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Use of capillary zone electrophoresis for analysis of imidodipeptides in urine of prolidase-deficient patients

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Abstract

Prolidase deficiency (PD) is characterized by massive urinary excretion of imidodipeptides X-Pro and X-Hyp. We report the applicability of capillary zone electrophoresis to urinary imidodipeptide determination. The protocol is fast, simple, reliable, only small amounts of sample are required and there is minimal sample preparation. Electropherograms of urine samples from control subjects and four patients with prolidase deficiency were compared. The presence of imidodipeptides normally absent in urine was evident in patients' urine. Further analysis of urine samples enabled identification of excreted imidodipeptides and the pattern of excretion appeared to be heterogeneous for different patients. This method appears to be useful for identification of imidodipeptides in biological samples, as an efficient aid in diagnosis of PD, and as a method for providing more information about this disease.

Keywords: Imidodipeptides

1. Introduction

Prolidase (EC 3.4.13.9), a ubiquitous imidodipeptidase which hydrolyses dipeptides containing C-terminal prolyl or hydroxyprolyl residues is important in the end stages of the catabolism of endogenous and dietary proteins. Deficiency of prolidase results from an autosomal recessively inherited disorder, prolidase deficiency (PD) and affected individuals excrete massive amounts of imidodipeptides X-Pro and X-Hyp into the urine. The disorder is associated with variable clinical features including recurrent infections, chronic skin ulceration and mental retardation [1]. The mechanisms involved in pathogenesis of the disease are still poorly understood.

Thus far, diagnosis of PD has been based on the determination of urinary amino acids by chromatography [2–4] and assay of the enzyme activity in erythrocytes [5], leukocytes or in skin fibroblast cultures and plasma [5,6]. However, some of the methods involved are time consuming and may have limitations in terms of sensitivity, complexity and exact identification of imidodipeptides [5,6].

More recently, high-performance liquid chromatography (HPLC) coupled to atmospheric pressure ionization mass spectrometry (API-MS) has been applied to identify a series of imidodipeptides present in urine samples and in serum of patients with prolidase deficiency [7–10]. This approach has proved to be very useful because it has the advantage of automated processing of large numbers of samples but a laborious sample pretreatment is still necessary.

This paper demonstrates the applicability of high-

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performance capillary electrophoresis (HPCE) as an alternative technique to estimation of urinary dipeptides. HPCE is a powerful method for the analysis of biological samples [11–13]; as with HPLC it shares the advantage of automation and in addition with this technique only very small amounts of sample are needed for each analysis.

We used capillary zone electrophoresis (CZE) for simultaneous detection of all imidodipeptides excreted into the urine of prolidase-deficient patients. The method we developed is fast, simple, reliable and sample preparation is minimal. The purpose of this study was thus to provide an efficient and rapid aid for diagnostic screening of prolidase deficiency.

This was of particular interest to us because after the first report of PD in 1968 [14] only 40 additional cases have been described to date. Although a rare condition, we were able to confirm the diagnosis in 8 Italian patients (25% of total cases), one of whom was asymptomatic at the time of diagnosis. In the light of this, we think that PD may be more common than suspected and could, at times, be misdiagnosed.

2. Experimental

2.1. Chemicals, origin of urine samples and their treatment

Standard imidodipeptides and creatinine were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical-reagent grade. Urine samples, collected over a 24-h period, were obtained from healthy volunteers (controls, aged between 20 and 50 years) and from patients (all males) affected by prolidase deficiency (two siblings aged 14 and 18 years and two unrelated individuals aged 31 and 55 years) [5]. Prior to analysis all samples were kept at −20°C.

A 1-ml aliquot of each urine sample was treated with Norite A for 5 min at room temperature, filtered on a 0.20-μm nitrocellulose filter and lyophilized. The residue was taken up in 100 μl of 0.1 *M* phosphate buffer, pH 2.5 and centrifuged at 5000 *g* for 5 min. In most cases there was no precipitate visible after this treatment.

2.2. Electrophoretic instrumentation and running conditions

All analytical runs were performed using a Biofocus 3000 system (Bio-Rad, Richmond, CA, USA) equipped with a fast-scanning UV-Vis detector. Samples were injected by pressure (5 s, 0.069 MPa/s) from the cathodic end of a fused-silica capillary (24 cm×25 μm I.D.) coated internally with a covalently-attached hydrophilic linear polymer [15]. The capillary was enclosed in a cartridge format and thermostatted at 20°C by liquid cooling. Migration was carried out at 7 kV for 20 min and on-column detection was performed by measuring UV absorbance at 200 nm.

Micropreparative separations were performed using the HP 3D CE system (Hewlett-Packard, Waldbronn, Germany) with a built-in diode array detector. The system included an HP ChemStation for system control, data collection and data analysis. The capillary used and the experimental conditions were essentially as described above for the analytical runs. Fractions were fully automatically collected by applying a pressure of 0.005 MPa to the capillary for a few seconds. For collection, the outlet vial was exchanged to an HPCE-microvial (Hewlett-Packard) containing 10 μl of 2% trifluoroacetic acid.

