ELSEVIER

Journal of Chromatography A, 768 (1997) 57–66

JOURNAL OF CHROMATOGRAPHY A

Complete resolution of imidodipeptide mixtures in urine of prolidase-deficient patients using micellar electrokinetic chromatography

Rudi Grimm[a], Giuseppe Zanaboni[b], Simona Viglio[b], Katharine Dyne[b], Maurizia Valli[b], Giuseppe Cetta[b], Paolo Iadarola[b,*]

[a]*Analytical Division, Hewlett-Packard GmbH, D-76337 Waldbronn, Germany*

[b]*Dipartimento di Biochimica, Università di Pavia, Via Taramelli 3/B, I-27100 Pavia, Italy*

Abstract

The use of capillary zone electrophoresis as an efficient method for the identification of urinary imidodipeptides of prolidase-deficient patients has already been reported. However, owing to the complexity of the components excreted, the resolution of electrophoretic patterns obtained was poor. Here we examine the use of micellar electrokinetic chromatography to enhance peak resolution in order to obtain better insight into the electropherograms of patients' urine. The usefulness of sodium dodecyl sulphate as surfactant is reported: refined electropherograms were achieved using 35 m*M* sodium borate, pH 8.3 containing 65 m*M* sodium dodecyl sulphate. Almost all peaks were baseline separated, collected and sequenced. This allowed us to define the exact imidodipeptide composition of patients' urine. The possibility of identifying and thus quantifying each single peak means that comparison of urinary imidodipeptide excretion patterns from different patients can be made and the hypothesis that peptide patterns can be correlated with differing clinical severity can be investigated.

Keywords: Imidodipeptides; Peptides

1. Introduction

Among various modes of high performance capillary electrophoresis (HPCE), capillary zone electrophoresis (CZE) has become one of the best separation techniques for analysis of charged compounds because of its high resolving capacity [1–5]. Recently, CZE has also been successfully employed for separating complex mixtures of components present in biological samples such as urine or serum, and its usefulness for diagnosis and studies of metabolic disorders has been demonstrated [6–12]. Micellar electrokinetic capillary chromatography (MEKC), which is an adaptation of CZE, is even more promising as it can be used for selective separation of both neutral and ionic compounds [13–18]. This technique involves the addition of surfactants to the operating buffer in an higher amount than the critical micelle concentration. The solute is then partitioned between the micelle and the surrounding aqueous medium and separation is based on the differential migration of the phases. Because the micelles possess ionic and hydrophobic sites of interaction simultaneously, MEKC provides a sophisticated means for achieving a very high selectivity of solutes with closely related structures.

Previously, we developed a reliable and sensitive CZE method for simultaneous detection of imidodipeptides X–Pro and X–Hyp present in urine of prolidase-deficient patients [19]. At that time we

*Corresponding author.

PII S0021-9673(96)00983-1

suggested the applicability of this technique as an efficient and rapid aid for diagnostic screening of prolidase deficiency (PD). However, owing to the high number and complexity of components excreted, the quality of electrophoretic patterns obtained was poor. As a consequence the heterogeneous composition of urinary imidodipeptides postulated for affected patients with differing clinical levels of severity was evidenced, at least at a qualitative level, only after whole urine samples were submitted to sequence and mass spectrometric analyses [19].

The aim of this investigation was to test the use of MEKC to enhance peak resolution in order to obtain a better understanding of the electrophoretic pattern, and hence of the composition of total urinary compounds excreted by each patient.

The results show that MEKC is a versatile means of separation which can be successfully applied to complex mixtures of solutes having almost the same charge. Although the potential of other surfactants has been explored, the utilization of aqueous sodium dodecyl sulphate (SDS) as the micellar phase offers the best separation efficiency.

2. Experimental

2.1. Reagents

Reagent-grade water obtained from a Milli-Q purification system from Millipore (Bedford, MA, USA) was used to prepare all solutions. All other reagents were of analytical grade and were used without further purification.

2.2. Origin of urine samples

Urine samples, collected over a 24-h period, were obtained from healthy volunteers (controls aged between 20 and 50 years) and from patients affected by prolidase deficiency (two male siblings aged 14 and 18 years and three other unrelated individuals aged 31, 40 and 55 years). All samples were prepared as previously described [19].

2.3. Electrophoretic instrumentation and running conditions

All analytical and micropreparative runs were performed using the HP 3D CE system (Hewlett–Packard, Waldbronn, Germany) with a built-in diode-array detector. The instrument included an HP CHEMSTATION for system control, data collection and data analysis. Fused-silica capillaries of 75–102 cm (68–95 cm effective length)×50–100 μm I.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA). A capillary coated on its internal surface with a covalently-bonded hydrophilic linear polymer [20] was obtained from Bio-Rad (Richmond, CA, USA) and was used without further modifications. Capillary dimensions were 28 cm effective length× 25 μm I.D. Stock solutions of 50 m*M* sodium phosphate at pH 2.5 and 3.85, 20 m*M* sodium formate, pH 4.45 and 35 m*M* sodium borate, pH 8.3 were used for the CZE runs. Tween 20 (10, 20 and 50 m*M*) or SDS (65 m*M*) were added to the buffers used for MEKC. Separations were carried out at 20 kV and samples were injected hydrodynamically with the inlet as the anode and outlet as the cathode. Analytes were monitored at 200 nm.

