)thio]ethyl sodium salt), PMSF (phenylmethylsulfonyl fluoride), and sodium 1-heptane-sulfonate were from Sigma, NH_4OH and methanol, reagent grade, were from Prolabo and acetonitrile (for HPLC and far UV) was from Carlo Erba. Dowex 50 W × 2 was from Fluka. The HPLC column was an ultrasphere ODS 5 μm (4.6 mm diameter, 250 mm high) purchased from Beckman. The standard was gly-pro (Sigma G-3200) and gly-L-^{14}C pro was especially prepared (by the Service des Molecules Marquées, CEN, Saclay, France). The dipeptides alanyl-proline (ala-pro) and glycyl-leucine (gly-leu) were from Sigma.

Subjects

The study was carried out on 27 elderly people hospitalized in a geriatric department. The cases (85 + 7 years old, 6 men and 9 women) had skin pressure sores of the sacrum or calcaneus. The 12 people in the control group were 2 men and 10 women, 88 + 6 years old. All 27 people were suffering from usual general disorders and all were given meals under the same conditions.

Urine sampling

24 hour urine samples were collected at room temperature. Several preservatives were tested: thimerosal (2.5 mmol/l), PMSF (5 mmol/l), and acetonitrile (191 mmol/l).

Urine preparation

The urine samples were subjected to ion exchange chromatography Dowex 50 W × 2 to eliminate components other than amino-groups or polypeptides. The resin was used in the H^+ form, poured into 5 mm diameter/50 mm high columns. The urine sample (1 ml) was adjusted to pH 7 with 4 mol/l NH_4OH, diluted to 6 ml with water and run onto the column. The column was washed in 2 ml water and eluted with 6 ml 2 mol/l NH_4OH containing 20% methanol. The eluate was dried under a stream of air and taken up in 1 ml water.

HPLC chromatography

Samples were analyzed by ion-pair reversed-phase high performance liquid chromatography on a Beckman ultrasphere ODS (C-18, 5μm spherical, 80 Å pore) cartridge using a Kontron high-performance liquid chromatograph with two T-414 LC pumps and a 432 variable-wavelength UV detector. The pumps were monitored by 457 HPLC controller software from Kontron. The sample was introduced via a Rheodyne model 7125 sample injection valve using a 100μl loop. The HPLC was connected directly to a Flo-one β detector (Packard Ins.) using Luma-Flow III (Lumac, Olen, Belgium) as scintillator reagent. The chromatogram was monitored and plotted by the Flo-One software.

Sodium 1-heptane sulfonate was used as the ion-pairing reagent (Frey et al., 1993) and the absorbance was monitored at 210nm. Linear gradients were prepared by mixing solution A (19.8mmol/l sodium 1-heptane sulfonate, adjusted to pH 2.3 with sulfuric acid) and solution B (solution A containing 50% acetonitrile). The two solutions were filtered through 0.2μm filters and degassed under vacuum before use. The shapes of the gradients used are shown in Fig. 1. The flow rate was 0.8ml/min. The gly-pro fraction was quantified with reference to dosed addition (200nmol) to the 1ml sample before ion exchange chromatography. Measurement therefore included two HPLC separations, one with and one without the dosed addition (Fig. 2).

Hydroxyproline determination

Hydroxyproline (Hyp) was determined by the method of Stegeman (Stegeman, 1958) on a 1ml urine sample, that had been hydrolysed with 6mol/l HCl (final concentration) for 24h at 100°C.

Creatinine determination

Creatinine was measured with a Vitros 250 apparatus (Ortho Clinical Diagnostics) (Granouillet et al., 1996). The results are expressed as μmol gly-pro/mmol creatinine.

Statistical methods

Controls and cases were compared by Mann-Whitney. U test for difference in medians. The cut off values of the tests were etermined by ROC (Receiver Operating Characteristic) curves. The solftware was NCSS statistical system for windows (Number Cruncher Statistical Systems, J. L. Hintze, Kaysville, Utah 84037, USA).

Results

The gly-L-^{14}C pro tracer (900,000dpm) showed that gly-pro was eluted at about 45min (Fig. 1), by 13% acetonitrile. A mixture of gly-pro, ala-pro and gly-leu was tested (Fig. 3) and gly-pro did not coeluate with the other dipeptides. Preservatives did not improved the results. Routine determinations were carried out using gly-pro (200nmol) as dosed addition.