2.3. Identification of imidodipeptides

Imidodipeptides excreted in urine were identified both by sequence and mass spectrometry analysis. Sequence analyses were performed on the HP G 1005 A protein sequencing system (Hewlett-Packard, Palo Alto, CA, USA) using the routine 3.0 chemistry according to the manufacturer's protocol.

Electrospray ionization mass spectrometry (ESI-MS) experiments on the whole urine samples were performed with a HP-5859 A system (Hewlett-Packard) and matrix-assisted laser desorption time-of-flight (MALDI-TOF) analyses of creatinine were carried out using the HP G 2025 A MALDI-TOF-MS instrument with α-cyano cinnamon acid as matrix. Samples were diluted 2:1 with matrix. Laser energy directed onto the samples was between 0.5 and 1.2 μJ.

3. Results and discussion

The simplicity of sample preparation, coupled with high sensitivity and selectivity allows us to consider capillary electrophoresis as a primary analytical technique for the analysis of imidodipeptides in body fluids and, in particular, as an aid in screening of prolidase deficiency.

Fig. 1A illustrates a standard profile obtained using a urine sample from a healthy volunteer separated by CE, under the experimental conditions reported in this paper. The electropherogram consists of a major peak (peak I) with a migration time of 4.19 min and a few additional minor components. This pattern was found to be highly reproducible as shown by profiles B and C in Fig. 1, obtained from

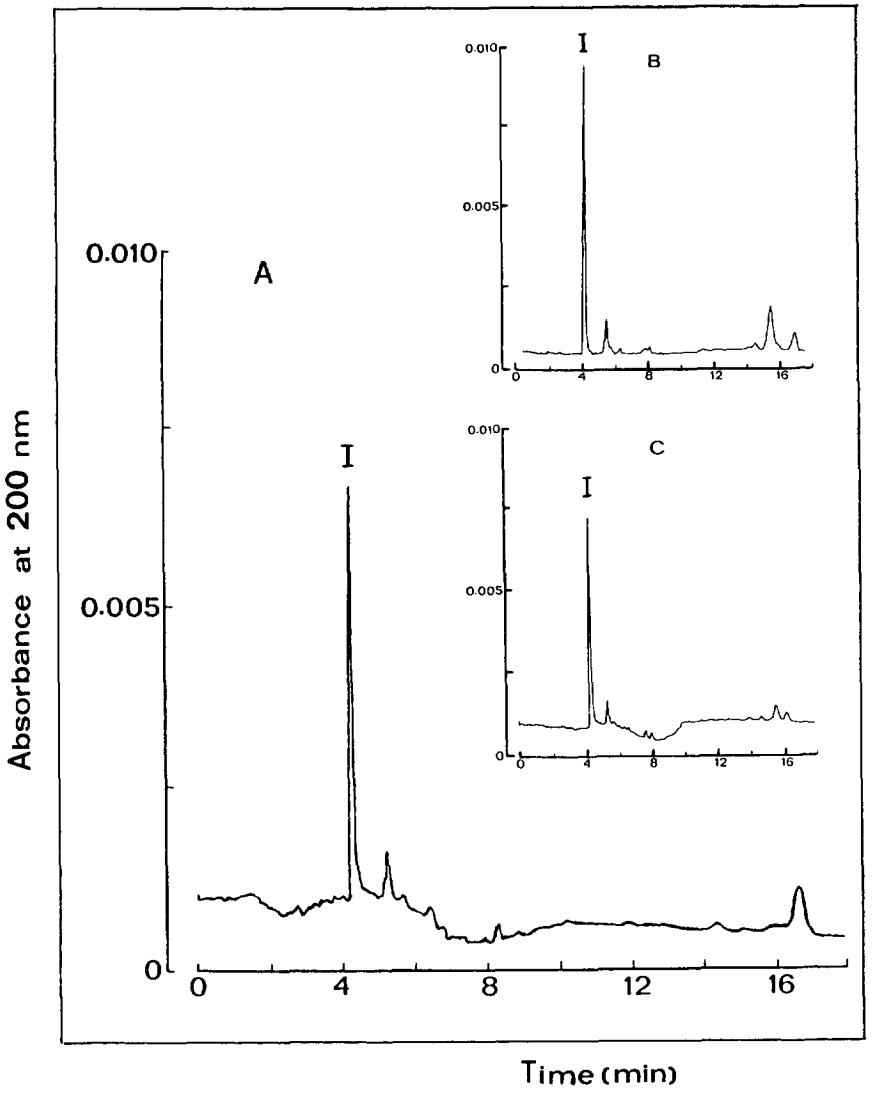

Fig. 1. (A, B, C) Direct analysis of urine samples from healthy volunteers by CZE. Capillary: coated fused-silica (24 cm×25 μm I.D.); carrier, 100 m*M* phosphate buffer (pH 2.5); applied voltage, 7 kV; detection UV absorbance at 200 nm. The samples were injected by pressure (5 s, 0.069 MPa/s) from the cathodic end of the tube. Temperature: 20°C.

runs performed on two other control samples. Additional runs performed on both fresh and stored (−20°C) urine samples from laboratory personnel (10–15 people) yielded essentially the same results (data not shown). In some cases urine samples had been frozen and thawed several times before analysis and results were reproducible.

