

Gradient high-performance liquid chromatographic analysis of enkephalin peptides, their metabolites and enzyme inhibitors using combined ultraviolet and electrochemical detection.

I. Optimization of separation and detection

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Abstract

Coulometric electrochemical detection (ED) was compatible with gradient HPLC analysis of enkephalin peptides, their metabolites and several enzyme inhibitors with combined UV detection. The compounds were separated using a reversed-phase octylsilane column with 35 mM phosphate buffer (pH 2.1)–acetonitrile eluent. Hydrodynamic voltammograms were determined for [D-Ala²]-methionine enkephalinamide (MEA) and its metabolites, and the response of MEA consisted of the oxidation of the tyrosine and methioninamide residues with a total transfer of five electrons at the highest potential used. Based on the voltammograms, ED was optimized for the selective determination of MEA and other tyrosine-containing peptides with detection limits between 0.4 and 0.6 pmol per 50 µl injection. In addition, simultaneous UV detection at 205 nm allowed the determination of all the aromatic peptides and enzyme inhibitors with detection limits between 1.5 and 30.0 pmol.

Keywords: Peptides; Enzyme inhibitors; Enkephalin peptides; Methionine enkephalin; Leucine enkephalin; Methionine enkephalinamide; Leucine enkephalinamide

1. Introduction

In recent years, there has been growing interest in using peptides as therapeutic drugs [1]. Methionine- and leucine-enkephalin are two naturally occurring opiate-like peptides that might be useful as analgesic agents [2]. However, orally and topically given enkephalins are poorly absorbed due to relatively large size and low lipophilicity of these peptides and due to enzymatic hydrolysis at the absorption site

(Fig. 1) [3,4]. Enkephalin analogs with improved metabolic stability have been developed, e.g., [D-Ala²]-methionine enkephalinamide (MEA) (Fig. 2), but they are still susceptible to metabolism [3,4]. The absorption of enkephalin peptides or other peptides might be improved with simultaneous application of suitable enzyme inhibitors [4], e.g., aminopeptidase inhibitor bestatin, enkephalinase inhibitors thiorphan and SCH 39370, and angiotensin converting enzyme inhibitor captopril (Fig. 2). Since these inhibitors may have pharmacologic effects by themselves [5], it is important to determine their absorption as well.

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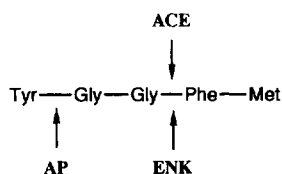


Fig. 1. Enzymatic degradation of methionine enkephalin by aminopeptidases (AP), enkephalinase (ENK), and angiotensin converting enzyme (ACE).

Excellent analytical methods are needed in these pharmacokinetic studies, since samples may contain the intact peptide, its metabolites, and enzyme inhibitors in a biological matrix, and the concentration of these compounds is often low.

Enkephalin peptides and their metabolites have been analysed simultaneously using reversed-phase HPLC with gradient elution and UV detection at 214 or 215 nm [3,6,7]. However, this method is not suitable for trace analysis, since the detection limits of enkephalins have ranged from 10–100 pmol per injection. Pre-column derivatization with fluorescence detection has offered detection limits between 0.1 and 1.0 pmol for enkephalins and their tyrosine-containing metabolites [8,9]. Similar detection limits have been obtained using liquid chromatography with electrochemical detection (LC–ED) [10–13].

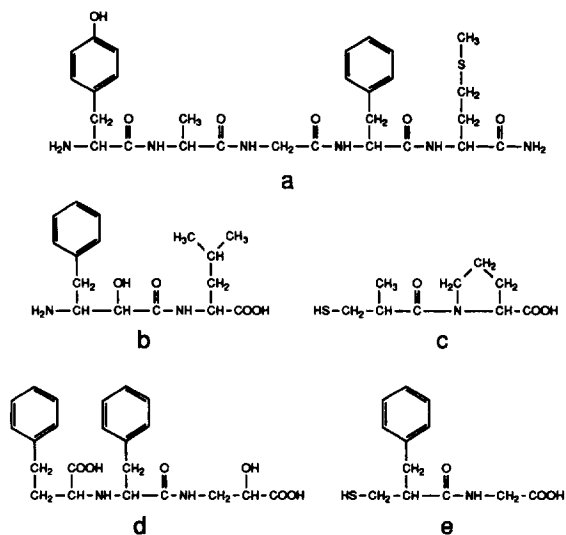


Fig. 2. Structures of (a) [D-Ala³]-methionine enkephalinamide, and enzyme inhibitors (b) bestatin, (c) captopril, (d) SCH 39370 and (e) thiorphan.

When using LC–ED, derivatization is not needed, since the phenolic group of the tyrosine residue is electroactive and can be oxidized using carbon electrodes. However, in the metabolic studies of enkephalins, two isocratic LC–ED methods have been used, one for the parent peptide and its largest metabolites, and the other for the smallest metabolites [10,11]. Therefore a gradient LC–ED method for the simultaneous analysis of enkephalins and their tyrosine-containing metabolites would be advantageous, if the inherent sensitivity of ED detection could be preserved.