To ensure run-to-run reproducibility of separations, the capillary column was purged with fresh buffer for 3 min before each injection. 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 HP microvial (Hewlett–Packard) containing 10 μl of 2% trifluoroacetic acid.

2.4. Identification of imidodipeptides

Imidodipeptides excreted in urine were identified by sequence analysis of the collected peaks. Sequence was 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.

3. Results and discussion

When aliquots of urine from prolidase-deficient patients were analyzed by CZE, separation profiles

as shown in Fig. 1 (panels B→F) were observed. As documented in a previous paper each electropherogram consists of a peak corresponding to endogenous creatinine plus several additional peaks indicated as imidodipeptides X–Pro and/or X–Hyp [19]. The electropherograms reported in Fig. 1 performed using 50 m*M* sodium phosphate pH 2.5 as run buffer, although heavily overlapping, were the best

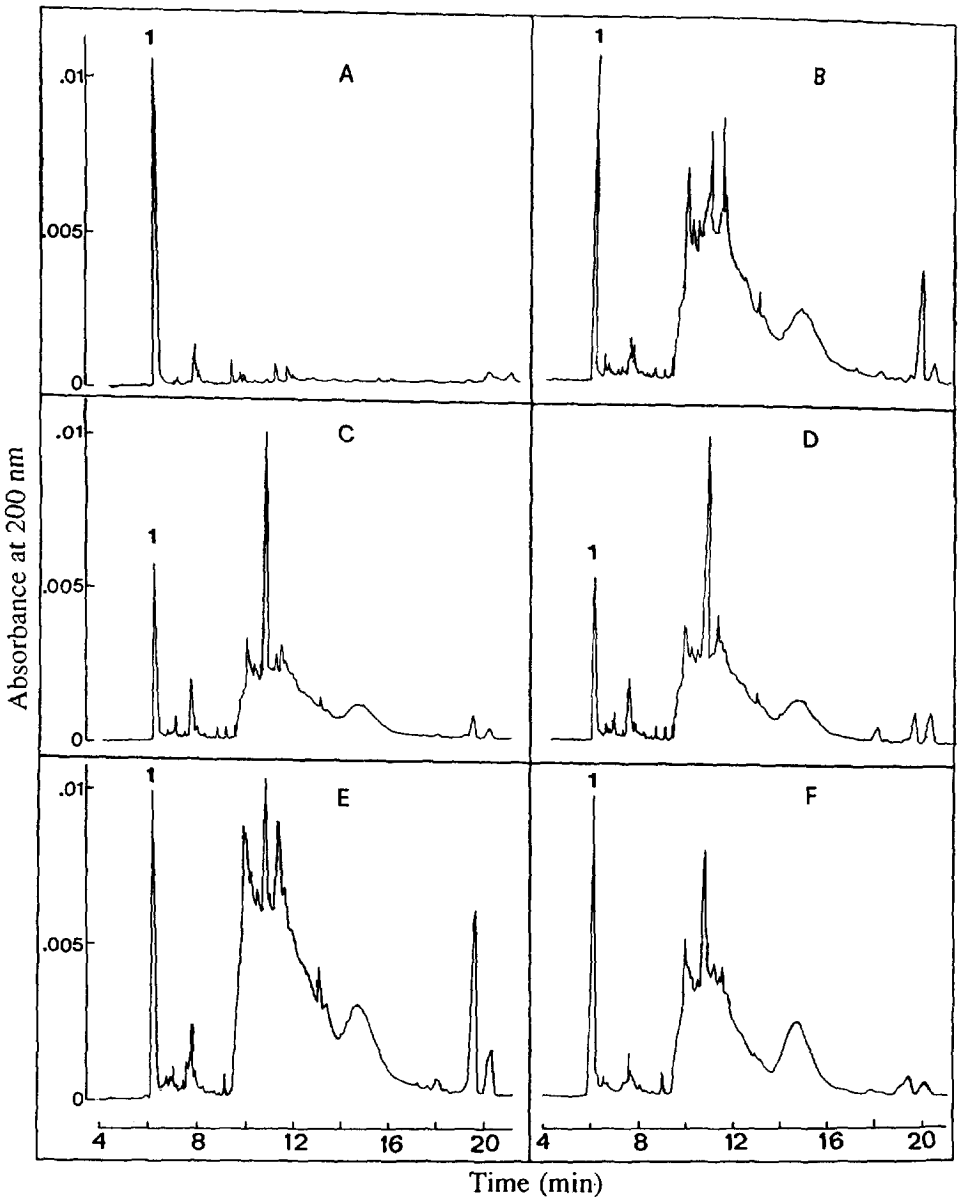

Fig. 1. Capillary zone electrophoresis of urine samples from a healthy volunteer (24 year old male, panel A) and five PD patients: G.D. (31 year old male, panel B); C.F. (18 year old male, panel C); C.M. (14 year old male, panel D); C.Ma. (55 year old male, panel E) and B.C. (40 year old female, panel F). Peak 1 of each panel: endogenous creatinine. Peaks with retention times between 9 and 20 min are the imidodipeptides X–Pro and X–Hyp identified as previously reported [19]. Experimental conditions: fused-silica capillary 70 cm effective length×50 μm I.D., run buffer: 50 m*M* sodium phosphate, pH 2.5; applied voltage: 20 kV; detection: UV absorbance at 200 nm. Positive to negative polarity.