Peak I was easily identified as endogenous creatinine after determining the migration time (4.19±0.05 min) of standard creatinine (Fig. 2A), by co-injection of standard creatinine with urine samples (Fig. 2B) and by MALDI-TOF mass spectrometry of the automatically collected CE fraction corresponding to peak I. An ion of m/z 114.3 (corresponding to the $[M+H]^+$ ion for creatinine, molecular mass 113.12 Da) was observed on the mass chromatogram in addition to the ions from the matrix (Fig. 2C). Minor components present in the electropherograms were not further investigated.

Aliquots of urine from prolidase deficient patients were analysed using the same CE conditions established for the reference samples. Fig. 3 (panels A–D) reports the electropherograms obtained from four different patients. Panels A and B show electropherograms from two brothers (C.M. and C.F.) while profiles C and D were obtained from two other unrelated individuals (G.D. and C.Ma. both males). As expected, these samples gave rise to a more

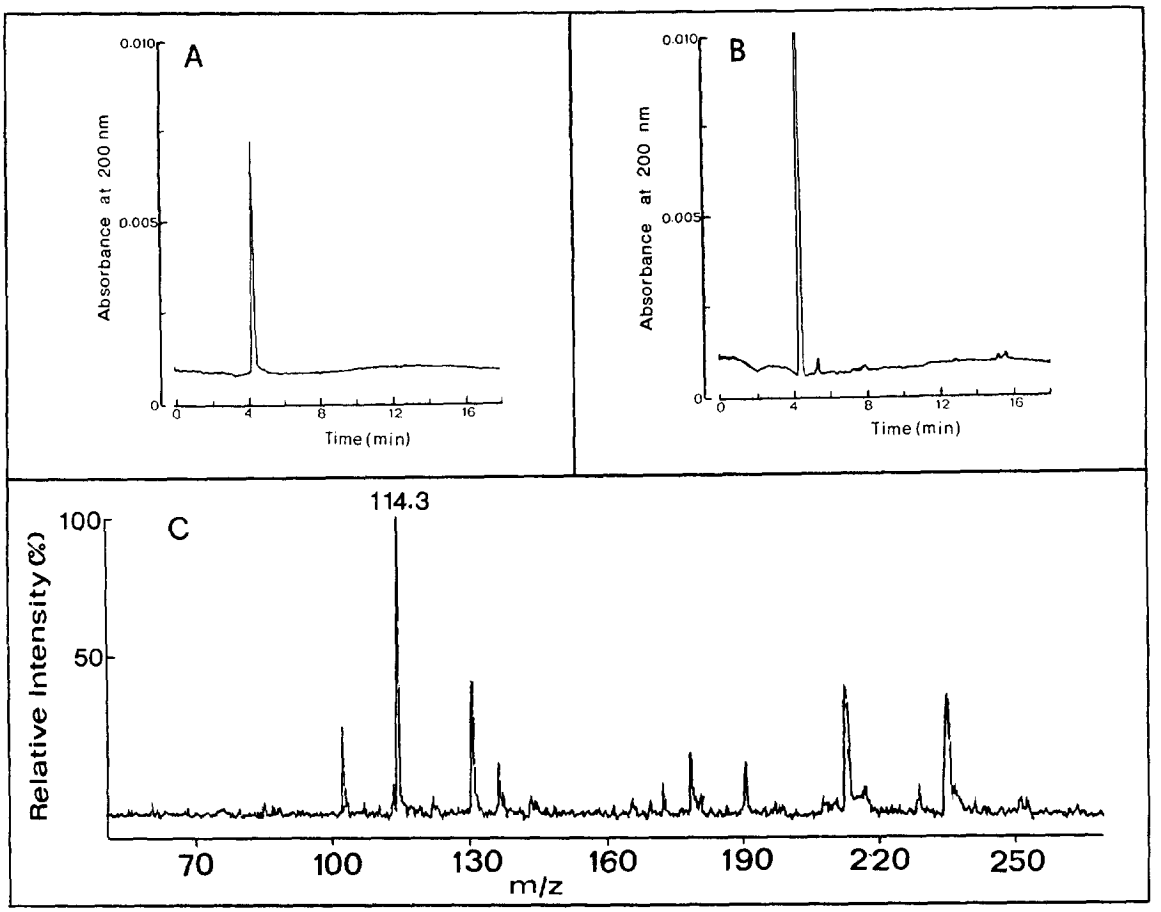

Fig. 2. (A) Capillary electrophoresis of standard creatinine solution (100 m*M* in phosphate buffer pH 2.5). (B) Electropherogram obtained from co-injection of standard creatinine with a control urine sample. (C) MALDI-TOF-MS of peak I automatically collected from a capillary electrophoretic run performed on urine sample of a control. The ion at m/z 114.3 was identified as creatinine.

