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Detection of neuropeptides using on-capillary copper complexation and capillary electrophoresis with electrochemical detection

Capillary electrophoresis with electrochemical detection using a carbon fiber electrode in conjunction with on-capillary copper complexation was evaluated for the determination of peptides in standard and biological matrices. Peptides composed of 2–10 amino acids were investigated. A comparison was made between the responses obtained for peptides containing the oxidizable residue tyrosine and those obtained for their respective copper complexes. Electrochemical detection of non-tyrosine-containing peptides and a cyclic peptide was also demonstrated. A separation of leucine (Leu)-enkephalin and five metabolites was developed and then used for the investigation of Leu-enkephalin metabolism in plasma. The appearance of the des-tyrosine (des-Tyr) Leu-enkephalin, which cannot be detected directly at a carbon electrode, was monitored using the on-capillary complexation technique. Direct injection of the plasma sample was possible using this methodology.

Keywords: Biuret reaction / Derivatization / Enkephalins / Capillary electrophoresis / Electrochemical detection / Neuropeptides / Copper
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1 Introduction

Neuropeptides play an important role in the regulation of many biological functions and diseases. Neuropeptide degradation is controlled by proteolytic enzymes whose activity is thought to be affected by disease states such as asthma, chronic stress, mental illness, and trauma [1–8]. A better understanding of the metabolic pathways of circulating neuropeptides could lead to the design of more effective drugs capable of inhibiting or stimulating the metabolic processes. For example, opioid peptides are known to regulate many immune functions, including stress and pain. However, this relationship is still poorly understood because it is bidirectional, which means that the peptides can have inhibitory and/or stimulatory effects, depending on their circulating concentrations [5, 9]. Plasma peptidases control the concentration of endogenous opiates, effectively modulating the immune response. Peptide metabolism, then, can be used as an indirect measure of endogenous enzyme activity and may indicate immune system deficiencies [2]. Leu-enkephalin has been widely utilized as a model compound to study

peptidase activity, yet even this small peptide has three possible degradation pathways and several possible metabolites [1–4, 6, 8]. The variety of metabolic pathways for Leu-enkephalin and other neuropeptides requires a general yet selective method of analysis if we are to understand the role these compounds play in biological signaling and physiological processes.

Capillary electrophoresis (CE) has become a routine tool for the separation of peptide mixtures. As with most analytical techniques, one of the limiting factors to high sensitivity and selectivity in CE is the detection method employed. Commercial CE instrumentation usually employs UV detection due to its ease of use; however, detection limits and selectivity are poor. Laser-induced fluorescence detection can be highly selective and sensitive, yet only specific wavelengths are available for excitation, and derivatization of the analyte is usually required. Mass spectrometry is gaining wider use as a detection method for CE. However, this method requires the use of volatile buffers and, although selectivity is high, routine analyses of biological fluids can be problematic. Cost is also an issue, although mass spectrometers continue to become more affordable.

Extensive work by Weber's [10–17] group has shown that copper complexation combined with liquid chromatography and electrochemical detection (EC) at carbon electrodes is a general, sensitive, and selective technique for peptide analysis. The detection scheme relies upon the ability of copper to complex with the peptide amide back-

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Abbreviations: CE-EC, capillary electrophoresis with electrochemical detection; CE-UV, capillary electrophoresis with UV detection

bone. Single electrode detection can be used to monitor the one-electron oxidation of the Cu(II) complex. This complex undergoes a chemically reversible oxidation to Cu(III), which can be detected at a second electrode in series. Thus, the methodology is both selective and widely applicable to a variety of peptide structures. The complexation reaction takes place with peptides that are electroactive and nonelectroactive at a carbon electrode, including cyclic peptides and peptides without a terminal amine. Copper complexation has been employed with capillary liquid chromatography for the determination of bradykinin and vasopressin in rat brain dialysates [18]. Concentration detection limits in the low μM range were achieved using on-column preconcentration of preformed copper complexes and single electrode detection.

