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Effect of different surfactants on the separation by micellar electrokinetic chromatography of a complex mixture of dipeptides in urine of prolidase-deficient patients

R. Grimm[a], G. Zanaboni[b], S. Viglio[b], K.M. Dyne[b], G. Cetta[b], P. Iadarola[b,*]

[a]*Analytical Division, Hewlett-Packard, D-76337 Waldbronn, Germany*
[b]*Dipartimento di Biochimica "A. Castellani", Università di Pavia, Via Taramelli 3/B, 27100 Pavia, Italy*

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Abstract

Prolidase deficiency is a severe disorder characterized by massive excretion of metabolites with closely related structures. At present, micellar electrokinetic chromatography is the separation method which provides the highest selectivity of structurally similar solutes. However, the structure of a surfactant can greatly affect the selectivity of separation depending on factors such as the length of hydrophobic alkyl chain or the nature of the hydrophilic group. Here we investigated the effect of three non-ionic and four anionic detergents for obtaining the best separation conditions for resolving imidodipeptide mixtures. The effect on resolution of variables such as temperature, surfactant concentrations and organic solvents was also examined. The greatest resolution was obtained at the lowest temperature studied (10°C) using 50 m*M* sodium borate, pH 9.3 containing 50 m*M* pentanesulfonate and 10% (v/v) methanol. Under these experimental conditions almost all excreted components were baseline separated and identified. © 1997 Elsevier Science B.V.

Keywords: Dipeptides; Imidodipeptides; Prolidase

1. Introduction

Recently, capillary electrophoresis (CE) has become one of the most important methods for the separation of peptide mixtures [1–5]. Among various separation modes, capillary zone electrophoresis (CZE) is certainly the best for obtaining resolution of charged molecules [6–11]. Application of this technique at both an analytical level and for micropreparative purposes allows one to obtain information concerning the purity and structural properties of peptides tested [5,12–15]. Since the separation mechanism in CZE is based on the difference between electrophoretic mobilities of the analytes in an electric field, the key parameters that need to be altered in order to optimize separation performance are the pH of the electrolyte and its ionic strength. However, structurally similar peptides cannot be completely separated since their electrophoretic mobilities are almost identical. In this case the addition of ionic or non-ionic surfactants to the background electrolyte at concentrations above the critical micelle concentration (CMC) provides another dimension of selectivity for peptide separation. In fact, in micellar electrokinetic chromatography (MEKC) the solute is partitioned between the micelle and the

*Corresponding author.

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surrounding aqueous medium and separation is based on the differential migration of the phases. Thus MEKC provides a sophisticated means for achieving a very high selectivity of solutes with closely related structures [16–23].

We have previously shown that MEKC is a versatile method for the separation of complex mixtures of imidodipeptides X-Pro and X-Hyp excreted into the urine of prolidase-deficient (PD) patients [24]. Although the potential of polyoxyethylenesorbitan monolaurate (Tween 20) as a surfactant was also partially explored, the use of aqueous sodium dodecyl sulfate (SDS) appeared to offer satisfactory separation efficiency. However, owing to the complexity of the components excreted and to their subtle differences in size, charge and hydrophobicity the separation was not complete even in the presence of a micellar phase.

To overcome these problems we systematically investigated the effect of different micellar agents for obtaining the best separation conditions for imidodipeptide mixtures. The effects on resolution of variables such as temperature, surfactant concentration and organic solvents were also examined. The improvement in peak resolution allowed a better understanding of the electrophoretic pattern and hence of the composition of total urinary compounds excreted by each patient.

The results from all experiments are compared.

2. Experimental

2.1. Urine samples

Urine samples, collected over a 24-h period, were obtained from the patients affected by PD as previously described [24]. A 1-ml aliquot of each urine sample was prepared for CE runs as described elsewhere [25].

2.2. Chemicals and buffers

Reagent-grade water used to prepare all solutions was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

The background electrolytes sodium phosphate 100 m*M* (pH 2.5) or sodium borate (pH 9.3) were from Merck (Darmstadt, Germany). They were used with appropriate addition of surfactants, cyclodextrins and/or organic modifiers. The borate solutions used were prepared by titrating 50 m*M* boric acid with the indicated detergent concentrations to pH 9.3 using 1 *M* NaOH. All ionic surfactants used: cholic acid (sodium salt), taurocholic acid (sodium salt), 1-pentanesulfonic acid (sodium salt) and SDS were from Sigma (St. Louis, MO, USA). α- and β-cyclodextrins were also purchased from Sigma. Non-ionic surfactants polyethyleneglycoldodecyl ether (Brij 35) and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Merck; polyethyleneglycol *tert.*-octylphenyl ether (Triton X-100) from BDH (Poole, UK). All surfactants were used at a concentration higher than their CMC (see Table 1).