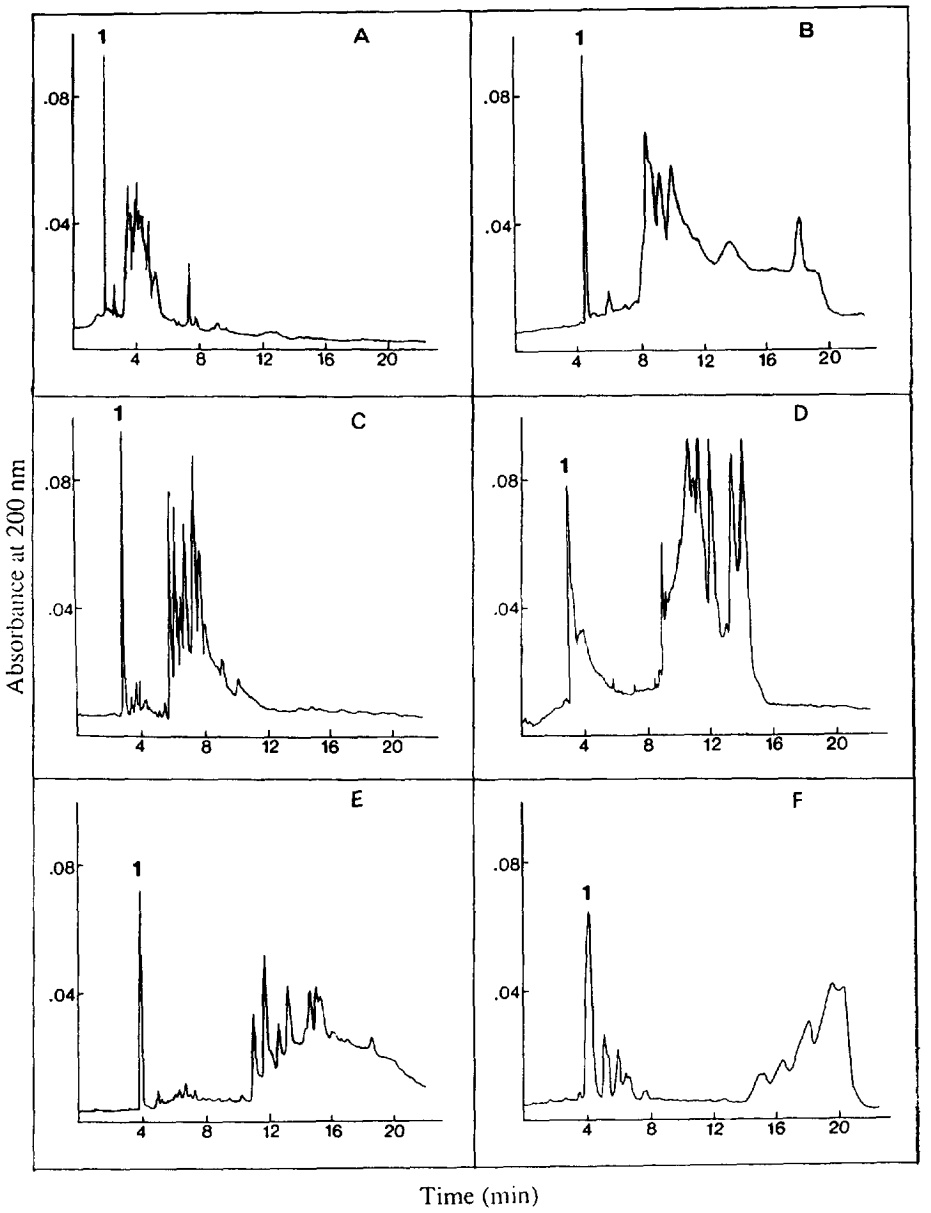

Fig. 2. Capillary zone electrophoresis of urine sample from patient G.D. Panels A and B: fused-silica capillaries 50 cm effective length×75 μm I.D. and 95 cm×100 μm I.D. respectively. Run buffer used was 50 m*M* sodium phosphate, pH 2.5; applied voltage: 20 kV; detection: UV absorbance at 200 nm. Panels C→F: coated capillary 28 cm effective length×25 μm I.D. Run buffer: 50 m*M* sodium phosphate, pH 2.5 (panel C), 50 m*M* sodium phosphate, pH 3.85 (panel D), 20 m*M* sodium formate, pH 4.45 (panel E) and 35 m*M* sodium borate, pH 8.3 (panel F). All other experimental conditions are the same as those described in the legend to Fig. 1. Peak 1 of each panel: endogenous creatinine. All X–Pro and X–Hyp imidodipeptides excreted by patients are contained in a cluster of peaks heavily overlapping with retention times variable depending on experimental conditions.

obtained with a 70 cm effective length×50 μm I.D. fused-silica capillary. Attempts to improve resolution using working buffers of increasing pH failed because, as also suggested by other authors [20], differences in net charge of imidodipeptides diminished (data not shown). Thus, in order to improve resolution of the components, we investigated use of different capillaries on a urine sample from patient G.D. (panel B of Fig. 1). The electropherograms obtained when two fused-silica capillaries of different size and length were used (50 cm effective length×75 μm I.D. and 95 cm effective length×100 μm I.D., respectively), are shown in panels A and B in Fig. 2. The overall pattern of peaks was very similar to that already reported in Fig. 1 and in terms of resolution it appeared there was no improvement. On the other hand, a sensitive increase of the separation factor was not achieved either when we used, at different pH values (2.5, 3.85, 4.5 and 8.3 respectively), a capillary (28 cm effective length×25 μm I.D.) coated on its internal surface with a hydrophilic linear polymer. As shown on panels C→F of Fig. 2 this capillary appears to offer little advantage, apart from a better resolution (at least at acidic pH) of the central portion of the electrophoretic pattern which contains as many as ten peaks. All attempts led to incomplete separation. Similar results were obtained using urine from the other patients considered (data not shown). The lack of baseline resolution for the urinary dipeptides contained in this complex mixture is certainly dependent on their similar electrophoretic mobilities (μ) due to minute differences in charge and molecular mass. The data reported