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IMPROVED DETECTION LIMITS IN THE ANALYSIS OF TYROSINE-CONTAINING POLYPEPTIDE HORMONES BY USING ELECTROCHEMICAL DETECTION

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SUMMARY

The pairing of reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ED) represents a potent combination for maximal resolution and sensitivity. These techniques have been utilized for the separation and detection of peptide fragments following their timed incubation in human serum. Using an operating potential of +1.06 V, HPLC–ED has been applied to such tyrosine-containing peptides as leucine enkephalin, luteinizing hormone-releasing hormone, and several $\psi[CH_2S]$ pseudopeptides. Improvements of at least 20-fold or greater over UV detection limits have been realized. Using a diode array UV spectrometer upstream of the electrochemical detector, the parent peptides or their more abundant fragments may be further characterized.

INTRODUCTION

The need for improved resolution and sensitivity in high-performance liquid chromatography (HPLC) has spurred a wide variety of technological improvements in the areas of hardware, columns and detectors. In connection with our studies of peptide hormone degradation, we have turned to electrochemical detection (ED), combined with reversed-phase C_{18} columns, to achieve sensitivity greater than that available with standard UV detection. The utility of this approach is outlined below using several peptide hormones and analogs of leucine enkephalin.

EXPERIMENTAL

Chemicals

HPLC-grade methanol and ammonium acetate were obtained from Fisher Scientific. A 0.25 *M* ammonium acetate buffer solution was prepared in deionized water, adjusted to pH 4.1 with redistilled acetic acid. The buffer was filtered through a Millipore 0.45-μm HA filter and degassed with helium. Peptides used in this study were either a gift from the National Institutes of Health or were prepared in this laboratory by standard procedures of solid phase peptide synthesis[1]. The internal standard used for the ED studies was carbobenzoxy-tyrosine (Cbz-tyrosine).

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Chromatographic conditions

The apparatus used was a DuPont Model 850 gradient liquid chromatography system with a constant temperature column compartment, fitted with a Rheodyne injector. UV detection was accomplished with an LKB photodiode array spectrophotometric Model 2140 detector, interfaced to an IBM personal computer and color monitor. ED was carried out using an amperometric detector with a glassy carbon electrode (Model LC-4, Bioanalytical Systems), set at an oxidation potential of + 1.06 V *vs.* Ag/AgCl reference electrode. Columns were either a DuPont Zorbax ODS analytical (250 × 4.6 mm) column fitted with a 3-cm guard column (packed with 9 μm ODS support) or a Spherisorb C_{18} column (250 × 4.6 mm).

Elution was either isocratic or with step gradients using 35% or 55% methanol in ammonium acetate buffer at a flow-rate of 1.5 ml/min and a column compartment temperature of 50°C, unless otherwise stated.

RESULTS AND DISCUSSION

Peptide hormones are rapidly degraded *in vivo*, usually by preferential enzymatic hydrolysis at one or more highly vulnerable amide bond cleavage sites[2]. If these primary cleavage sites can be readily determined, this knowledge is potentially quite useful for designing more stable, potent, and eventually, orally active peptides. HPLC has proven to be an ideal system for quantifying the rates of degradation (determination of half-lives, $t_{1/2}$, by monitoring the parent peak *vs.* time) and for determining the sites of cleavage (through collection and identification of the peptide fragment peaks). These studies can be performed using purified proteolytic enzymes, blood serum, or tissue homogenates that model the *in vivo* physiological parameters of interest.

It has previously been established that, in the presence of human serum, leucine enkephalin is rapidly degraded into inactive fragments primarily by cleavage of the 1–2 and 3–4 amide bonds[3,4]. We have repeated these studies *in vitro* using both UV detection and ED. The half-life values were 14.7 and 12.5 min, as determined with UV and ED, respectively, with the UV study based on a starting peptide concentration of 0.9 m*M* *vs.* 45 μ*M* for the ED-based experiment. Since these $t_{1/2}$ values are in good agreement, not only does this serve to confirm that the degradation enzymes were not subject to saturation in the UV studies, but it also demonstrates the utility of ED in those instances where the experiment may be hormone-limited.

The operating parameters for ED were established by running a series of hydrodynamic voltammograms (HDV) on the amino acid models, tyrosine and tryptamine, and on the peptides, leucine enkephalin and luteinizing hormone-releasing hormone (LH-RH):

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 LH-RH
1 2 3 4 5 6 7 8 9 10

These HDV studies (Fig. 1) confirmed that ED at 1.06 V should provide a proper balance between sensitivity and signal-to-noise ratio for monitoring the peptides of interest. The literature contains several examples of other peptides for which ED has been applied under similar conditions[5–8].