2.3. CE systems

Part of the experiments were carried out on a HP 3D CE system (Hewlett-Packard, Waldbronn, Germany) with a built-in diode array detection (DAD) system. The CE system included an HP ChemStation for system control, data collection and analysis. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 56 cm effective length×50 μm I.D. were used. All experiments were performed at 10, 15, 20 and 25°C at 25 kV, samples were pressure-injected by applying 10 kPa for 2 s and data were recorded at 200, 280 and 330 nm. In addition, spectra were recorded from 190–400 nm in parallel. Between runs the capillary was flushed for 6 min with electrolyte buffer.

Another series of CE analyses were performed with a Beckman P/ACE 2100 instrument (Palo Alto, CA, USA) equipped with System Gold software for collection and analysis of data. Fused-silica capillaries and experimental conditions were the same as described above. Samples were pressure-injected by applying 25 kPa for 5 s and on-column detection was performed by measuring UV absorption at 200 nm. For each new background electrolyte the capillary was flushed with 0.1 *M* NaOH, washed with water and finally filled with new electrolyte. The system was then allowed to equilibrate for 15 min before starting injection.

Table 1
Structure and concentration of surfactants used

Surfactant	Structure	Concentration
Nonionic:		
Polyoxyethylenesorbitan monolaureate (Tween 20)	$(OCH_2CH_2)_nOH$; $CHCH_2(OCH_2CH_2)_nOCO(CH_2)CH_3$; $HO(CH_2CH_2O)_n$; $(OCH_2CH_2)_nOH$	10 - 150 mM
Polyethyleneglycoltert. octylphenylether (Triton X-100)	$CH_3C(CH_3)_2CH_2C(CH_3)_2-C_6H_4-(OCH_2CH_2)_nOH$	1.5 - 15 mM
Brij-35	$CH_3(CH_2)_{17}(OCH_2CH_2)_nOH$	10 - 100 mM
Anionic:		
Sodiumdodecylsulfate	$CH_3(CH_2)_{11}OSO_3^-$	65 mM
Pentanesulfonic acid	$CH_3CH_2CH_2CH_2CH_2SO_3^-$	50 mM
Bile salts:		
Cholic acid	H_3C, OH, $CHCH_2CH_2COO^-$, CH_3, HO, H, OH	25 mM
Taurocholic acid	H_3C, OH, $CHCH_2CH_2COHNCH_2CH_2SO_3^-$, CH_3, HO, H, OH	25 mM

3. Results and discussion

We have previously shown that the efficacy of non-ionic surfactant Tween 20 for the separation of complex mixtures of components in biological samples was very low at acidic pH [24]. Use of a polar additive such as SDS significantly improved resolution of these compounds although complete separation was only achieved after performing a second CE run i.e., the cluster of peaks not resolved in the first run was collected and reinjected on a different capillary [24]. Currently, while overcoming some of these limitations, we are extending our investigation to other non-ionic and ionic buffer additives to

evaluate their potential for analysis of urinary imidodipeptides. These peptides comprise a series of closely related structures so that the differences in selectivity of various surfactants can be investigated and aid in understanding of the factors that influence separation. The use of some bile salt micelles will also be discussed.

All the surfactants used are listed in Table 1.

Although the use of non-ionic micelles is still rare, we decided to make a careful study of these surfactants because they have well known advantages [16] over other additives: i.e., they show weaker interaction with peptides than cationic and anionic surfactants and, in particular, they do not contribute to the conductivity of the background electrolyte which means less Joule heat and, therefore, higher resolution. On the basis of prior experience [24], all runs were performed at a pH of 2.5 and/or 9.3. The buffer systems chosen were 100 m*M* sodium phosphate, pH 2.5 (system 1) and 50 m*M* sodium borate, pH 9.3 (system 2). The detergent Brij 35 was used in a range of concentrations between 10 m*M* and 100 m*M* while the concentrations of Tween 20 ranged between 10 m*M* and 150 m*M*. Triton X-100 was used in the range 0.1–1% (v/v), approximately 1.5–15 m*M*, because electrolytes containing higher concentrations of this surfactant are very viscous and difficult to work with.