Previous work utilizing the biuret reaction has relied mainly upon precolumn or postcolumn derivatization with copper to form the complexes [10–14, 17, 18]. Our group has demonstrated on-capillary complexation with capillary electrophoresis and EC detection for a limited number of peptides [19]. More recently, optimized conditions for the complexation procedure have been evaluated by our group using CE with UV detection (CE-UV) [20]. The findings revealed that copper complexation can be accomplished precapillary and on-capillary (or both combined), but on-capillary complexation offers significant advantages over the former approach. These include direct injection of the sample and the best overall separation efficiency.

Our previous work utilizing on-capillary complexation with EC detection focused on only a small number of peptides [19]. In this work, tyrosine (Tyr)-containing peptides, non-Tyr-containing peptides, a cyclic peptide, and peptides capable of binding more than one copper were used to further investigate the applicability of this methodology. Tyr-containing peptides can be detected by direct oxidation of the Tyr residue at a carbon electrode, as well as by complexation. The separation and detection of an enkephalin peptide mixture is also presented. To demonstrate the usefulness of the method for the analysis of a biological sample, the metabolism of Leu-enkephalin in plasma was investigated.

2 Materials and methods

2.1 Reagents

All peptides were obtained from Sigma (St. Louis, MO, USA) and Bachem (Bubendorf, Switzerland). Peptides studied were angiotensin I, angiotensin II, angiotensin III, angiotensin 1–7, oxytocin, Leu-enkephalin, des-Tyr Leu-enkephalin, TyrGlyGly, TyrGly, GlyPheLeu, and PheLeu.

Stock solutions (1 mM) were prepared in NANOpure water (Labconco, Kansas City, MO, USA) and refrigerated until use. Hydrophobic peptides (GlyPheLeu and PheLeu) were dissolved in 25 mM boric acid, pH 9.8. Anhydrous cupric sulfate, L(+)-tartaric acid disodium salt, boric acid and sodium dodecyl sulfate (SDS) were purchased from Sigma. Copper-containing buffers were prepared by dissolving the appropriate amount of cupric sulfate and tartaric acid in borate buffer and then adjusting to the desired pH with 1 M NaOH (Fluka, Buchs, Switzerland). A 50 mM SDS solution was used for plasma analysis (Section 3.5).

2.2 CE-EC system

The CE-EC apparatus has been described previously [21]. Polyimide-coated fused-silica capillaries (25 μm ID \times 360 μm OD \times 70 cm) were obtained from Polymicro Technologies (Phoenix, AZ, USA). A Spellman CZE 1000R power supply (Plainview, NY, USA) was used to apply the separation voltage. The electrochemical cell consisted of a 33 μm carbon fiber microelectrode (Avco Specialty Materials, Lowell, MA, USA), an Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN, USA), and a platinum wire auxiliary electrode housed in a rubber septum. The carbon fiber electrode (exposed length 100–200 μm) was aligned using micromanipulators. The electrode was withdrawn from the capillary to facilitate cleaning and storage between experiments. All measurements were obtained using a BAS LC-4CE amperometric detector (Bioanalytical Systems). The analog signal output from the electrochemical detector was converted to a digital signal with a DA-5 data acquisition interface and evaluated using BAS Chromgraph software (Bioanalytical Systems).

CE-EC requires isolation of the electrochemical cell from the separation voltage. To isolate the electrochemical detector from the separation voltage, a decoupler was constructed by securing the capillary in polyetheretherethylene (PEEK) tubing, scoring the polyimide coating, and applying pressure to form a fracture in the capillary wall [22]. Since small inner diameter capillaries (25 μm) were used, the tip of the capillary was placed in 48% HF to form a conical shape to aid in electrode alignment [23].