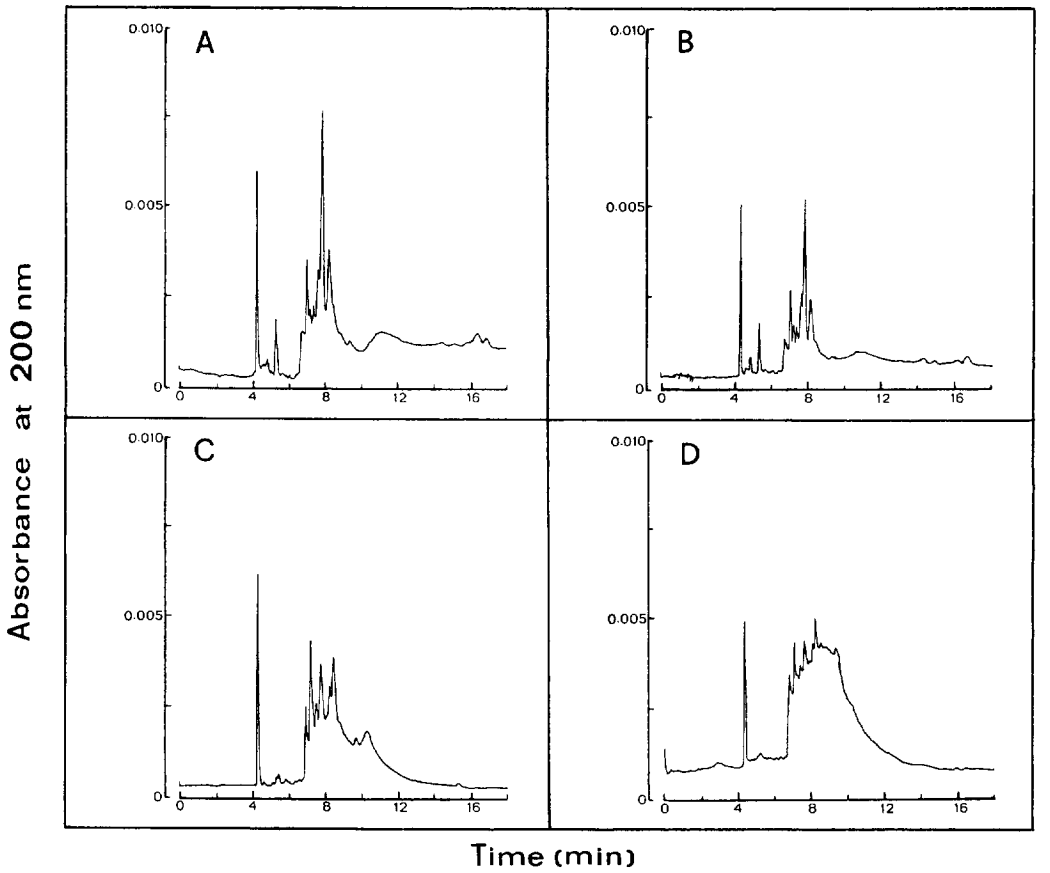

Fig. 3. Direct capillary electrophoresis of urine samples obtained from two male siblings, C.M. and C.F. (panels A and B respectively) and two other unrelated individuals, G.D. and C.Ma. (panels C and D respectively) affected by prolidase deficiency. The capillary and conditions used are the same as those described in the legend to Fig. 1 .

complicated pattern than the reference. In all cases at least eight peaks (in addition to peak I, also present in normal urine) were discernible.

The qualitative identification of these peaks as a complex mixture of the imidodipeptides X-Pro and X-Hyp was initially achieved by comparing the profiles from the patients with the electropherograms of standard imidodipeptides injected into the CE system individually (data not shown) and as a mixture. Fig. 4 shows the electropherograms obtained when a mixture of the following standard dipeptides was run: Gly-Pro, Ala-Pro, Val-Pro, Leu-Pro, Ile-Pro, Phe-Pro, Trp-Pro and Pro-Pro.

The electrophoretic profile obtained after co-injecting this mixture with control urine is shown in the inset of Fig. 4. As in the case of urine samples from patients, the separation was not complete but this was not surprising as the charge to mass ratio is very similar for all the dipeptides tested. Under the experimental conditions used all peaks migrated in the range between 6.15 and 12 min. Owing to the complexity of the mixture and because this was not the main aim of our work, no attempt was made to improve the separation profile.

We were very interested to note the heterogeneity of the electrophoretic patterns obtained for urinary dipeptides from our patients because, to our knowledge, this is the first work in which the results clearly show, at a qualitative level, that urinary excretion of dipeptides is heterogeneous for different patients. The specificity of CE in detecting this heterogeneity is shown in Fig. 3 where it can be seen