Thus, when MEKC was performed on aliquots of urine from patient G.D. [25] using buffer systems 1 and 2, a complete set of electropherograms was achieved for each. Representative examples of separation profiles obtained for each surfactant at low pH (panels A, C and E on the left) and high pH (panels B, D and F on the right) are reported in Fig. 1. It can be seen that the efficiency and resolution for the three surfactants considered were better at pH 9.3. The explanation could be that at this pH imidodipeptides exhibit a net negative charge and the fraction of these analytes not incorporated into the micelles will migrate towards the negative electrode at a retarded velocity with respect to electroosmotic flow (EOF). This effect produces greater differences in the mobility between analytes improving separation. At the other extreme of pH (pH 2.5) dipeptides travel toward the negative electrode with a velocity given by the sum of their electrophoretic mobilities and the EOF and there is insufficient charge difference between them to show differential migration. Thus the pH of the background electrolyte, rather than the surfactant structure or its concentration, seems to play a predominant role in determining resolution of X-Pro imidodipeptides. Experimentally, a dramatic difference of selectivity was observed between the two background electrolytes, employing Tween 20, Brij-35 and Triton X-100 as additives. As shown in panel A (Fig. 1), in the presence of Tween 20, in the range of concentrations between 10 m*M* and 150 m*M*, the separation of the urinary imidodipeptides was very poor at pH 2.5. In contrast, at pH 9.3 separation of the mixture was improved, but still only partially, in the presence of 50 m*M* Tween 20 (panel B). Increasing the Tween 20 concentration to 150 m*M* did not result in further improvement of the resolution. These results may provide additional knowledge concerning the ability of this additive to allow separation of peptide mixtures. In a case where closely related peptides were involved, other authors [17] have previously shown that the separation factor increased with an increase of concentration of Tween 20 and, in another case [22], that peptides with the same chain length could be resolved at low pH with high concentration of the same additive.

The electrophoretic profile of panel D compared to that illustrated in panel C shows the increase in resolution achieved when the pH is changed from 2.5 to 9.3 and Brij 35 was used as an additive. This emphasized the need of a basic pH to obtain good separation. Besides the lack of a complete resolution of compounds investigated, a similarity in the peak pattern with the pattern obtained with Tween 20 at the same pH was observed.

Separation performance using Triton X-100 was also considered. As shown in panels E and F (Fig. 1), little evidence of separation was obtained with both buffer systems and individual peptides could not be differentiated because they exhibited poor peak shape. As higher concentrations of Triton X-100 had little effect on the relative mobility of imidodipeptides and were difficult to work with, no further work on this micellar system was carried out. Several attempts to further improve the separation obtained using Tween 20 and Brij 35 at pH 9.3 produced contrasting results. Although addition of methanol or acetonitrile to the electrolyte containing Tween 20 did not substantially affect resolution (data