in Table 1 which refer to imidodipeptides X–Pro excreted in urine from PD patients, as deduced from the sequence performed directly on whole urine samples [19], confirm the assessed similarity. In fact, the μ values (calculated according to the model for theoretical prediction of peptide mobility in CZE proposed by Rickard et al. [21] and reviewed by Castagnola et al. [22]), ranged between 1.93 and $3.47 \cdot 10^{-8}$ $m^2 V^{-1} s^{-1}$ but the majority showed an average value of 2.2244×10^{-8} $m^2 V^{-1} s^{-1}$.

Thus, these peptides which contain Pro or Hyp as the C-terminal and have subtle variations in their charge-to-mass ratio, could be used as test substances to verify the well known ability of surfactants to separate closely related species.

The use of micellar buffer modifiers to supplement the differences in electrophoretic mobilities of peptides has been described by several authors [20,23–30]: different ionic and non-ionic surfactants have been shown to provide additional separation mechanisms to conventional CZE. In fact, owing to possible interactions between the hydrophobic moiety of the peptides and the inner core of the micelles, the separation factor increases and selectivity improves appreciably. This is especially true with short-chain peptides which moderately interact with the micelle [29].

Attempts to perform MEKC on the mixture of the components excreted from PD patients were initially made employing Tween 20 as neutral surfactant. The results obtained when urine samples from patients B.C. and G.D. were run using 50 m*M* sodium phosphate (pH 2.5) containing 10 m*M* Tween 20 as working buffer are shown in Fig. 3 (panels A and B respectively). The use of this surfactant did not improve the separation sufficiently in relation to expectations; in fact the overall pattern of peaks in Fig. 3 is similar to the corresponding electropherograms in Fig. 1. The addition of Tween 20 to the run buffer and its interaction with imidodipeptides did

Table 1
Molecular masses and electrophoretic mobilities of imidodipeptides X–Pro identified in urine of prolidase-deficient patients

Imidodipeptides X–Pro	Molecular mass	Electrophoretic mobility[a] $\mu \times 10^{-8}$ ($m^2 V^{-1} s^{-1}$)
Trp–Pro	301	1.93
Tyr–Pro	278	2.01
Phe–Pro	262	2.07
Glu–Pro	244	2.15
Asn–Pro	229	2.19
Ile–Pro	228	2.20
Leu–Pro	228	2.20
Thr–Pro	216	2.26
Val–Pro	214	2.27
Pro–Pro	212	2.28
Asp–Pro	230	2.32
Ser–Pro	202	2.33
Ala–Pro	186	2.43
Gly–Pro	172	2.52
His–Pro	252	3.47

[a] Calculated according to the model proposed by Rickard et al. [21] and reviewed by Castagnola et al. [22].