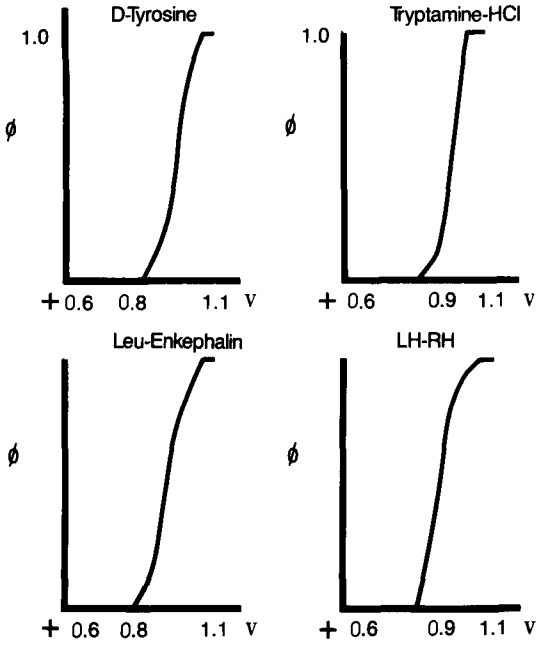


Fig. 1. Hydrodynamic voltammograms of D-tyrosine, tryptamine–HCl, leucine enkephalin, and luteinizing hormone-releasing hormone (LH-RH) with normalized electrochemical detector response (ϕ) plotted *vs.* applied potential in volts.

Using a Zorbax C_{18} reversed-phase column and an isocratic system consisting of 0.25 *M* ammonium acetate (pH 4.1)–methanol (65:35), the degradation process was studied for leucine enkephalin and a series of backbone modified peptide analogues in which each of the amide linkages has in turn been replaced by the thiomethylene ether moiety[9]. A strong ED signal (100–750 nA) was readily obtained using 4.5 nmol (in 100-μl aliquots) of peptide or pseudopeptide, suggesting that upon substantial peptide degradation by proteolytic enzymes, the remaining tyrosine-containing analog or fragment peaks can be readily identified for $t_{1/2}$ determinations.

In a direct comparison of the sensitivity of ED *versus* UV detection, the ED mode would be expected to be far better. Thus, while a 50-μl injection of leucine enkephalin (7.5 nmol) was barely detectable at 254 nm, the same solution gave a readily measurable ED signal (18 nA) even when diluted up to 2000 times (3.7 pmol). Although those ratios would likely change if the peptide structure and the UV wavelength were altered, the greatly increased sensitivity of ED is readily apparent.

Fig. 2 demonstrates an actual degradation study of a relatively stable analogue, [D-Ala^2]-leucine enkephalin, using the ED mode. The $t_{1/2}$ was determined to be 126 min, and both the internal standard (Cbz-tyrosine), and the peptide analogue are easily monitored at 1.06 V using starting concentrations of $1.2 \cdot 10^{-5}$ *M* and $6.2 \cdot 10^{-6}$ *M*, respectively.

A detailed analysis of the variation in biological half-lives and the nature of the peptide fragments for enkephalin pseudopeptide analogues has been reported