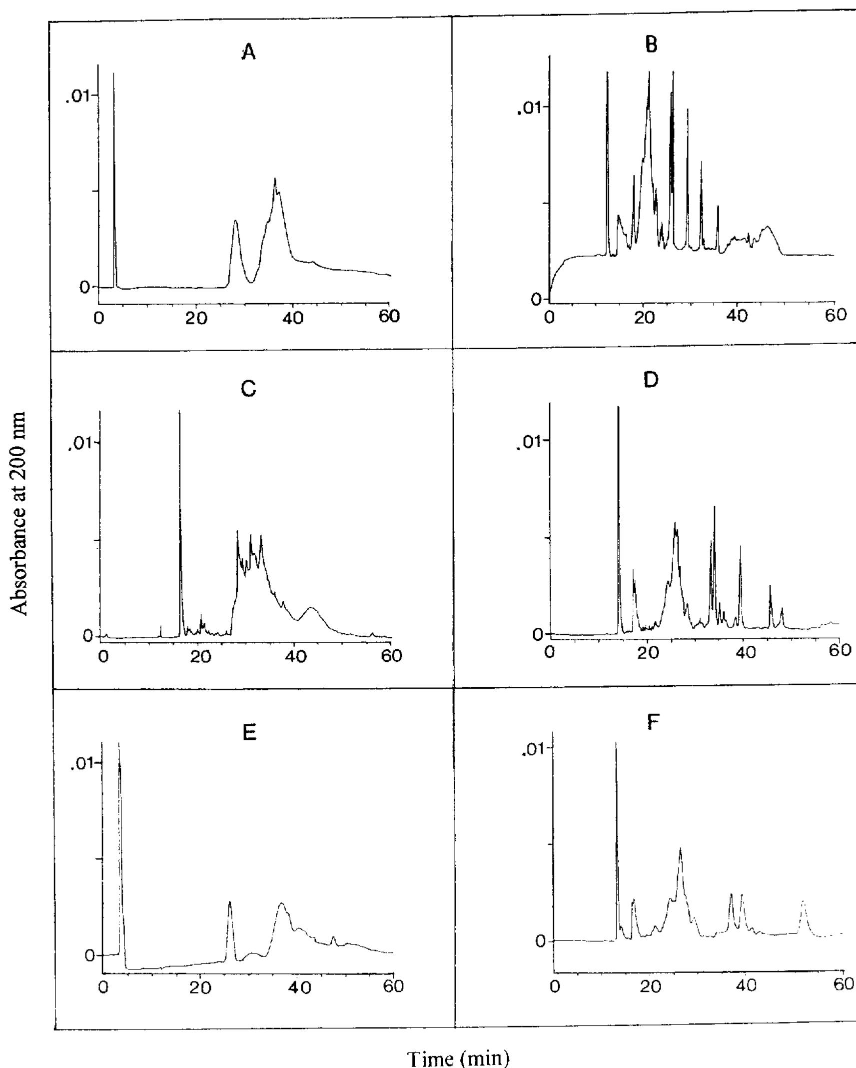

Fig. 1. Micellar electrokinetic chromatogram of urine from patient G.D. [24] performed in: 100 m*M* sodium phosphate, pH 2.5 containing 100 m*M* Tween 20 (panel A), 50 m*M* Brij 35 (panel C) and 10 m*M* Triton X-100 (panel E), and in: 50 m*M* sodium borate, pH 9.3 containing the same detergents (panels B, D and F, respectively). The first peak of each panel is endogenous creatinine; for identification of all other imidodipeptides see Table 2. Experimental conditions: fused-silica capillary 56 cm effective length×50 μm I.D.; voltage: +25 kV applied to injection end; detection: UV absorbance at 200 nm; temperature 20°C. Positive to negative polarity.

not shown), an increase in selectivity was observed when 20% (v/v) acetonitrile was added to Brij 35-containing electrolyte (Fig. 2, panel A). As shown in panel B of Fig. 2, a smaller resolution was evidenced when acetonitrile was replaced by 15% (v/v) methanol. It is interesting to note that, although both organic solvents employed improved the shape of peaks, complete separation of all compounds present in the mixture was never achieved. Therefore, using a background electrolyte pH of 9.3, which showed the best separation selectivity, we also studied the effect of anionic surfactants and bile salts on resolutions of imidodipeptides.

The separation profiles of X-Pro imidodipeptides obtained using 50 m*M* borate pH 9.3 containing 65 m*M* SDS (panel A), 50 m*M* pentanesulfonate (panel

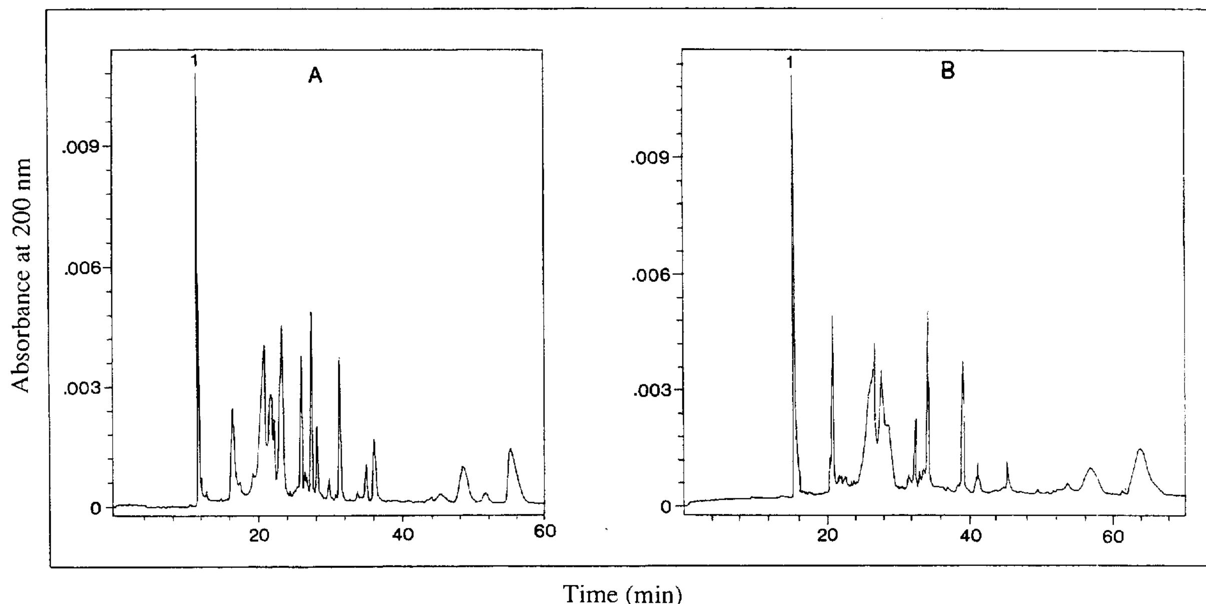

Fig. 2. Micellar electrokinetic chromatogram of urine from patient G.D. performed in 50 m*M* sodium borate, pH 9.3 containing 50 m*M* Brji 35 and 20% (v/v) acetonitrile (panel A) or 15% (v/v) methanol (panel B). The first peak (peak 1) is endogenous creatinine. All other experimental conditions are the same as those described in Fig. 1.