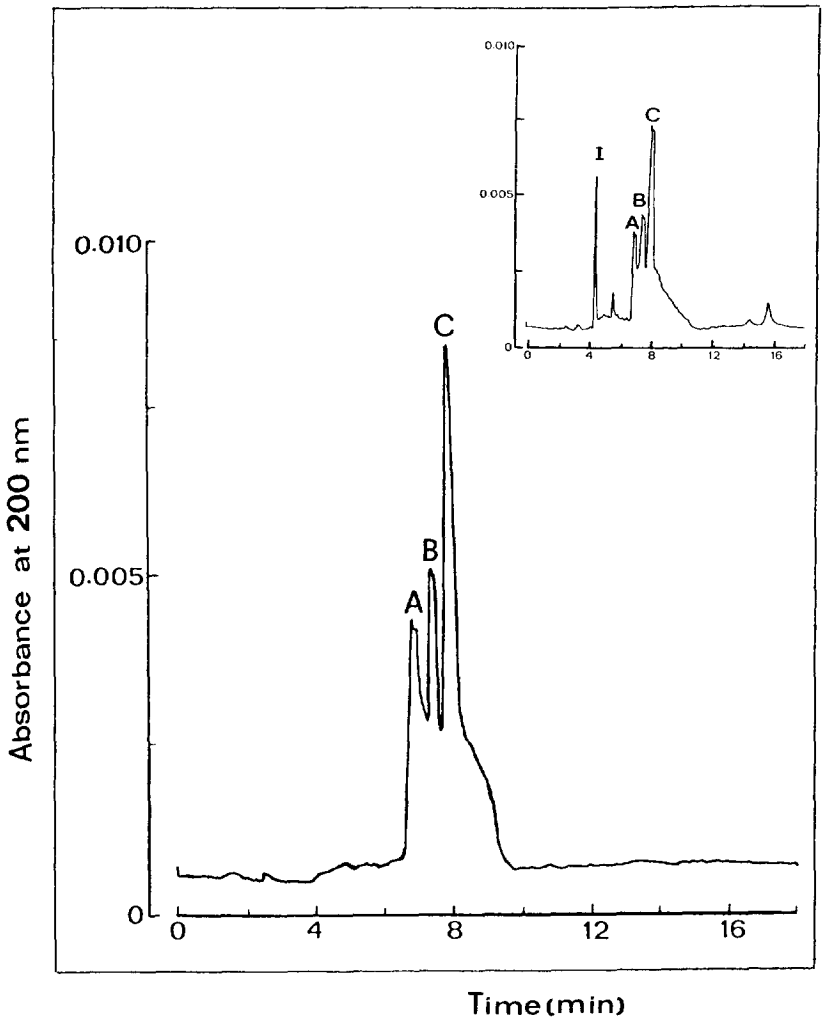

Fig. 4. Capillary electrophoresis of a mixture of eight standard imidodipeptides (the concentration of each dipeptide was 1 m*M* in 100 m*M* phosphate buffer, pH 2.5). The material present under each peak, determined by individually injecting the imidodipeptides, was as follows: Peak A: Gly-Pro, Ala-Pro; Peak B: Val-Pro, Ile-Pro, Pro–Pro; Peak C: Leu-Pro, Phe-Pro, Trp-Pro. Inset: electrophoretic pattern obtained from co-injection of standard dipeptides with a control urine sample. The capillary and the experimental conditions are the same as those described in the legend to Fig. 1 .

that the profiles in A and B are very similar to each other but they differ from electropherograms depicted in panels C and D where some components seem missing. It should be pointed out that patterns in Fig. 3A and Fig. 3B belong to two brothers each affected by a clinically severe form of PD. The severity of the disease is greater for the patient whose pattern is shown in Fig. 3C while the clinical symptoms are milder for the patient depicted in Fig. 3D. A previous attempt to correlate severity of symptoms of these patients with their prolidase activity failed as only small differences in residual enzymatic activity, assayed both in cultured fibroblasts and in hemolysates prepared from patients' red cells, were found [5]. Thus, in an attempt to speculate whether a correlation between the electrophoretic pattern of the patients' urinary imidodipeptides and severity of symptoms exists, we identified all the dipeptides excreted into urine of our four patients. Aliquots (3 μl out of 500 μl) of urine samples from