The urine samples from the elderly people with and without pressure sores were analysed. There was no significant difference between the results for the men and the women. Mann-Whitney U test showed a difference ($p = 0.01$) between the patients with and without pressure sores. The urine of patients with pressure sores had significantly more gly-pro than the controls (Fig. 4).

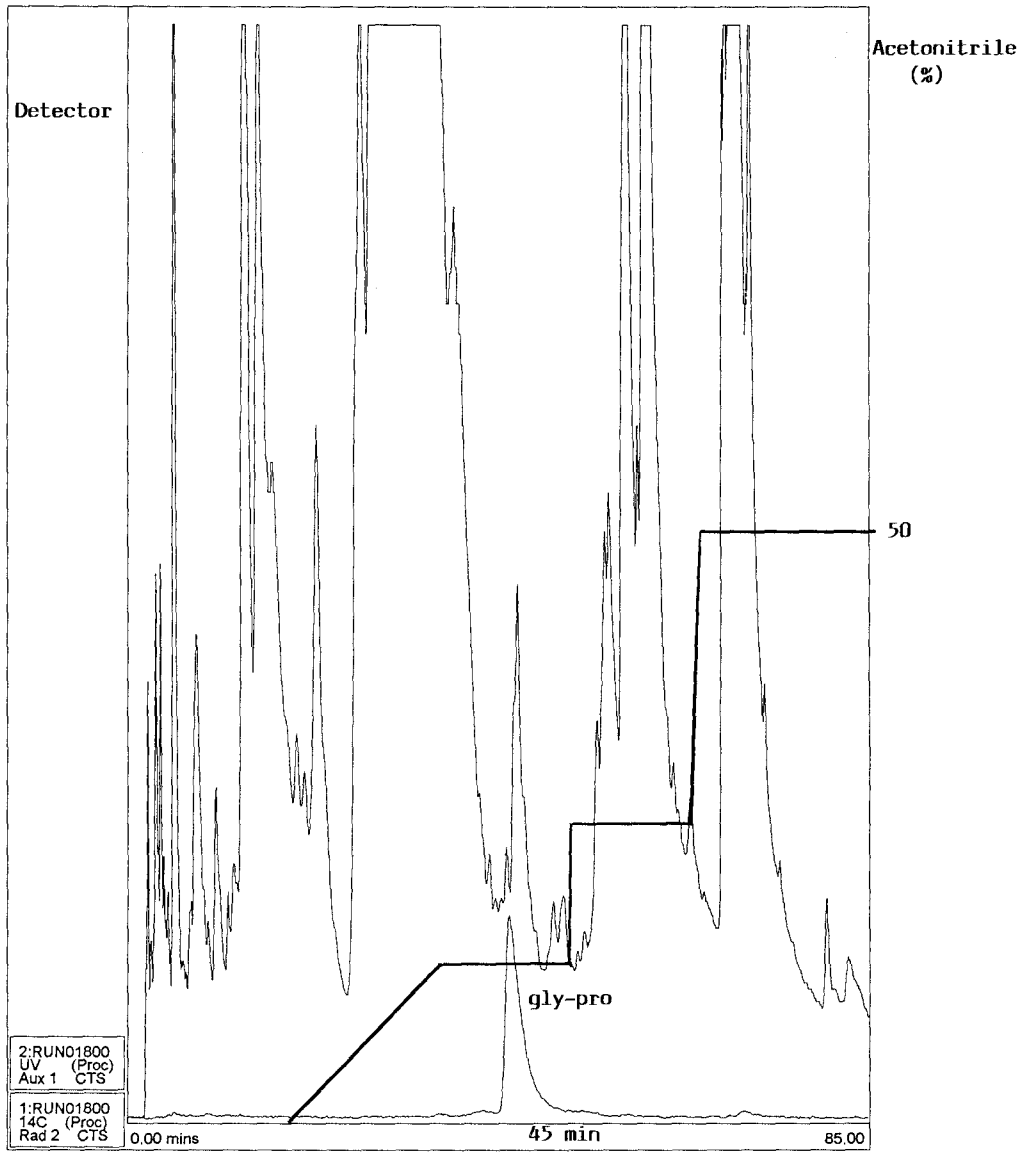

Fig. 1. HPLC chromatogram of a urine sample with added gly-L-^{14}C pro (lower graph). Column ultrasphere Beckman (C-18, 5μm); gradient elution from 19.8mmol/l sodium heptane sulfonate, pH 2.3, with acetonitrile as the gradient former

A cut-off at 7μmol/mmol of creatinine gave this parameter a positive predictive value of 70%.