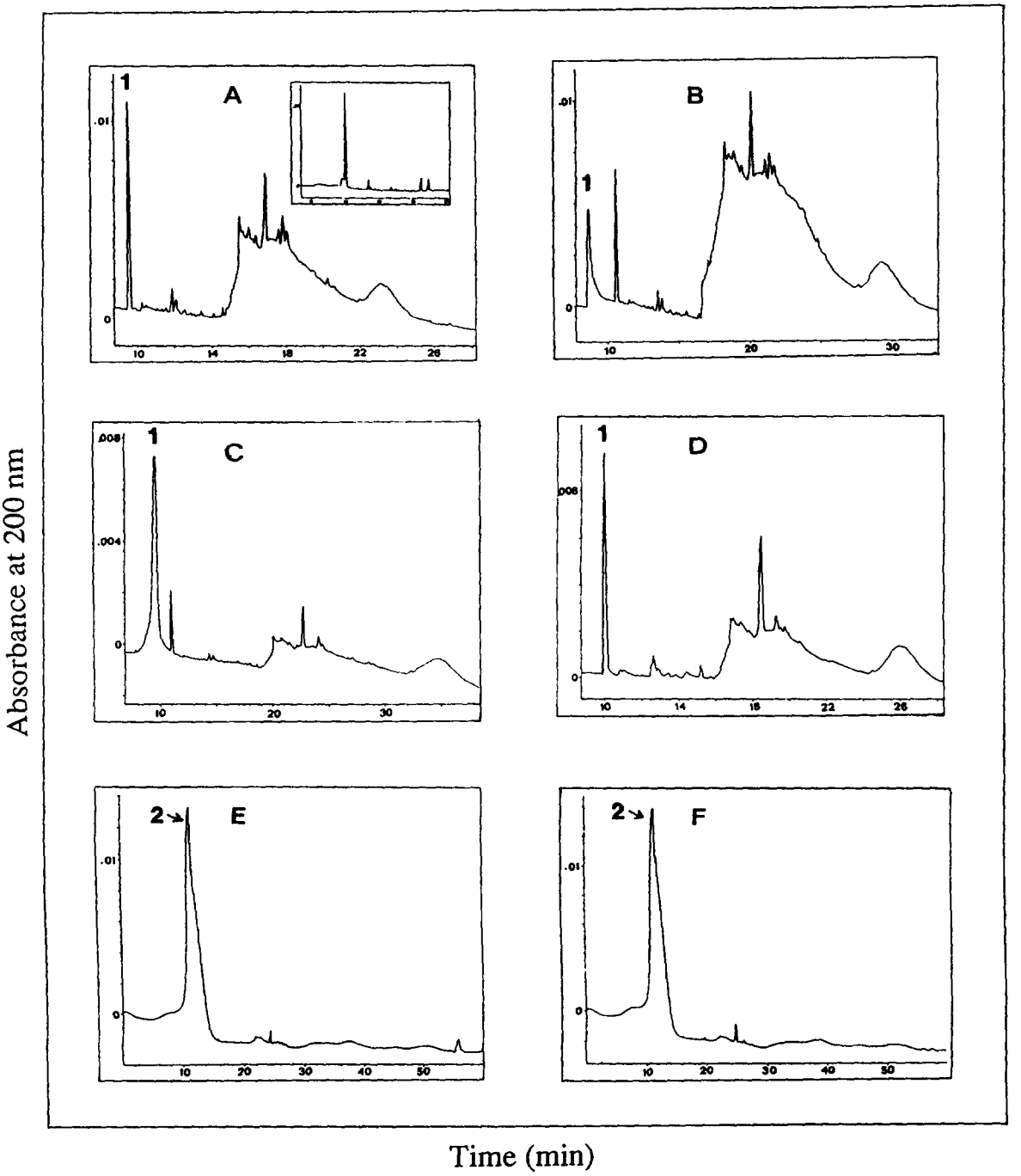

Fig. 3. Micellar electrokinetic chromatography of urine samples from patients B.C. and G.D. in 50 m*M* sodium phosphate pH 2.5 containing Tween 20 at a concentration of 10 m*M* (panels A and B respectively), 50 m*M* (panels C and D) and 100 m*M* (panels E and F). In panels A→D peak 1 is endogenous creatinine. All imidodipeptides are represented by the cluster of peaks with retention time ranging between 14 and 30 min. In panels E and F peak 2 contains both creatinine and all unresolved imidodipeptides. All experimental conditions are the same as those described in the legend to Fig. 1. The inset of panel A shows the electropherogram of urine sample from a control run using the same experimental conditions.