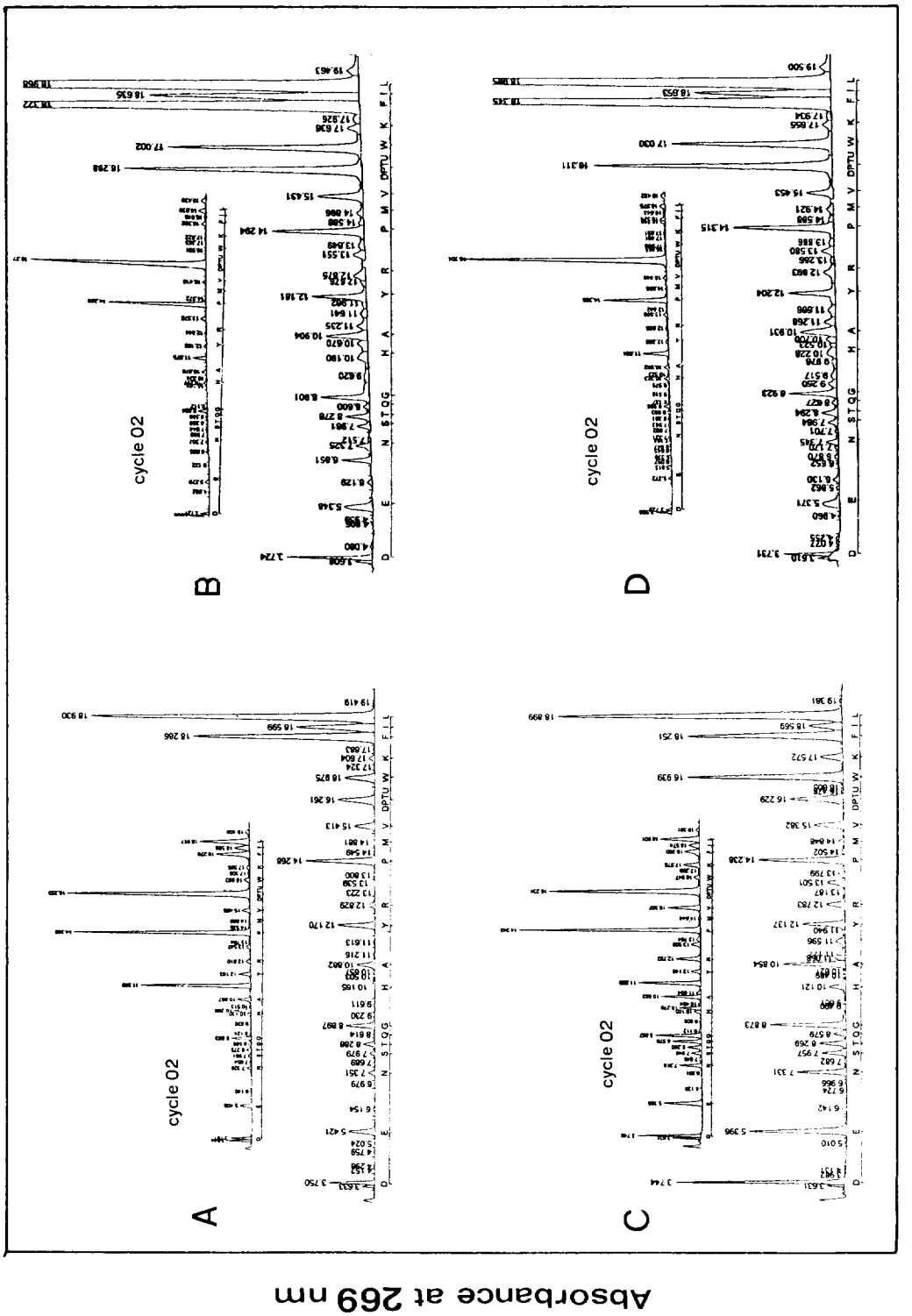

Fig. 5. PTH-amino acids obtained in the first cycle of sequencing of whole urine samples from patients C.M. (A), C.F. (B), G.D. (C) and C.Ma. (D). Inset of panels A–D shows the pattern obtained in the second cycle of degradation for each patient. PTH-Hyp was identified as the peak eluting at 11.57 min between PTH-Ala and PTH-Tyr. Experimental conditions are reported in the text.

each patient were submitted both to ESI-MS and to NH_2-terminal sequence analysis. In each case an aliquot of normal urine was used as reference sample.

For automated Edman degradation, urine samples were directly loaded onto the hydrophobic part of the biphasic sequencer support using the loading station and submitted to three cycles of degradation. The first cycle of sequencing resulted in the presence of a large number of phenylthiohydantoin-amino acids (PTH-amino acids) in all urine samples except the reference (Fig. 5). In the second cycle only the clear signals for PTH-Pro and PTH-Hyp were present (inset of Fig. 5) while no signals were observed in the third cycle. In the case of control urine very little background interference (in the range between 0.5 and 2.0 pmol) was observed (Fig. 6).

ESI-MS analysis performed on whole urine samples confirmed the nature of these dipeptides containing C-terminal prolyl and hydroxyprolyl residues. The mass spectrum of each urine sample exhibited a number of ions in the range from m/z 173 to 340 (Fig. 7); their identification was based on the mass spectra of standard imidodipeptides. The total num-