The possible link between urinary gly-pro and collagen breakdown was assessed by hydroxyproline in the same urine samples. There was also a significant difference ($p = 0.01$) between the controls and the patients with pressure sores (Fig. 5). In addition, the linear regression parameters indicated no correlation between the amounts of gly-pro and hyp in the urine ($r = 0.43$, $0.01 < p < 0.05$).

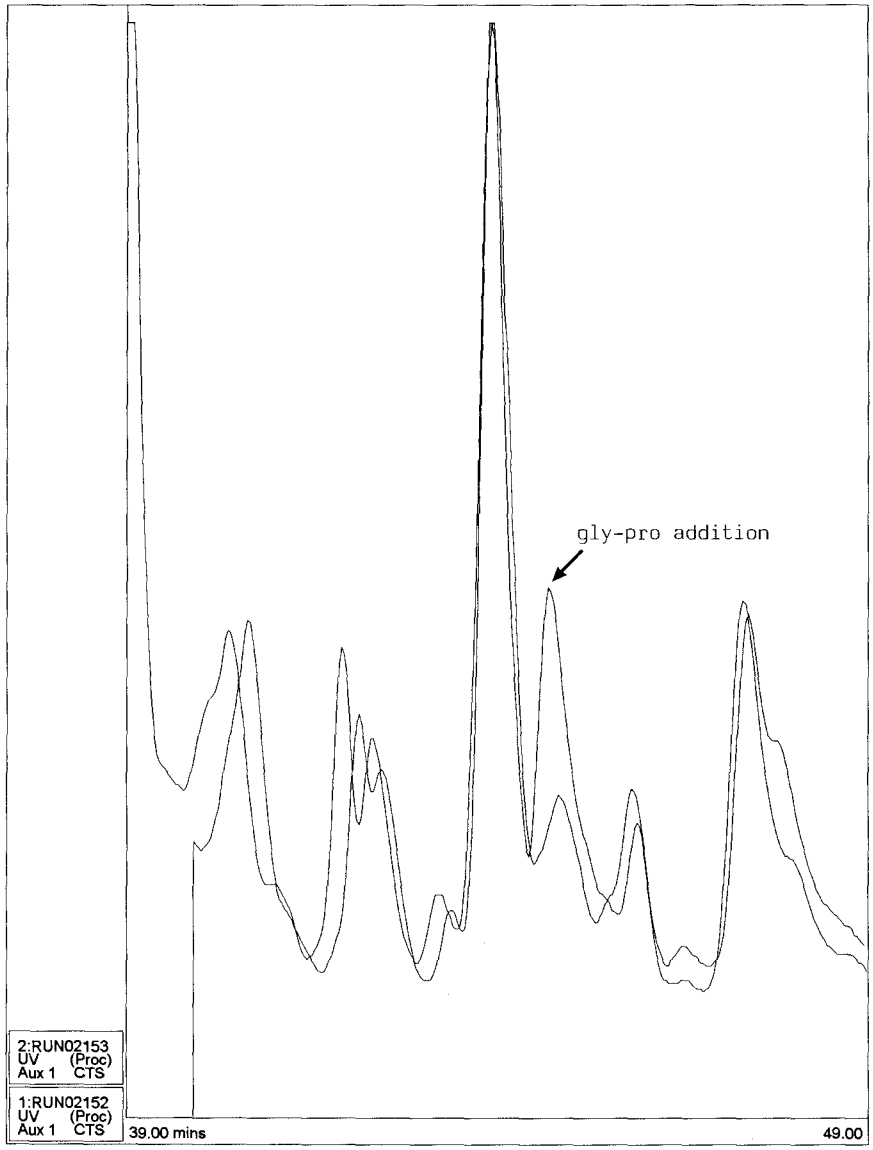

Fig. 2. Chromatogram of the gly-pro area with and without added standard gly-pro. The chromatography conditions are the same as in Fig. 1

Discussion

The use of HPLC allowed the gly-pro in urine samples to be measured by UV detection without prior derivatization. Quantitation was improved by spiking urine samples with dosed addition of gly-pro. As adding preservatives to the urine gave no improvement, fresh urine without preservatives was used because it is difficult to check the preservative concentration when the urines are collected on the ward.