not result in a better separation of the compounds of the complex mixture examined: it appeared that small differences in hydrophobicity of the different imidodipeptide side-chains do not contribute towards enhancing resolution of peaks present in the central portion of the electropherograms. The effect of increasing the concentration of Tween 20 was also investigated. Unfortunately, the separation factor did not increase, as suggested by other authors [29] when the concentration of Tween 20 was raised to 50 m*M* (panels C and D). A further increase to 100 m*M* resulted in an even worse separation of the mixtures' components (panels E and F). We decided to use SDS in an attempt to investigate whether a different surfactant could improve resolution. Experiments using 35 m*M* sodium borate, pH 8.3 containing 65 m*M* SDS as running buffer resulted in good separation and peak shape of the components present in urine from PD patients: as shown in Fig. 4 (panels B–F), apart from a cluster of peaks (with retention times between 5.5 and 6.5 min) which still partially overlapped, at least ten other components appeared as peaks that were completely baseline separated. With respect to CZE the MEKC separation mode showed up a larger number of peaks in each electropherogram. These findings could be promising of

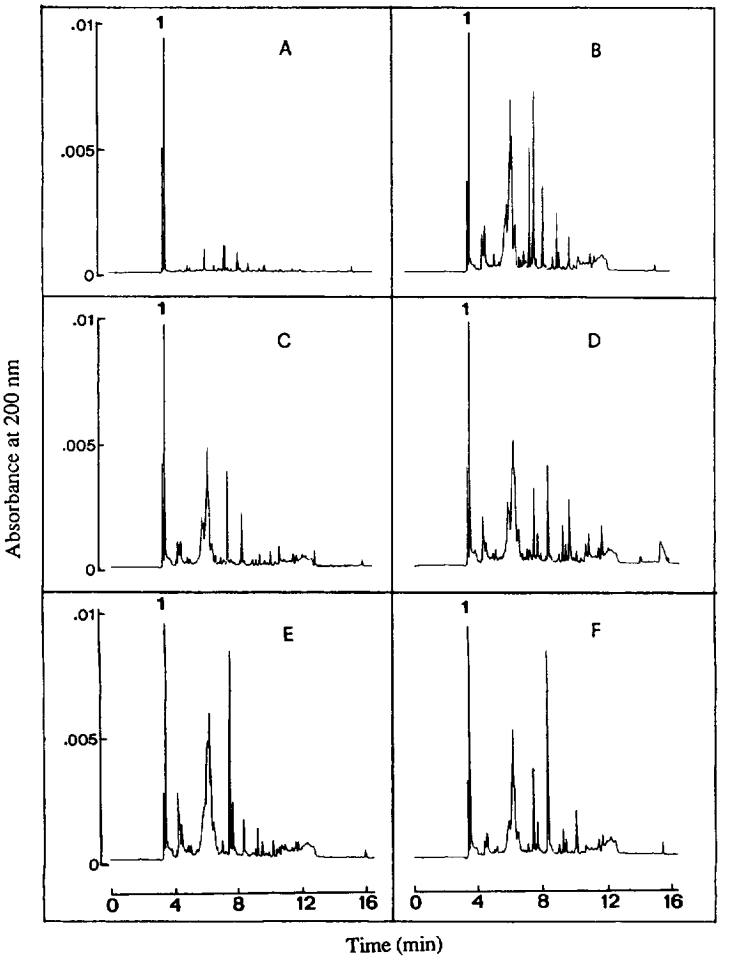

Fig. 4. Micellar electrokinetic chromatography in the presence of SDS of urine samples from a healthy volunteer (panel A) and the five PD patients already indicated in the legend to Fig. 1 (panels B→F). Peak 1 of each panel: endogenous creatinine. For identification of all other peaks see the text. Experimental conditions: fused-silica capillary 70 cm effective length×50 μm I.D. Run buffer: 35 m*M* sodium borate, pH 8.3 containing 65 m*M* SDS. All other conditions are the same as those described in the legend to Fig. 1.

a better insight into the electropherograms of the patients tested.

After performing several consecutive runs to verify the reproducibility of elution times for each peak, a systematic investigation was performed using G.D.'s urine sample. We carried out a micropreparative run (Fig. 5) on a 68 cm effective length×100 μm I.D. fused-silica capillary and all the separated fractions were automatically collected. In addition, the peaks of the cluster, indicated by arrows in Fig. 5 were completely resolved by applying this material to a fused-silica capillary of the same effective length as that used in the first run but with 50 μm I.D. and using the same experimental conditions. As shown in the inset of Fig. 5 the final separation achieved was excellent.