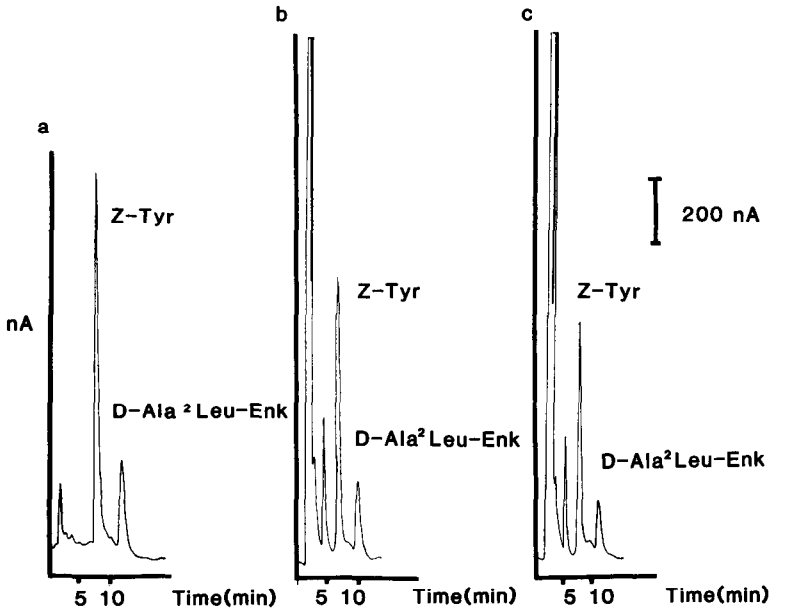


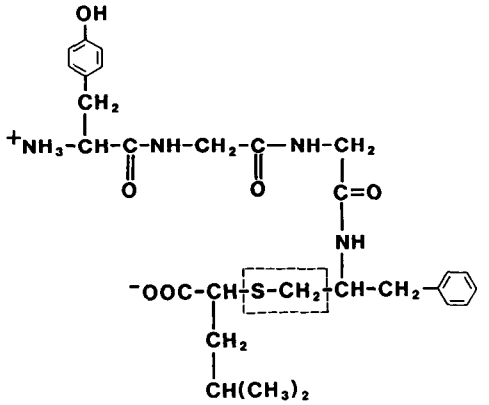
Fig. 2. Human serum degradation study of [D-Ala²]-leucine enkephalin, using ED (1.06 V), monitored at (a) 0, (b) 5, and (c) 20 min. HPLC conditions: methanol–0.25 *M* ammonium acetate buffer (pH 4.1) (35:65); C_{18} reversed-phase column (25 cm) plus 3-cm guard column; 50°C.

elsewhere[10]. Nevertheless, the utility of ED for these studies was so compelling that closer examination of related analytical parameters is appropriate.

One difficulty that has been noted with ED is the attainment of flat baselines when using gradient elution conditions[11]. This is particularly difficult with compounds having widely different hydrophobic properties since isocratic conditions result in abnormally long chromatographic runs. Gradients in reversed-phase HPLC have been successfully employed with ED by maintaining comparable ionic strengths in both organic and buffer mobile phases[12].

Another alternative is to utilize step gradients. This technique proved especially helpful in the $t_{1/2}$ determination of one of our more lipophilic leucine enkephalin pseudopeptides, Tyr-Gly-Gly-Pheψ[CH_2S]Leu (Fig. 3). Normally this analogue is far more strongly retained on our C_{18} reversed-phase columns than other enkephalin derivatives[13]. Fig. 4 demonstrates the chromatogram of our usual internal standard, Cbz-tyrosine ($1.2 \cdot 10^{-5}$ *M*), along with the pseudopeptide ($6.2 \cdot 10^{-5}$ *M*). By stepping from 35% to 55% methanol at 15 min, the more strongly retained analogue now appeared at 28.0 min. The use of the step gradient substantially reduced the analysis time for the degradation of this compound and did not appear to interfere with the quantitation of the results from the serum treatment. With three separate aliquots, the measured peak areas relative to internal standard (at t = 15 min) used to determine the percent degradation gave a calculated sample standard deviation of less than 1.5%.

Another common method used to reduce retention times of lipophilic compounds is to vary temperature. By changing the column compartment temperature



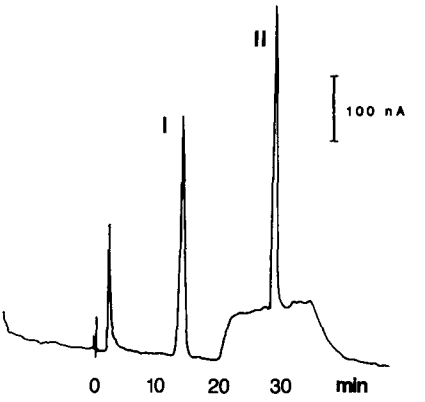


Fig. 3. Structure of Tyr-Gly-Gly-Pheψ[CH_2S]Leu.

Fig. 4. Use of ED with step gradient in reversed-phase HPLC. Separation of carbobenzoxy-tyrosine (I: 12 min) and Tyr-Gly-Gly-Pheψ[CH_2S]Leu (II: 28 min) on a Zorbax C_{18} 250 × 4.6 mm column. Conditions: mobile phase, methanol–0.25 *M* ammonium acetate buffer (pH 4.1) (35:65) for 15 min, with step to methanol–0.25 *M* ammonium acetate (55:45).

from 26°C to 50°C and simultaneously increasing flow-rate from 1.0 to 1.5 ml/min, the retention time for [D-Ala^2]-leucine enkephalin on reversed-phase HPLC was reduced from 25 min to 9 min (Table I). The corresponding retention volumes were 25 ml and 13.5 ml, respectively. Virtually all of the chromatographic determinations cited in this study have been carried out at 50°C in order to decrease analysis time and to reduce peak broadening.

In addition to monitoring the disappearance of the parent peptides, it is important to detect and identify new fragment peaks that appear following incubation with serum or enzyme mixtures. Although the sensitivity of ED provides a convenient method for discerning some minor fragment peaks, it is less ideal in terms of peak characterization or homogeneity assessment. For this, we turned to the use of a diode array spectrometer.

Fig. 5a represents a typical reversed-phase HPLC profile of three peptidic components as visualized in a non-optimized ED mode. The increased sensitivity of ED

TABLE I

THE EFFECT OF TEMPERATURE AND FLOW-RATE ON THE RETENTION TIME OF [D-Ala^2]-LEUCINE ENKEPHALIN

Conditions: Zorbax C_{18} column; ammonium acetate (0.25 *M*) (pH 4.1)–methanol, 1.06 V (*vs.* Ag/AgCl).