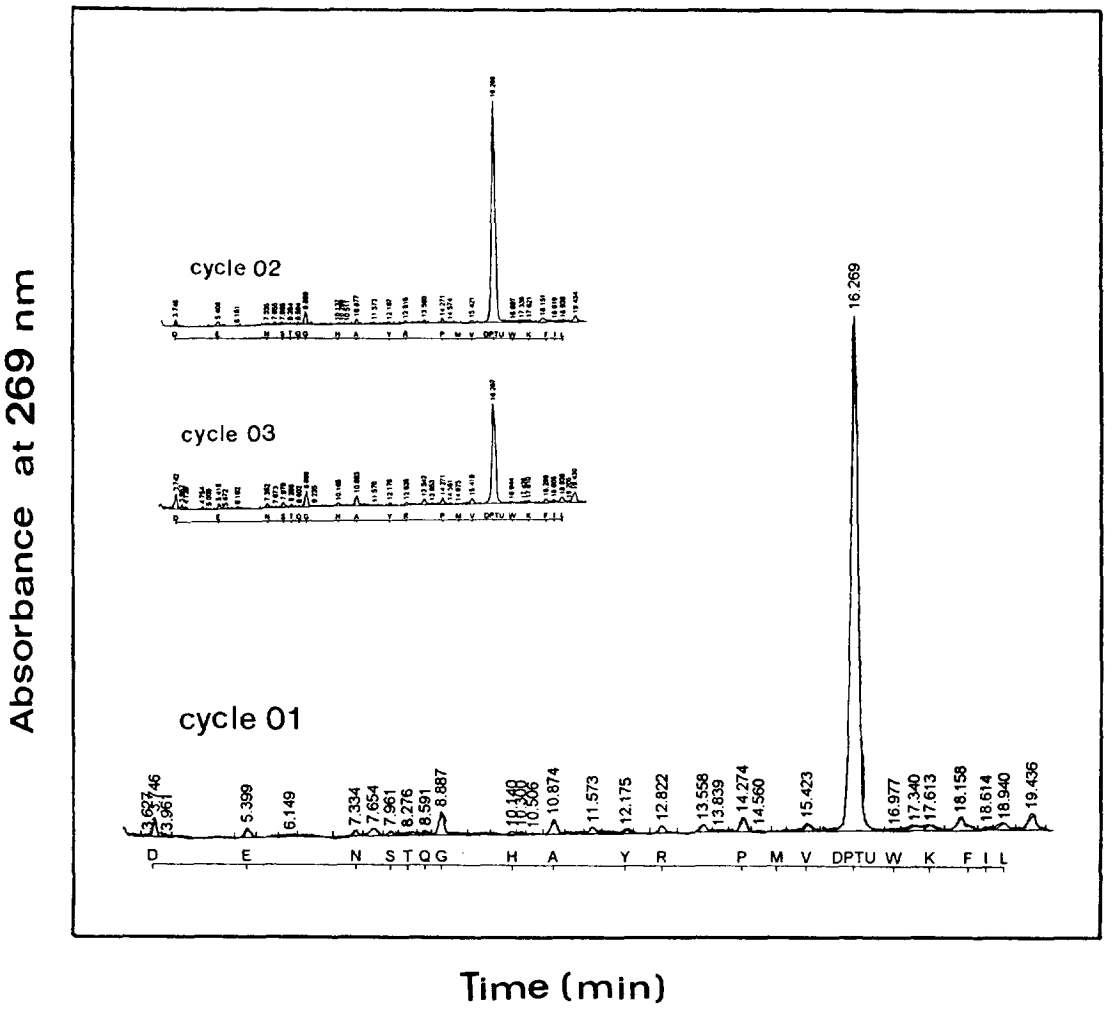

Fig. 6. Pattern obtained in the first cycle of sequencing of urine sample from control. Inset: second and third cycles of degradation. Experimental conditions are reported in the text.