B) 25 m*M* sodium cholate (panel C) and 25 m*M* sodium taurocholate (panel D) are presented in Fig. 3. Each electropherogram exemplifies the typical separation profile achieved at 25°C for each surfactant.

As shown, the various profiles displayed at 200 nm were very similar confirming that the chemical structure of surfactants was not the only crucial separation variable for this mixture of compounds. However when attention was focused on the elution region (indicated by arrows in Fig. 3) containing most of these peptides it was seen that selectivity could be influenced by the length of the hydrophobic alkyl-chain of surfactant. In fact, owing to its longer chain, SDS interacts with imidodipeptide analytes more strongly than pentanesulfonate. In the case of pentanesulfonate, imidodipeptides showed slightly higher distribution constants between the micellar phase and the aqueous phase; the values of distribution constants calculated as suggested by Terabe et al. [26] were in the range between 29.2 and 708.5 for SDS and 40.5 and 852 for pentanesulfonate. As a consequence in the presence of pentanesulfonate selectivity was improved and the peaks contained in the imidodipeptide elution region of panel B appeared sharper compared to the corresponding region of panel A. An observation seems consistent with the results shown in Fig. 2. Clear differences in selectivity between sodium cholate and sodium taurocholate were seen by looking at the separation patterns in panels C and D (Fig. 3), the selectivity being better with sodium taurocholate. This difference in resolution is likely to be associated with the nature of the surfactant hydrophilic group which is known to be one of the important parameters that can affect selectivity [27]. Thus the presence of a sulfonate group as the polar moiety of the micelles either in pentanesulfonate and in sodium taurocholate and the similar electrophoretic profiles obtained in both cases suggest that there may be a similarity in the way that the compounds investigated partition with the micelles.

The need to optimize experimental conditions in order to obtain high-resolution separations led us to investigate the role of modifiers such as α- or β-cyclodextrins and other variables such as organic