2.3 CE separations

Each day the capillary was flushed successively with 0.1 N NaOH, NANOpure water, and run buffer for 10 min each. The run buffer for the data presented in Sections 3.1–3.3 consisted of 25 mM boric acid, 3 mM tartaric acid, and 1 mM copper sulfate, pH 9.8. For the enkephalin separation and plasma analysis (Sections 3.4 and 3.5), the

run buffer was composed of 100 mM boric acid, 3 mM tartaric acid, and 1 mM copper sulfate, pH 9.8. The buffer was degassed each day by sonicating for 15 min. On-capillary complexation was achieved by injecting peptide samples that were dissolved in 25 mM boric acid, pH 9.8, directly onto the capillary [20]. Injections were made at 10 psi for 2–5 s using a pressure system built in-house.

2.4 Plasma analysis

Blood samples were drawn at the University of Kansas Health Center, and the plasma was separated out using sodium EDTA as the anticoagulant. Plasma was used the day of blood withdrawal and was diluted 1:10 with NANO-pure water to slow enzyme activity. Leu-enkephalin (1 mM in water) was mixed 1:10 with the diluted plasma and placed in a shaking water bath at 37°C. Aliquots were withdrawn at 15 min intervals and injected directly onto the capillary. Prior to injection, 50 mM SDS was flushed through the capillary for 3 min to remove plasma proteins adsorbed on the capillary wall [24].

3 Results and discussion

3.1 Native peptides vs. copper complexes

On-capillary complexation requires a high pH buffer that contains copper. The sample is injected directly in the capillary and the complexation reaction takes place inside the capillary [19, 20]. This approach eliminates the dilution of the sample with reagent, which is undesirable when analyzing biological fluids. In these studies, Leu-enkephalin, a small, linear neuroactive peptide containing tyrosine, was used to compare the response due to direct oxidation at a carbon electrode with that obtained for its copper complex. Angiotensin peptides were employed to evaluate the on-capillary complexation method for larger, structurally similar compounds.

Two overlaid CE-EC electropherograms of Leu-enkephalin and its copper complex are shown in Fig. 1. The responses for both the native peptide and the copper complex were linear from 980 nM–100 μ M ($r^2 = 0.999$ and $r^2 = 0.999$, respectively). However, the sensitivity (slope of the calibration curve) for the copper complex was 198 pA/ μ M compared to 94 pA/ μ M for the native peptide. The limit of detection (LOD) for Leu-enkephalin was also improved by a factor of two using copper complexation. The copper complex had an LOD of 500 nM (S/N = 3) while the native peptide had an LOD of 980 nM (S/N = 3). The increase in sensitivity and lower LOD are due to a combination of factors. The anodic signal generated from the oxidation of the copper complex is an additive effect from the Tyr and Cu(II) oxidation [13]. The position of Tyr in the peptide also affects the electrochemical signal. Peptides, such as Leu-enkephalin, containing a Tyr at the

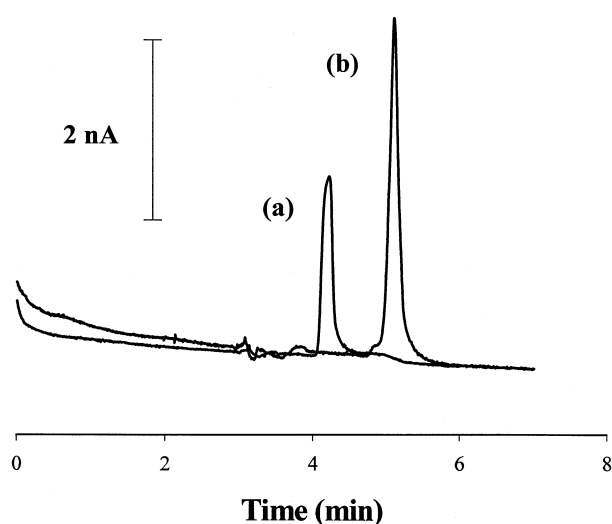


Figure 1. Electropherogram comparing the electrochemical response for (a) Leu-enkephalin and (b) the Leu-enkephalin copper complex formed on-capillary. Separation conditions are noted in Section 2.3. Peptide concentration, 20 μ M; detection potential, +800 mV vs. Ag/AgCl.