There appeared to be a decrease in gly-pro degradation in elderly people with skin trophic disorders. This phenomena indicated a decrease of prolidase

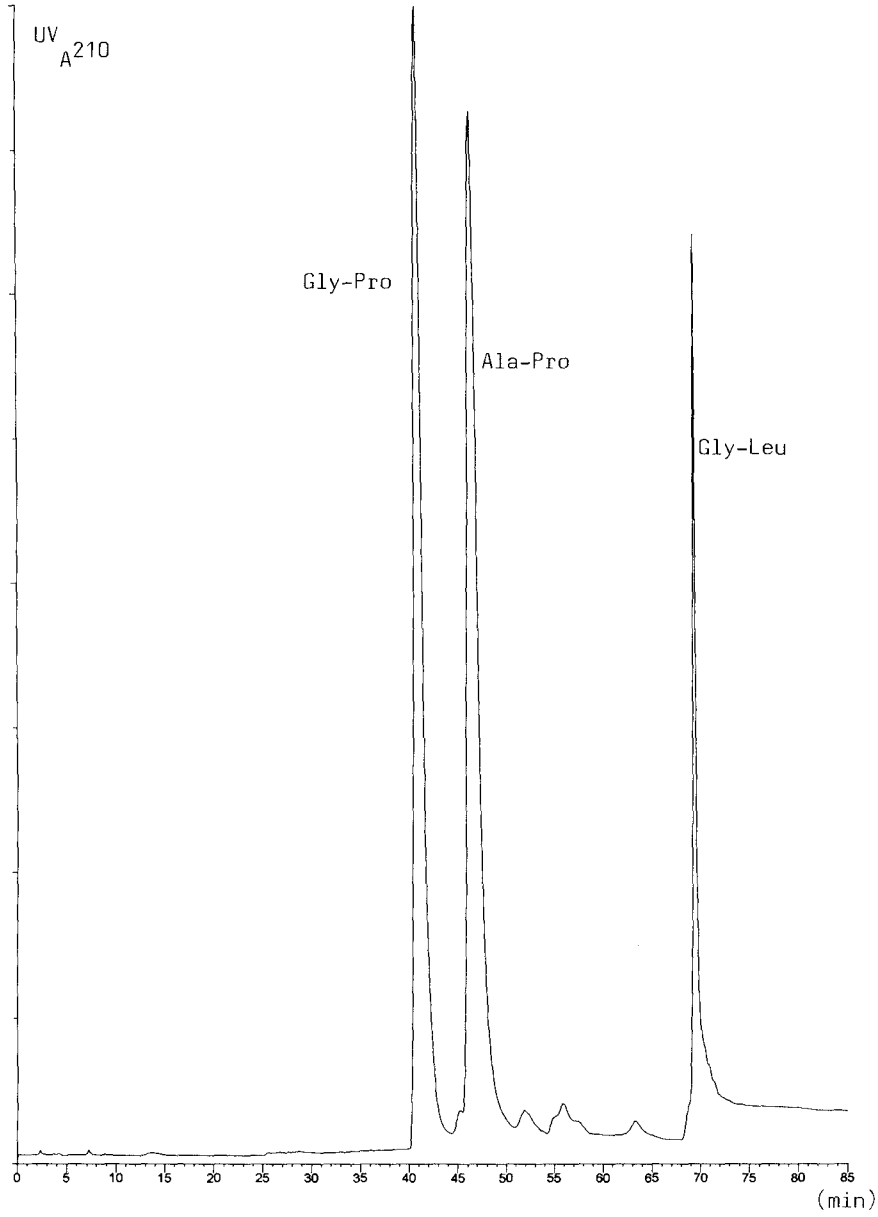

Fig. 3. Chromatogram of a mixture of gly-pro, ala-pro, gly-leu. The chromatograph conditions are the same as Fig. 1

activity. Such a decrease was observed in elderly people mainly in patients with senile dementia (Yoshida et al., 1996). In addition, the over expression of prolidase mRNA in scar tissue (Senboshi et al., 1996) allowed to understand the appearance of refractory ulcers correlated with prolidase deficiency. The elderly people with skin trophic disorders did not seem to suffer from a major prolidase deficiency, but this minor disturbance could interfere with proline recycling, thus limiting protein synthesis. The restriction could involve all proteins, particularly collagen, and contribute to a loss of extracellular matrix