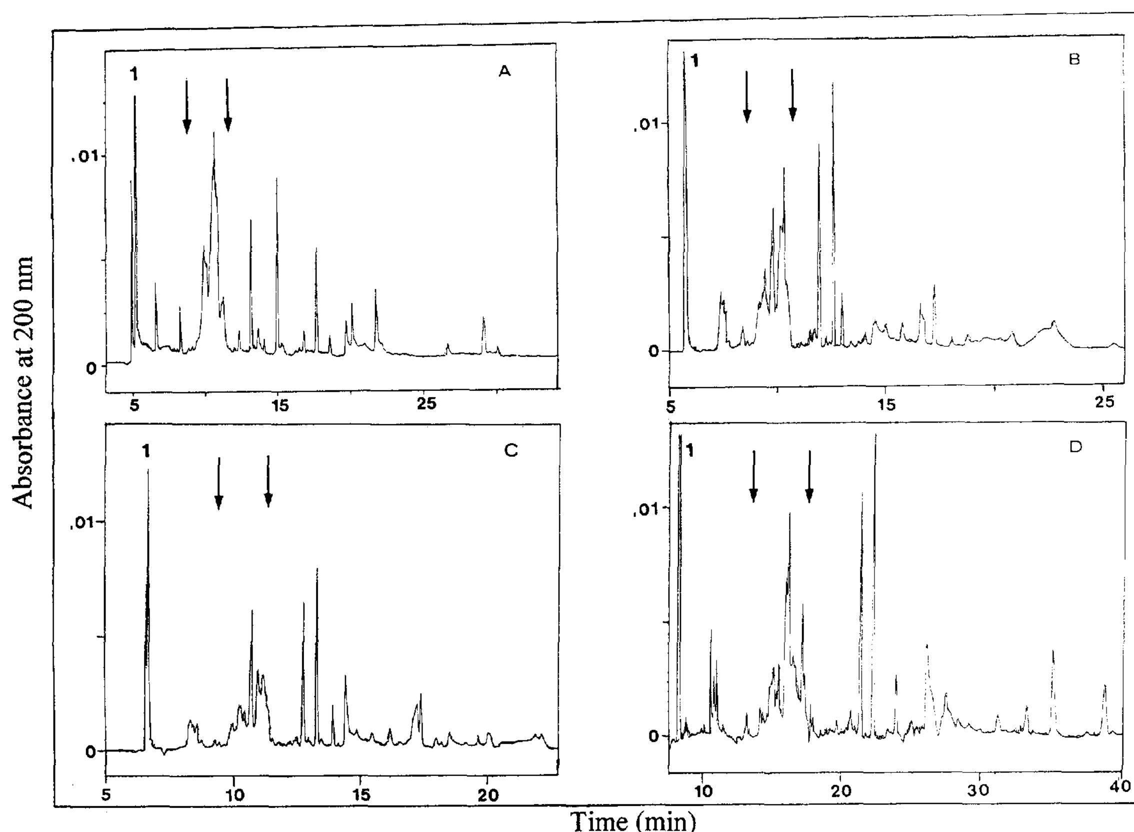

Fig. 3. Micellar electrokinetic chromatogram of urine from patient G.D. performed in 50 m*M* sodium borate, pH 9.3 containing the following surfactants: 65 m*M* SDS (panel A), 50 m*M* pentanesulfonate (panel B), 25 m*M* sodium cholate (panel C) and 25 m*M* sodium taurocholate (panel D) at 25°C. The first peak (peak 1) is endogenous creatinine. The arrows indicate the elution region containing most of the excreted X-Pro/Hyp dipeptides. All other experimental conditions are the same as those described in Fig. 1.

solvents and temperature. The addition of α- or β-cyclodextrins (in a range of concentrations between 10 and 20 m*M*) in binary mixtures with each of anionic surfactants already considered did not improve separation sufficiently in relation to expectations. The overall resolution of peaks was only slightly enhanced when α-cyclodextrin was added to SDS-containing buffer at pH 9.3 and, upon addition of β-cyclodextrin to the same electrolyte system, the final profile was even worse than that of Fig. 3.

Practically no influence on resolution was shown for α- and β-cyclodextrins in the presence of the other three detergents studied (data not shown). These results show that none of the analyte molecules examined could be included, by hydrophobic interaction, into the cavities of cyclodextrins thus supporting the idea that the polar head groups may be more important than the hydrophobic core in controlling the selectivity for the dipeptides.

Further experiments were conducted using the solutions of the four anionic surfactants in mixtures with methanol. The observation that this additive improved efficiency led to the optimization of methanol conditions; three different percentages of methanol: 5, 10 and 20% (v/v) were compared for their influence on peak resolution. The greatest increase in resolution was observed when 10% (v/v) methanol was added to 50 m*M* pentanesulfonate and when the separation was performed at 10°C (Fig. 4). In fact, when the temperature was varied as an additional parameter to modulate peptide separation, the best separation was obtained at the lowest temperature,