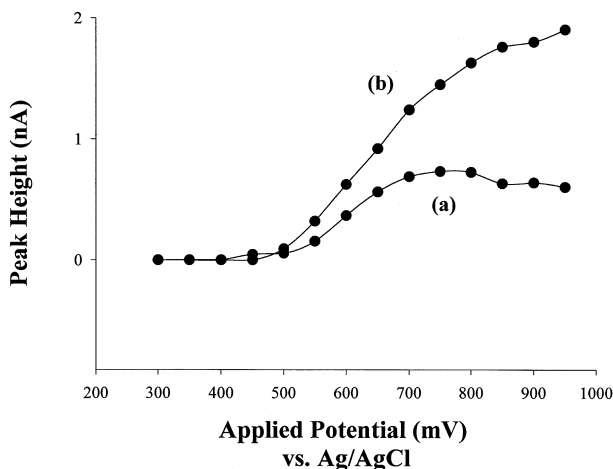
amine terminus have higher sensitivities than peptides with Tyr in nonterminal positions [13]. On-capillary complexation provides the added benefit of peak stacking [20]. Because the derivatization reaction takes place inside the capillary, the sample can be made at a lower ionic strength than the run buffer.

Similar trends in response for copper complexes were observed with longer peptides from the angiotensin family: angiotensin I, angiotensin II, angiotensin III, and angiotensin 1–7 (Table 1). These peptides are linear and range in size from ten to seven amino acids. On-capillary complexation occurs rapidly and completely. In addition, all four compounds contain a Tyr residue, which permits direct comparison between the electrochemical signals of the native peptide and the copper complex using CE-EC.

Hydrodynamic voltammograms of angiotensin II and its complex are presented in Fig. 2. Upon complexation, the electrochemical signal generated from oxidation of the peptide complex is substantially increased over that of the native peptide. The E_{\max} value is also shifted to a higher potential, in this case from +750 to +950 mV (vs. Ag/AgCl). Table 1 lists the E_{\max} and respective response for four angiotensin peptides and their copper complexes. Table 1 should be interpreted as a response comparison between peptides and their respective copper complexes. Comparison of response between the different peptides is inapplicable because experiments were performed on different days, which required realignment of the electrode (see Section 2.2). The increase in response at E_{\max} ranged from 61% for angiotensin III to 278% for angiotensin I.

Table 1. Electrochemical response of angiotensin peptides and their respective complexes formed on-capillary

	E_{\max} (mV) vs. Ag/AgCl for native peptide	Response at E_{\max} (pA/ μM)	E_{\max} (mV) vs. Ag/AgCl for Cu complex	Response at E_{\max} (pA/ μM)	% Increase in response at E_{\max}
Angiotensin I (10 aa)	+750	7.3	+950	27.6	278
Angiotensin II (8 aa)	+750	13.8	+950	35.9	160
Angiotensin III (7 aa)	+800	23.3	+950	37.6	61
Angiotensin 1–7 (7 aa)	+650	4.2	+900	8.7	107

**Figure 2.** Hydrodynamic voltammograms for (a) angiotensin II and (b) the angiotensin II copper complex. Separation conditions are noted in Section 2.3.