Each fraction of Fig. 5 was submitted to NH_2-terminal sequence analysis; the results obtained allowed us to identify the C-terminal prolyl and hydroxyprolyl imidodipeptides reported in Table 2. The extent of interaction between imidodipeptides and SDS-micelles could be presumed from the migration order of peptides. Ser–Pro and Asp–Pro (peaks 2 and 3 respectively) eluted faster because of their weak interaction with the micelles while Trp–Pro and His–Pro (peaks 8 and 9 respectively) are the peptides which have stronger interaction. It should be noted that, despite the very high resolving power of MEKC, most of these peaks still appeared to be partially overlapping, complete resolution only being possible with a second CE run. This was not surprising since, to some extent, it was a confirmation of the electrophoretic behaviour already shown by this mixture in the presence of Tween 20. Indeed the peculiar structural features of these peptides means that their full resolution is problematic. However the addition of SDS to the background electrolyte improves their separation from a series of other peaks (numbered 10 to 13) which completely overlapped with imidodipeptides in the CZE sepa-

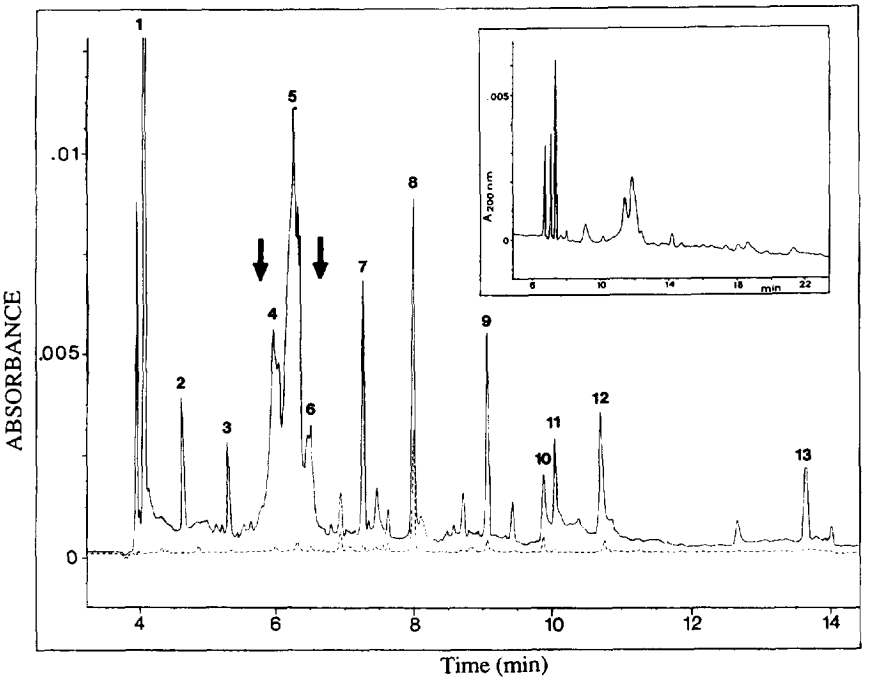

Fig. 5. Micropreparative micellar electrokinetic chromatography of urine sample from patient G.D. Experimental conditions: fused-silica capillary 68 cm effective length×100 μm I.D., run buffer: 35 m*M* sodium borate, pH 8.3 containing 65 m*M* SDS. Other conditions are the same as those described in the legend to Fig. 1. Peak 1: endogenous creatinine, peaks 2 to 9: X–Pro and X–Hyp imidodipeptides. For their identification see Table 2. (———) Absorbance at 200 nm. (- - -) Absorbance at 280 nm. The peaks numbered 2–13 were submitted to NH_2-sequence analysis. Inset shows the separation obtained when the cluster of peaks indicated by arrows was re-injected in the CE system. Experimental conditions are described in the text.

Table 2
Qualitative and quantitative analysis of imidodipeptides found in G.D.'s urine

Peak no.	Imidodipeptides found	Amount[a] (pmol)
2	Ser–Pro	3.51
3	Asp–Pro	3.98
4	Gly–Pro	8.52
	Phe–Hyp	3.80
5	Phe–Pro	19.85
	Ala–Pro	2.49
	Leu–Hyp	6.52
6	Pro–Pro	4.98
7	Leu–Pro	10.05
8	Trp–Pro	1.95
9	His–Pro	1.44

[a] The values reported refer to a 2-ml urine sample treated with Norite A for 5 min, filtered on a 0.20 μm nitrocellulose filter and lyophilized. The residue was then taken up in 100 μl of run buffer. The sequenced fractions were collected from two consecutive runs; a 42-nl aliquot was injected each run.

ration mode and even in the MEKC mode with Tween 20. Investigations are still in progress to identify these peaks, since although no sequence was obtained when submitted to NH_2-terminal analysis, their identification could be very important to extend our knowledge of the material excreted into urine by PD patients. It should be pointed out that, as these peaks are not present in urine from healthy individuals, they appear to be strictly related to this disorder.

The collection and identification of fractions from urine samples from other PD patients will enable us to compare excreted material and will allow us to obtain not only a reliable quantitative composition of imidodipeptides but also to definitively demonstrate that heterogeneity of urinary excretion between different patients, already postulated on the basis of a qualitative analysis [19] but still not well understood, does exist.