Temperature (°C)	*Flow-rate (ml/min)*	*Pressure (psi)*	*Methanol in mobile phase (%)*	*Retention time (min)*
26	1.0	2900	35	34
26	1.0	2900	40	25
50	1.5	2400	35	12
50	1.5	2400	40	9

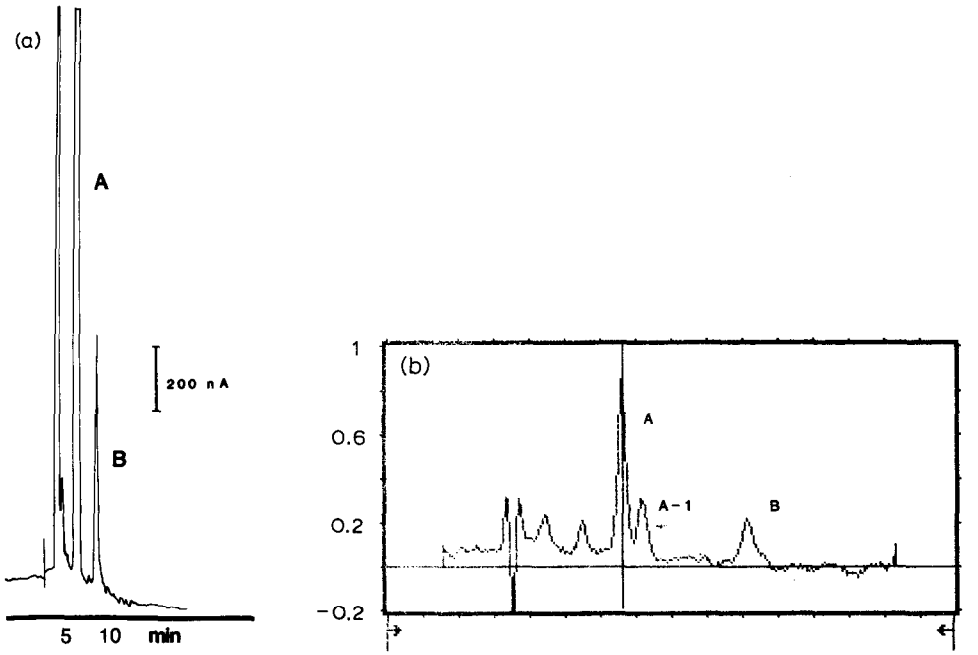


Fig. 5. (a), Reversed-phase HPLC profile of carbobenzoxy-tyrosine, leucine enkephalin, and LH-RH using ED. The first large peak represents the solvent front while peak A is an unresolved mixture of the internal tyrosine standard and enkephalin. Peak B represents LH-RH. The vertical scale sensitivity is 2000 nA. (b), Reversed-phase HPLC profile of carbobenzoxy-tyrosine (A), leucine enkephalin (A-1) and LH-RH (B) as in Fig. 5a using a diode array UV detector (253 nm). Note that the separate components in peak A are readily discernible in the diode array mode without the need to repeat the separation using more favorable ED parameters. Chromatographic conditions: Spherisorb C_{18} column 250 × 4.6 mm I.D.; mobile phase, methanol–0.25 *M* ammonium acetate buffer, pH 4.1 (35:65); column temperature, 50°C. Horizontal (time) scale runs from 1 (left) to 735 (right) sec. Full-scale deflection = 0.015.

results in two of the components, Cbz-tyrosine and leucine enkephalin, appearing as the unresolved off-scale peak A (5.3 min), while LH-RH elutes as peak B at 8.5 min. In contrast, the inhomogeneity of peak A is readily discernable in the UV mode as seen in Fig. 5b.

In summary, ED, coupled with the use of a UV diode array spectrometer, offers a number of advantages for peptide degradation studies. The high sensitivity of ED is complemented by its selectivity, which effectively reduces the number of interfering components which are present in the quenched serum samples. Although not all fragment peaks are ED active (at 1.06 V), the initial degradation of tyrosine-containing peptides should produce one fragment which is ED active. The combination of the diode array spectrometer with the electrochemical detector provides a new approach, reversed-phase HPLC with simultaneous ED and UV detection (RP-HPLC–UV–ED), for carrying out these assays, with an effective pairing of optimal sensitivity and partial characterization.

Although the potential orders of magnitude increase in sensitivity of ED over UV detection may seem incompatible with a series connection, the use of a 95:5 stream splitter should obviate this problem and minimize detector overload. A further

advantage of the stream splitter is that it facilitates subsequent identification (*e.g.*, by amino acid analysis) of degradation fragments collected in the UV mode, while the more destructive ED technique is applied to only 5% of the eluate volume.

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