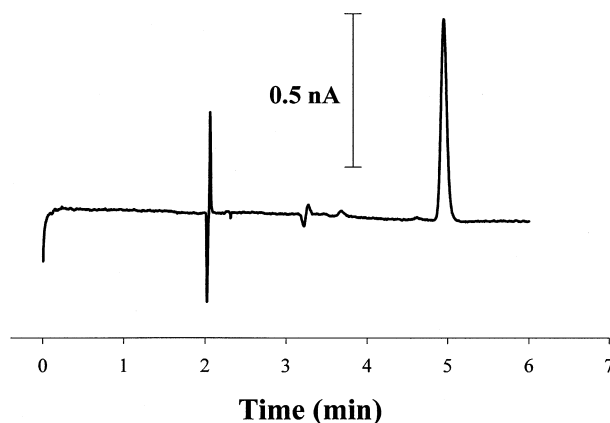
The differences in response can be explained by the overall contribution from copper complexation. Based on length alone, angiotensin I and II are capable of binding more than one copper. Weber *et al.* [25] have provided evidence based on molar absorptivity that angiotensin II binds two copper ions. The magnitude of the difference in electrochemical response for angiotensin I and II suggests complexation at multiple sites. The change in response due to complexation for angiotensin III and angiotensin 1–7 is less pronounced (than angiotensin I and II) at their respective E_{\max} values. This indicates that these peptides are probably binding only a single copper ion. Overall, on-capillary complexation provides reproducible, predictable electrochemical behavior for linear peptides containing tyrosine. The complexation kinetics were found to be rapid enough to perform on-capillary complexation.

3.2 On-capillary complexation and detection of a nonelectroactive peptide

Copper complexation also provides the means to detect peptides which are not electroactive [17]. In this paper, "nonelectroactive" refers to those peptides that are not directly oxidized at a carbon electrode. Subsequently, the

electrochemical signal is due only to the oxidation of the Cu(II) complex. Des-Tyr Leu-enkephalin was utilized as a model analyte to evaluate the on-capillary complexation technique for detection of a nonelectroactive peptide by CE-EC. Injection of the native peptide with no copper in the run buffer yields no signal. Adding copper to the run buffer results in a measurable response, as shown in Fig. 3. The response for the des-Tyr Leu-enkephalin copper complex was found to be linear between 5–100 μM ($r^2 = 0.997$). The sensitivity (slope of the calibration curve) was 43 pA/ μM and the LOD was estimated to be 1.0 μM at an S/N = 3 based on the response at 5 μM (S/N = 10).

The E_{\max} for the des-Tyr Leu-enkephalin complex is +550 mV (vs. Ag/AgCl) while the Leu-enkephalin complex has an E_{\max} of +800 mV (vs. Ag/AgCl), as determined from hydrodynamic voltammetry (Fig. 4). The Tyr-containing peptide copper complex provides a considerably greater electrochemical response than does the non Tyr-containing peptide copper complex, as expected [13, 25]. However, the significant difference in E_{\max} illustrates the possibility for tunable selectivity with electrochemical detection. At +500 mV, the des-Tyr Leu-enkephalin complex gives a greater response than the Leu-enkephalin complex, which could allow its selective detection in biological samples.

**Figure 3.** Electropherogram of the des-Tyr Leu-enkephalin copper complex formed on-capillary. Detection potential, +550 mV vs. Ag/AgCl.

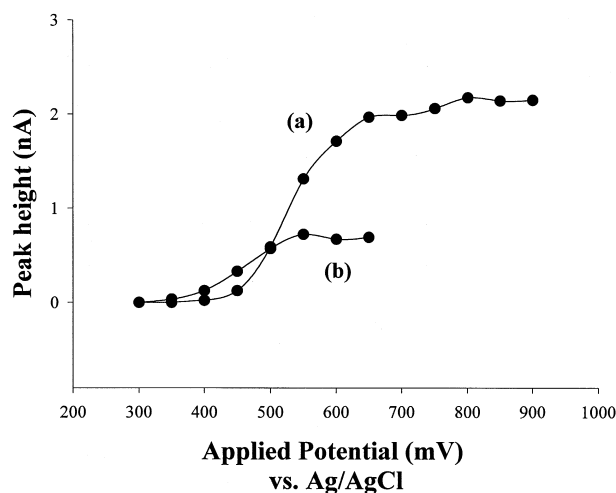


Figure 4. Hydrodynamic voltammograms comparing the response of (a) the Leu-enkephalin complex and (b) the des-Tyr Leu-enkephalin complex.