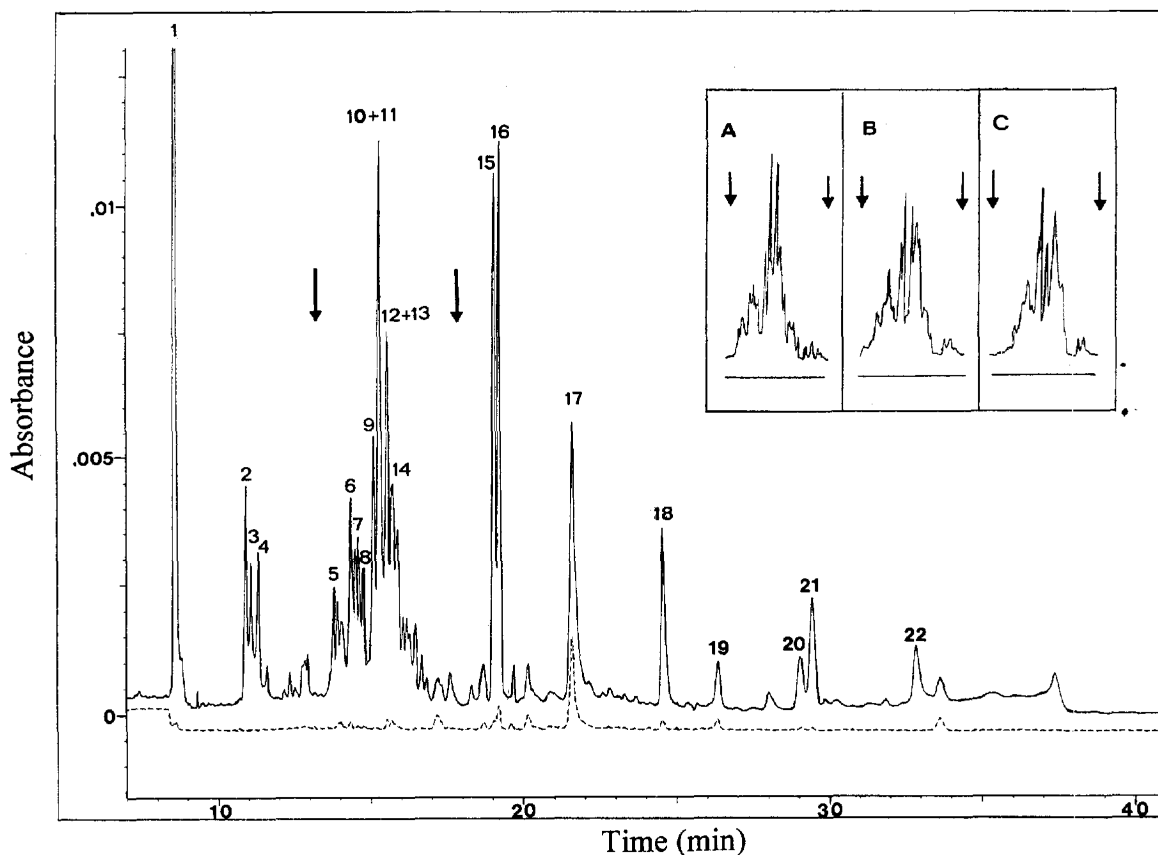

Fig. 4. Micellar electrokinetic chromatogram of urine from patient G.D. performed in 50 m*M* sodium borate pH 9.3 containing 50 m*M* pentanesulfonate and 10% (v/v) methanol. Temperature: 10°C. Inset shows the resolution obtained for the cluster of peaks indicated by arrows at temperature of 15°C (panel A), 20°C (panel B) and 25°C (panel C), respectively. The first peak (peak 1) is endogenous creatinine; for identification of peaks numbered 2 to 19 see Table 2. (—) Absorbance at 200 nm. (- - -) Absorbance at 280 nm. All other experimental conditions are the same as those described in Fig. 1.

similar to that previously reported for different peptide mixtures [18]. As the temperature increased to 15, 20 and 25°C, resolution decreased progressively (inset of Fig. 4, panels A, B and C, respectively). This decrease in resolution is the result of peak broadening and peak shape distortion caused by the *cis–trans* isomerization of the X-Pro bond which is temperature dependent as previously reported [28]. In the light of these results, separations were also performed using the Brij 35 system at 10°C. Although resolution was enhanced with respect to that obtained at 20°C, separation of peaks was not improved as much as expected (data not shown). The addition of 15% (v/v) acetonitrile to pentanesulfonate-containing buffer instead of methanol gave similar results. As expected, organic solvents reduced the EOF thus expanding the migration window available for the separation and allowing a better distribution of components. It is worth noting that the electrophoretic pattern shown in Fig. 4 represents the best separation ever obtained for the complex mixture of urinary compounds excreted by PD patients.

Although the number of peaks obtained was high, the resolution between adjacent peaks was good enough to permit their identification. Identification was performed either by comparison of migration times and, in some cases comparison of diode array spectra with the corresponding set of data obtained by analyzing authentic compounds. A complete list

Table 2
Identification of peaks found in urine from patient G.D.

Peak No.	Compound
1	Creatinine
2	Citrulline
3	Glutamine
4	Ser–Pro
5	Asp–Pro
6	Gly–Pro
7	Ala–Pro
8	Met–Pro
9	Val–Pro
10+11	Ile/Leu–Pro/Hyp
12+13	Phe–Pro/Hyp
14	Pro–Pro
15	His–Pro
16	Tyr–Pro
17	Trp–Pro
18	n.i.[f]
19	n.i.
20	n.i.
21	Uric acid
22	n.i.

[f] n.i.=Not identified.

of components separated is reported in Table 2. In addition to X-Pro and X-Hyp imidodipeptides, some of which were already previously identified [25], other compounds excreted in concentrations higher than that in normal subjects were evidenced. Peaks 2, 3 and 21 contain citrulline, glutamine and uric acid, respectively. The presence of large amounts of these metabolites, particularly citrulline and glutamine, in the urine of PD patients could suggest that prolidase deficiency is also associated with a generalized urea cycle disorder. However, as shown in Table 2, four peaks (namely peaks 18, 19, 20 and 22) are still unidentified; investigations are currently in progress and evaluation of their nature could extend our knowledge of this disease. Moreover, when MEKC with pentanesulfonate containing 10% (v/v) methanol was also performed on urine samples available from other patients [25], a clear difference between the electrophoretic patterns of the various patients was observed. This was particularly evident when the imidodipeptide-containing region of each electropherogram (indicated by arrows in Fig. 4) was expanded as shown in Fig. 5.