4. Conclusions

The CZE method we developed previously [19] was shown to be a simple and fast tool for diagnostic screening of prolidase deficiency. However, owing to the poor quality of electropherograms obtained, we were unable to achieve quantitative analysis of components excreted into urine. Here we extend our prior investigation by examining the potential of MEKC for separation of single X–Pro and X–Hyp peptides.

Although the presence of non-ionic surfactant Tween 20 in micelles did not provide further separation selectivity in addition to electrophoretic mobility, the use of SDS as surfactant improved the resolution and allowed us to exactly define the imidodipeptide composition of PD patient's urine. The possibility of identifying and quantifying other excreted components will help us to understand the pattern of material excreted by different patients and perhaps the pathogenic mechanisms leading to clinical changes in PD.

Acknowledgments

The authors gratefully acknowledge the valuable contribution of Mr. Marco Bellaviti (Department of Biochemistry, University of Pavia) for technical support in the CE experiments: This work was supported by grants from Regione Lombardia (Milan, Italy), area 5.2.5., progetto 723.

References

[1] H. Linduer, W. Hellinger, A. Dirschlinayer, H. Talasz, M. Wurm, B. Sarg, M. Jaquemar and B. Puschendorf, J. Chromatogr., 608 (1992) 69.
[2] G.A. Ross, P. Lorkin and D. Perreth, J. Chromatogr., 638 (1993) 69.
[3] E. Watson and F. Yao, J. Chromatogr., 608 (1992) 211.
[4] M.H.J.M. Lancenhuizen and P.S.L. Janssen, J. Chromatogr., 638 (1993) 311.
[5] J.B.L. Damm, G.T. Overklift, B.W.M. Vermenlen, C.F. Fluitsma and G.W.K. van Dedem, J. Chromatogr., 608 (1992) 297.
[6] B.L. Karger, A.S. Cohen and A. Gutmann, J. Chromatogr., 492 (1989) 585.
[7] W.G. Kuhr, Anal. Chem., 62 (1990) 403.
[8] E. Jellum, A.K. Thorsud and E. Time, J. Chromatogr., 559 (1991) 455.
[9] P. Meier and W. Thormann, J. Chromatogr., 559 (1991) 505.
[10] M. Zhu, R. Rodriguez, T. Wehr and C. Siebert, J. Chromatogr., 608 (1992) 225.
[11] P. Meinly and W. Thormann, J. Chromatogr., 608 (1992) 251.

[12] H. Soini, M.L. Riekkola and M.V. Novotny, J. Chromatogr., 608 (1992) 265.
[13] S. Terabe, K. Otsuka, K. Ichikava, A. Tsuchija and T. Ando, Anal. Chem., 56 (1984) 111.
[14] S. Terabe, K Otsuka and T. Ando, Anal. Chem., 57 (1985) 834.
[15] S.A. Swedberg, J. Chromatogr., 503 (1990) 449.
[16] E.L. Little and J.P. Foley, J. Microcol. Sep., 4 (1992) 145.
[17] M. Castagnola, D.V. Rossetti, L. Cassiano, R. Rabino, G. Nocca and B. Giardina, J. Chromatogr., 638 (1993) 327.
[18] N. Matsubara and S. Terabe, J. Chromatogr., 680 (1994) 311.
[19] G. Zanaboni, R. Grimm, K.M. Dyne, A. Rossi, G. Cetta and P. Iadarola, J. Chromatogr. B, 683 (1996) 97.
[20] H.J. Issaq, G.M. Janini, I.Z. Atamna, G.M. Muschik and J. Lukszo, J. Liq. Chromatogr., 15 (1992) 1129.
[21] E.C. Rickard, M.M. Strohl and R.G. Nielsen, Anal. Biochem., 197 (1991) 197.
[22] M. Castagnola, I. Messana and D.V. Rossetti, in P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996, p. 239.
[23] J. Lin, K.A. Kobb and M. Novotny, J. Chromatogr., 519 (1990) 189.
[24] N. Matsubara and S. Terabe, Chromatographia, 34 (1992) 493.
[25] T. Yashima, A. Tsuchiya, O. Morita and S. Terabe, Anal. Chem., 64 (1992) 2981.
[26] H.K. Christensen and S.H. Hansen, J. Chromatogr., 16 (1993) 2961.
[27] W. Nashabeh, K.F. Greve, D. Kirby, F. Foret, B.L. Karger, D.H. Reifsnider and S.E. Bullder, Anal. Chem., 66 (1994) 2148.
[28] T. Tadey and W.C. Purdy, Electrophoresis, 19 (1995) 574.
[29] N. Matsubara, K. Koezuka and S. Terabe, Electrophoresis, 16 (1995) 580.
[30] S. Hjerten, J. Chromatogr., 19 (1985) 191.