3.3 Response comparison between peptide-copper complexes

Table 2 compares the responses at +650 mV and the respective E_{\max} values (vs. Ag/AgCl) for several peptide-copper complexes. The peptides represent: linear peptides capable of binding more than one copper (angiotensin I and II), linear peptides capable of binding only one copper (Leu-enkephalin, des Tyr Leu-enkephalin, angiotensin III, angiotensin 1–7), electroactive peptides (angiotensin peptides, Leu-enkephalin, and oxytocin), a non-electroactive peptide (des-Tyr Leu-enkephalin), and a cyclic peptide (oxytocin). The shorter peptide copper complexes containing Tyr provide the greatest electrochemical response at their respective E_{\max} values [17]. Leu-enkephalin, angiotensin III, and angiotensin II copper complexes provide the greatest current response per μM . Not surprisingly, the only nonelectroactive peptide, des-Tyr Leu-enkephalin, gives the lowest signal at both values.

Table 2. Electrochemical response comparison for seven peptide copper complexes

	Response at +650 mV vs. Ag/AgCl (pA/ μM)	Response at E_{\max} vs. Ag/AgCl (pA/ μM)	E_{\max} (mV)	Amino acids
Angiotensin I	9.8	17.5	900	10
Angiotensin II	23.0	34.5	950	8
Angiotensin III	31.1	79.3	950	7
Angiotensin 1–7	14.3	23.1	950	7
Leu-enkephalin	27.8	30.7	800	5

Oxytocin contains a disulfide bridge between Cys¹ and Cys⁶ residues and shows the application of on-capillary complexation for the determination of cyclic peptides by CE-EC. The lack of a terminal amine due to the disulfide bridge actually provides a structure that is ideally suited for metal complexation [26]. The location of the disulfide group results in significantly lower pK_a values for the complexing amino groups compared to regular oligopeptides. Deprotonation occurs readily and the conformation of the binding sites provides a very stable Cu(II) complex [26]. Oxytocin also contains tyrosine, and the expected increase in response is observed upon complexation.

3.4 Separation and detection of Leu-enkephalin and metabolites

Leu-enkephalin is a potent, opioid peptide with many biological functions and has been used as a model substrate to study the activity of plasma peptidases [1–4, 8]. Current methods for monitoring Leu-enkephalin metabolism in plasma employ thin-layer chromatography with radiolabeled peptides. In addition to the inherent safety issues imposed with the use of radioactivity, the protocol is extremely laborious. Any method developed to replace the conventional method of analysis must be able to separate and detect all the possible metabolites of Leu-enkephalin. There are three significant enzyme degradation pathways for Leu-enkephalin in plasma [27]. Ideally, the analytical method would be capable of detecting all possible metabolites in a single, fast run.

Initially, we were concerned that the basic pH required for the biuret reaction, and resulting high EOF, would limit the usefulness of copper complexation for the development of a CE-EC method for the determination of Leu-enkephalin and its metabolites. Surprisingly, we found that addition of copper to the run buffer actually improved the separation of Leu-enkephalin and its metabolites at high pH [20]. A complete separation of Leu-enkephalin and its peptide metabolites using copper complexation and CE-EC in less than 8 min is shown in Fig. 5. Without copper complexation, the nonelectroactive metabolites (PheLeu, GlyPheLeu, and des-Tyr Leu-enkephalin) are not detected.