The electrophoretic profile (top left Fig. 5) obtained from urine of G.D., the patient affected by the most severe form of PD is in fact very different from that of the urine obtained from patient B.C. (lower left Fig. 5), whose clinical symptoms are milder. Similarly urine from patient C.Ma. (middle right Fig. 5) showed a different separation profile from that of patient C.F. (top right Fig. 5). Thus, the previously postulated [24] hypothesis of heterogeneity of dipeptide excretion between patients with differing clinical severity appears to be strongly supported by the results shown above.

4. Conclusions

Owing to the complexity of the mixtures examined, numerous attempts were made to find the best experimental conditions which allow the highest resolution of urinary components. We were successful in separating these complex mixtures and showed how the use of micellar-modified buffers may or may not result in peptide separation depending on the nature of surfactant and the experimental conditions used. Different non-ionic and ionic detergents were added to either 100 m*M* sodium phosphate, pH 2.5 or 50 m*M* sodium borate, pH 9.3 as background electrolytes. Although a good electrophoretic profile was obtained with 50 m*M* sodium borate, pH 9.3 and Brij 35 as surfactant at 10°C, the best resolution was observed using 50 m*M* sodium borate, pH 9.3 containing 50 m*M* pentanesulfonate and 10% (v/v) methanol. A further enhancement of resolution was obtained by performing separations at 10°C. The good separation of the urinary imidodipeptide mixtures and the identification of nearly all components excreted into the urine by PD patients, including metabolites other than imidodipeptides, may aid in better understanding of this disorder. In fact, the exact quantification of metabolites excreted into the urine may be useful for future therapies aimed at reintegrating amino acids and/or other metabolites.

Acknowledgments

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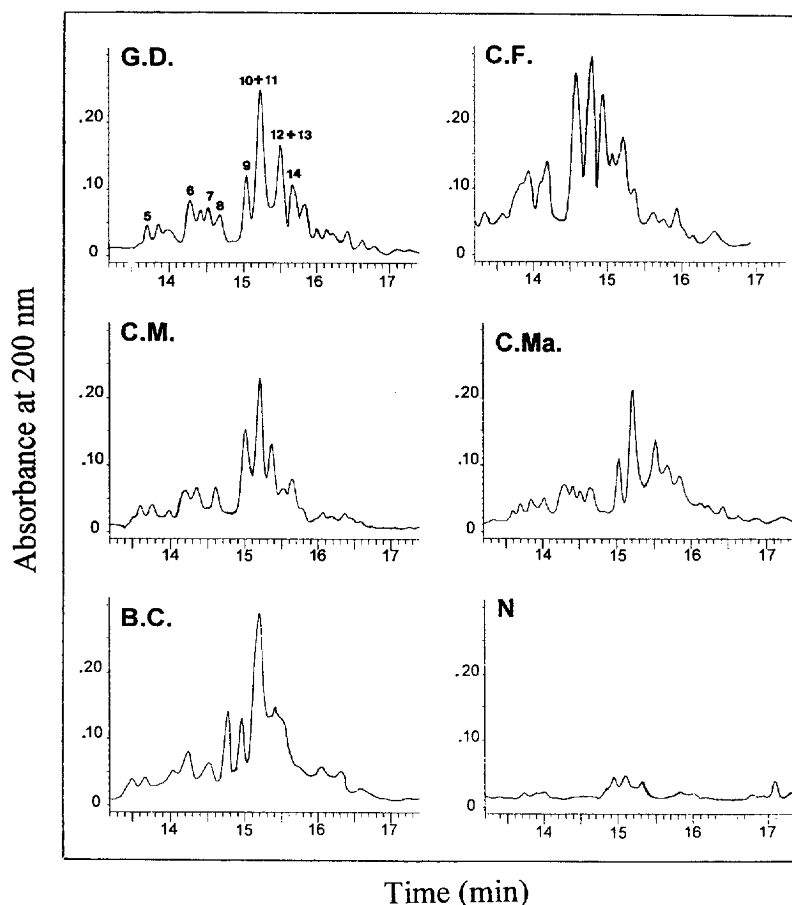

Fig. 5. Micellar electrokinetic chromatogram of urine samples from patients: G.D., C.F., C.M., C.Ma., B.C. [24] and from a control (N). For each electropherogram only the amplified region included between 12 and 17 min is reported. For identification of peaks numbered 5 to 14 in G.D.s profile see Table 2. All other experimental conditions are the same as those described in Fig. 1.

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