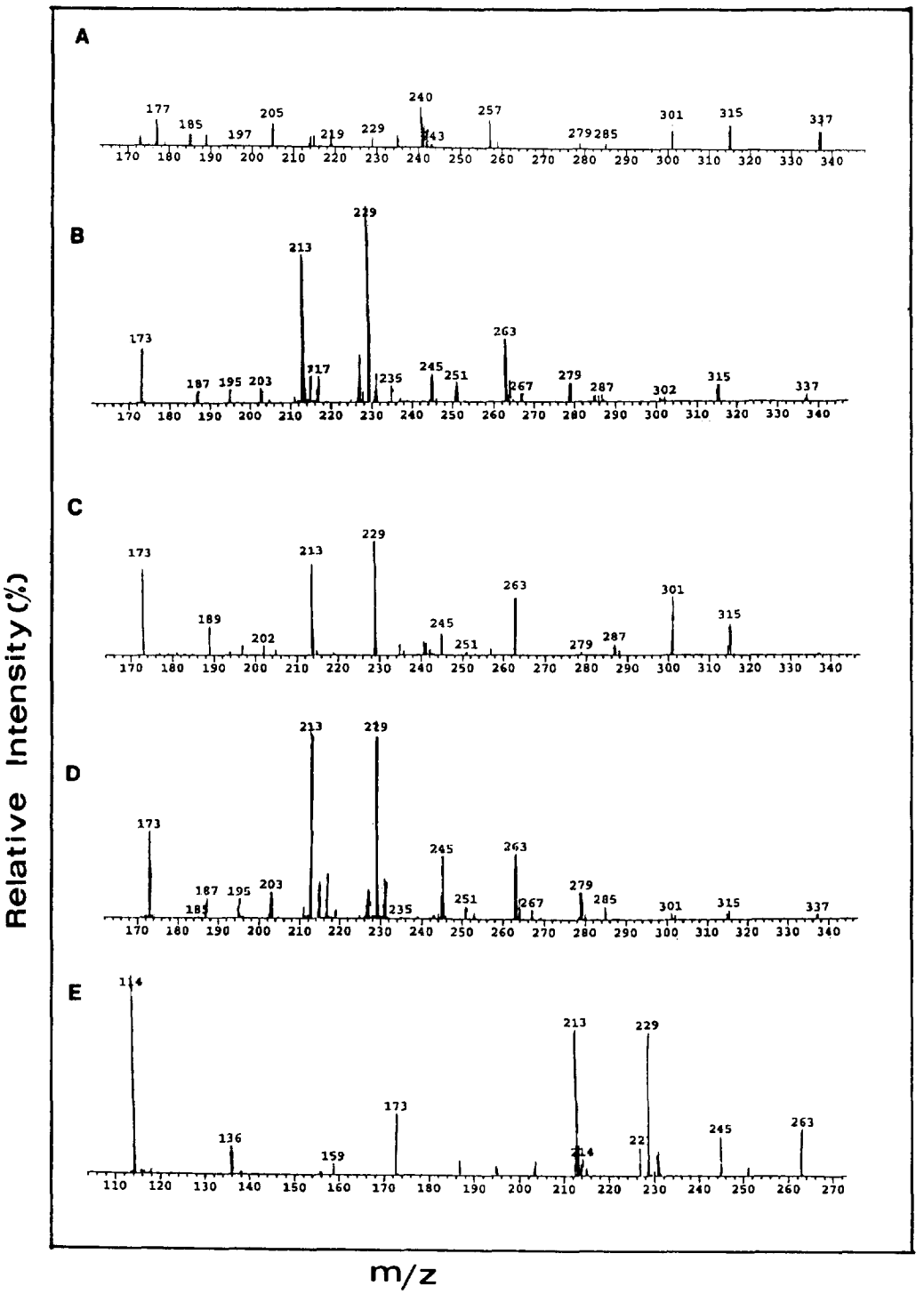

Fig. 7. ESI-MS analyses of whole urine samples from a control (A) and from patients C.M. (B), C.F. (C), G.D. (D), C.Ma. (E). For patients C.M., C.F. and G.D. the mass spectrometer was scanned from m/z 170 to 340 while in the case of patient C.Ma. the mass spectrometer was scanned from m/z 110 to 270.

Table 1
Identification of imidodipeptides X-Pro and X-Hyp in urine of patients with prolidase deficiency

Imidodipeptides X-Pro		Imidodipeptides X-Hyp	
Sequence	Ion mass	Sequence	Ion mass
Asp-Pro	231	Val-Hyp	231
Glu-Pro	245	Ile/Leu-Hyp	245
Ser-Pro	203	Arg-Hyp[b]	288
Thr-Pro	217	Phe-Hyp	279
Gly-Pro	173		
His-Pro[a]	253		
Ala-Pro	187		
Tyr-Pro	279		
Pro-Pro	213		
Val-Pro	215		
Trp-Pro	302		
Phe-Pro	263		
Ile/Leu-Pro	229		
Asn-Pro[b]	230		

[a] Not found in patients C.M., C.F. and C.Ma.
[b] Not found in patient C.M.

ber of imidodipeptides found is reported in Table 1; the results obtained are in good agreement with previous findings [3,7,16].

The C-terminal prolyl and hydroxyprolyl imidodipeptides found are almost the same for all four patients but at a qualitative level, some differences between them were detected. Interestingly dipeptides His-Pro and Arg-Hyp were not excreted into urine by patients C.M., C.F. and C.Ma. Moreover peptides Val-Hyp and Phe-Hyp were particularly abundant in C.M.'s urine with respect to the other three patients while the amount of Trp-Pro was very low. On the contrary, a large amount of dipeptides Asp-Pro, Glu-Pro and Asn-Pro was found in urine of patient G.D. As deduced from the sequence data, it was also noteworthy that, although in all patients tested the most abundant imidodipeptides found were Leu-Pro/Hyp, Phe-Pro/Hyp and Gly-Pro, discrepancies were found between the patients. The sum of the amounts of these dipeptides (calculated as the ratio between picomoles of the above mentioned dipeptides and total dipeptide content in the urine sample) accounted for 82%, 75% and 80% of the whole dipeptide content for patients C.F, C.M. and C.Ma., respectively. However in patient G.D., the sum of the amount of Leu-Pro/Hyp, Phe-Pro/Hyp and Gly-Pro accounted for only 39% of the total amount. Thus, although heterogeneity between these patients is evident, the correlation between the severity of PD and the pattern of urinary dipeptide excretion by different patients remains to be proved since the exact pathogenetic mechanisms leading to clinical changes in PD are still not well understood. In the case of dipeptide excretion, the most important variable refers to physical and dietary conditions of the patients studied which, as known, make the concentration of urinary substances vary greatly.

To obtain a reliable quantitative composition of excreted dipeptides further experiments and a larger number of patients are needed. However, recent advances in the development of fraction collection in capillary electrophoresis and subsequent off-line analysis of fractions will possibly offer a better insight into the electropherograms of each patient. Thus this analytical methodology would appear appropriate, with respect to other methods utilized so far, to provide additional information on PD.

4. Conclusions

The capillary electrophoretic method reported here is a useful aid for screening and diagnosis of prolidase deficiency as it allows easy detection of urinary dipeptides containing C-terminal prolyl or hydroxyprolyl residues. Imidodipeptiduria was investigated in four patients using this technique; it proved to be sensitive and fast with analysis times of less than 20 min being required.

Either fresh or stored urine samples may be used and the technique can be performed in most biochemical laboratories as commercial HPCE instruments are now available. This simple method combined with MS and peptide sequencing enabled semiquantitative evaluation and exact identification of dipeptides which may be of interest in further studies in PD patients in view of the heterogeneity of dipeptide excretion that appears to exist between patients with differing clinical severity.

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