The difference in oxidation potentials between the peptide complexes can be used for selective detection, as illustrated from the hydrodynamic voltammogram (HDV) of the mixture in Fig. 6. For example, at +500 mV only three peptide-copper complexes, Leu-enkephalin, des-Tyr Leu-enkephalin, and TyrGly, provide a measurable signal. As the detection potential is increased, the other peptide complexes provide a measurable signal. At +750 mV, five of the six peptides have reached their E_{\max} . The only pep-

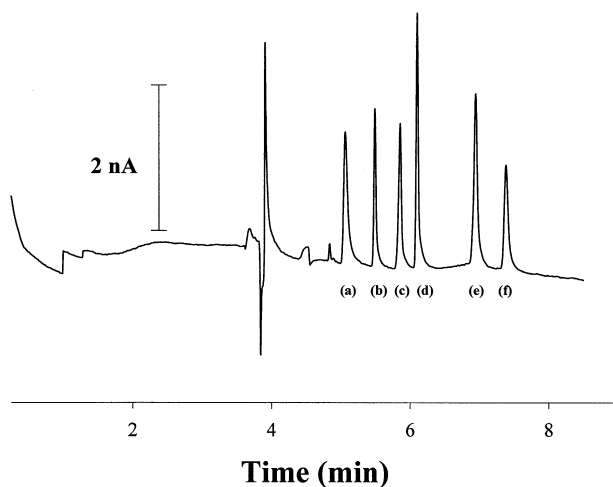


Figure 5. On-capillary complexation and separation of an enkephalin peptide mixture containing: (a) PheLeu, (b) GlyPheLeu, (c) TyrGly, (d) TyrGlyGly, (e) Leu-enkephalin, and (f) des-Tyr Leu-enkephalin. All peptides are at a concentration of 10 μM . Detection potential, +950 mV vs. Ag/AgCl.

ptide not detected at +750 mV is PheLeu. It has been shown that nonelectroactive dipeptides form copper complexes, but their oxidation potentials are considerably higher due to the instability of the Cu(II) complex [11]. Two dipeptides are produced as metabolites of Leu-enkephalin. TyrGly is inherently electroactive and the signal below +900 mV is due to the oxidation of the Tyr. PheLeu does not contain an electroactive moiety. Increasing the detection potential above +900 mV provides a measurable signal for the PheLeu-Cu(II) complex, and all six peptides can be seen, as shown in Fig. 5.

3.5 Leu-enkephalin metabolism in plasma monitored by CE-EC

An analytical method is useful only if it can be applied to complex, real samples such as plasma and other biological fluids. Copper complexation combined with CE and EC is capable of surmounting the problems associated with the conventional methods for monitoring Leu-enkephalin metabolism in plasma. Figure 7 shows overlaid electropherograms taken at different time points. The peak corresponding to Leu-enkephalin disappears over time and two major metabolites appear – the amino acid Tyr and des-Tyr Leu-enkephalin. Peaks corresponding to the other peptides were not seen, indicating that metabolism is predominantly due to the enzyme aminopeptidase N [27].

Results from a separate experiment illustrate the tunable selectivity of EC. An electropherogram obtained at +550 mV after Leu-enkephalin was incubated in plasma

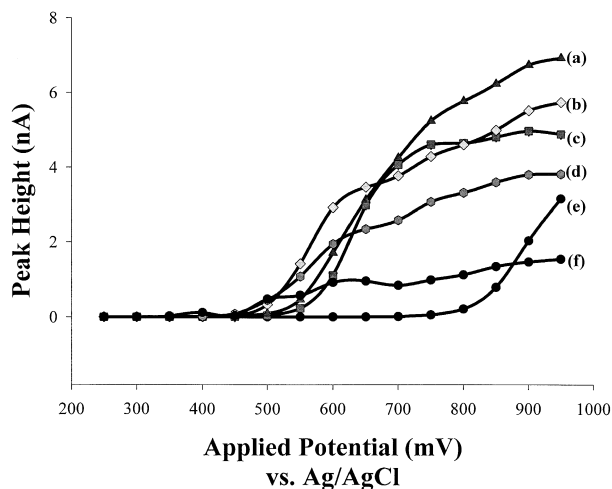


Figure 6. Hydrodynamic voltammograms of the enkephalin copper complex mixture: (a) TyrGlyGly, (b) TyrGly, (c) GlyPheLeu, (d) Leu-enkephalin, (e) PheLeu, and (f) des-Tyr Leu-enkephalin.