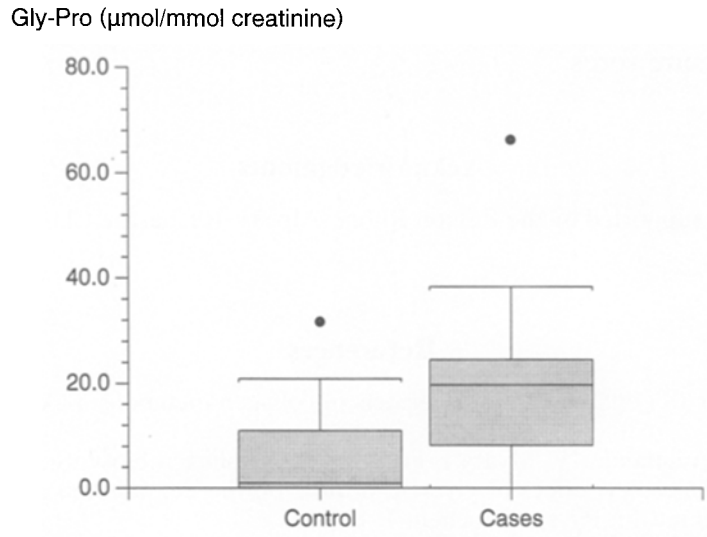

Fig. 4. Urine glycyl-L-proline concentration in elderly people. The top and bottom of the boxes are the upper and lower quartile. The line through the middle of the boxes indicates the median. The upper and lower adjacent values (interquartile range ×1.5) are shown by lines above and under the boxes. Dots are outside values

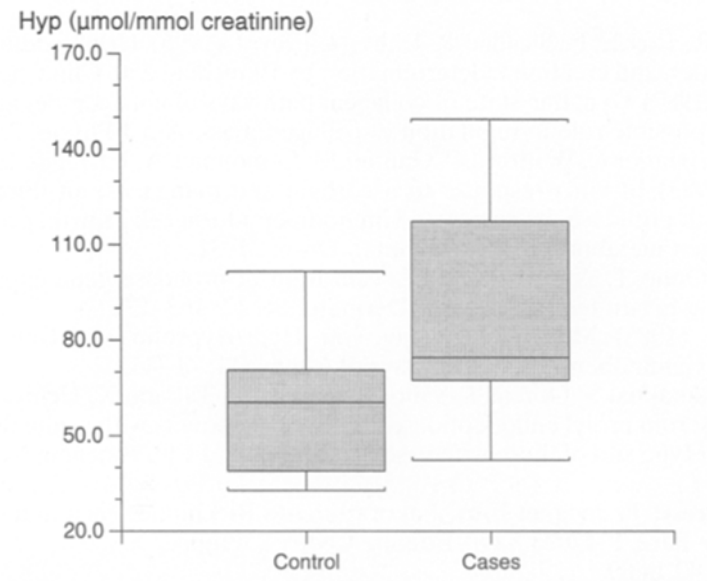

Fig. 5. Urine hydroxyproline concentration in elderly people

protein in elderly people. The increase in hyp indicated an increase in collagen breakdown which tended to stop at gly-pro.

The disturbance of proline recycling suggested that the disorder can be treated nutritionally by adding proline supplement to the diet. The therapeutic assays used in prolidase deficiency (Charpentier et al., 1981), including

supplementation with ascorbate and manganese might be also useful for treating pressure sores.

Acknowledgments

This work was supported by the Région Rhône-Alpes («Recherche Clinique») and «GIP Exercice».

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Received November 5, 1998

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Prof. Dr. Gert Lubec, Universitäts-Kinderklinik, Währinger Gürtel 18–20, A-1090 Wien. – Redaktion: Sachsenplatz 4–6, A-1201 Wien. – Hersteller: Satz und Umbruch: Best-Set Typesetter Ltd., Hong Kong; Offsetdruck: Novographic Druck G.m.b.H., Maurer-Lange-Gasse 64, A-1230 Wien. – Verlagsort: Wien. – Herstellungsort: Wien. – Printed in Austria.