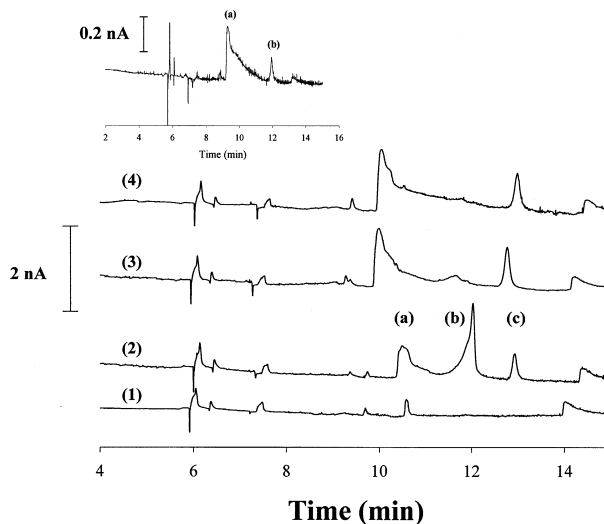


Figure 7. Metabolism of Leu-enkephalin monitored by copper complexation and CE-EC. (1) Plasma blank, (2) $t = 30$ min, (3) $t = 60$ min, (4) $t = 90$ min. Peak identification: a, Tyr; b, Leu-enkephalin; c, des-Tyr Leu-enkephalin. Detection potential, +800 mV vs. Ag/AgCl. Inset electropherogram: detection of des-Tyr Leu-enkephalin at +550 mV vs. Ag/AgCl. Peak identification: a, Tyr; b, des-Tyr Leu-enkephalin. Electropherogram was obtained after Leu-enkephalin was incubated with plasma for 90 min. Prior to injection, the capillary was flushed with 50 mM SDS.

for 90 min is also shown in Fig. 7 (inset). The E_{max} for des-Tyr Leu-enkephalin is +550 mV. Application of a higher detection potential did not result in an increased response for this peak, as would be expected if the peak corresponded to any of the other metabolites. This pro-

vides confirmation that the peak corresponds to des-Tyr Leu-enkephalin. Direct injection of the diluted plasma sample onto the capillary was possible, but the noise increased substantially with time, possibly due to adsorption of plasma proteins on the decoupler near the end of the capillary. Therefore, the capillary was regenerated between runs by flushing with a dilute SDS solution, as previously described [24]. SDS denatures the proteins and reduces their adsorption to the capillary wall. Flushing with SDS did not affect the separation or detection of the peptides.

The successful monitoring of Leu-enkephalin metabolism in plasma illustrates four important points. First, on-capillary complexation can occur even with complex biological samples. Second, direct injection of plasma is possible, although adsorption of proteins to the capillary wall and decoupler may lead to increased noise. Regeneration and cleaning of the capillary was necessary. In this case, the only sample preparation required was dilution of the plasma with water to slow down the enzyme activity. Third, the copper complexation reaction takes place inside the capillary; therefore, there is no dilution of the sample, either from sample preparation or derivatization. Finally, both Tyr and non-Tyr-containing peptides can be detected. Without the ability to detect non-Tyr-containing metabolites that do not normally give a response at a carbon electrode, it would be difficult to determine the degradation pathway of Leu-enkephalin in this example.

4 Concluding remarks

On-capillary copper complexation provides a selective, yet general, method for the determination of peptides using CE-EC. The reaction takes place inside the capillary, eliminating the need for post- or precapillary derivatization. The method is useful for electroactive, nonelectroactive, and cyclic peptides; it could provide a rapid analytical assay to monitor peptide degradation and, hence, enzyme activity in biological matrices. Direct injection of a biological sample was demonstrated, and the metabolism of Leu-enkephalin in plasma was successfully monitored. Future work will focus on applying the technique to larger peptides and peptides without terminal amines. The use of CE with dual electrode detection will provide the additional selectivity needed for the determination of peptides in more complex mixtures.

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