Gradient LC–ED is seldom used with carbon electrodes, since baseline drift is often expected to be large and make the method impractical. However, a stable baseline can be obtained, and excellent methods have been developed for several compounds, e.g., for *o*-phthalaldehyde derivatives of amino acids with low oxidation potentials [14], and *Alternaria* mycotoxins with quite high oxidation potentials [15]. Some gradient LC–ED methods have also been developed for peptides. Gradient LC–ED with carbon [16] or mercury–gold amalgam [17,18] electrodes has been used as a qualitative tool in peptide mapping. Recently, two gradient LC–ED methods were developed for the quantitative determination of peptides. In one of these studies, tyrosine-, tryptophan- and sulfur-containing peptides were determined using pulsed amperometric detection with a platinum electrode, but this method was not particularly sensitive [19]. In another study, a large number of peptides with and without electroactive residues were analysed using post-column derivatization with a copper solution and an ED detector with dual carbon electrodes [20,21]. Detection limits between 6 and 100 fmol were obtained with a microbore column, but the method is quite complicated for routine measurements.

In this study, a gradient LC–ED method was developed for enkephalin peptides and their tyrosine-containing metabolites using dual high-efficiency (coulometric) graphite electrodes without any derivatization. The chromatographic behaviour of the compounds was initially studied using isocratic elution, and a gradient method was developed for MEA, its metabolites and enzyme inhibitors bestatin and SCH 39370. Hydrodynamic voltammograms were determined for MEA and its metabolites using

the gradient method, and the number of electrons released from each compound was calculated. Based on the voltammograms, ED detection was optimized for the selective determination of MEA and other tyrosine-containing peptides. In addition, UV detection was combined with ED detection to allow the determination of all the aromatic peptides and enzyme inhibitors many of which are not electroactive. The effect of on-line pre-electrolysis of the mobile phase on ED and UV baseline drift during gradient elution was also studied. This study demonstrates that gradient HPLC with combined UV and ED detection is an excellent method for simultaneous determination of enkephalin peptides, their metabolites and several enzyme inhibitors.

2. Experimental

2.1. Chemicals

Amino acids and peptides were purchased from Sigma (St. Louis, MO, USA) with following exceptions. Tyr-D-Ala was obtained from Haartman Institute (University of Helsinki, Helsinki, Finland), and Tyr-D-Ala-Gly-Phe from Bachem (Bubendorf, Switzerland). Enzyme inhibitors bestatin and thiorphan were from Sigma. Captopril was kindly donated by Bristol-Myers Squibb (Princeton, NJ, USA), and SCH 39370 (N-[N-[1-(S)-carboxyl-3-phenylpropyl]-(S)-phenylalanyl]-(S)-isoserine) [22] by Schering-Plough (Kenilworth, NJ, USA). Hydroquinone was from Sigma.

Acetonitrile (HPLC Super grade) was purchased from Rathburn (Walkenburn, Scotland). Phosphoric acid 85% (H₃PO₄) and sodium dihydrogen phosphate dihydrate (NaH₂PO₄) (both of analytical grade) were from Merck (Darmstadt, Germany). Other chemicals were of analytical grade. Water was distilled and further purified with Millipore-Q UF Plus apparatus (Molsheim, France).

2.2. Preparation of standard solutions

Stock solutions of the amino acids, peptides, and enzyme inhibitors (0.25–1.0 mM) were prepared in water. SCH 39370 did not dissolve until the pH of the solution was adjusted to 6.6 with 0.1 M NaOH.

Working solutions were prepared in glutathione bicarbonated Ringer's (GBR) solution [23], since the HPLC method was developed for ocular penetration studies *in vitro*, where GBR solution is used as a medium. GBR solution contains inorganic salts, glucose (5 mM), and oxidized glutathione (0.15 mM), and the pH of the solution is 8.0–8.1. Both the stock and the working solutions were divided into aliquots, and stored frozen (–20°C) until used. However, working solutions of thiol-containing captopril and thiorphan were freshly prepared in water to avoid the oxidation of these compounds.

2.3. Equipment

HPLC was performed with two Beckman 114M pumps connected to a high-pressure mixing chamber, and a Beckman 420 system controller (Berkeley, CA, USA). Stainless steel reservoir filters were replaced with polyethylene filters (Upchurch, Oak Harbor, WA, USA) to avoid the leaching of electroactive metal ions, and standard PTFE tubes of the pumps were replaced with FEP tubes (DuPont, Wilmington, DE, USA) with a much lower oxygen permeability. A Scientific Systems LP-21 LO-Pulse pulse damper (State College, PA, USA) was connected after the mixing chamber. Samples were introduced with a Spectra-Physics 8775 autosampler (San Jose, CA, USA) with a 50- μ l sample loop. UV detection was performed with Waters 486 UV detector (Milford, MA, USA). The accuracy of the mobile phase mixing was determined without a column using the method described by Beinert [24]. At the same time, the dwell volume between the mixing chamber and column inlet was determined to be 1.5 ml by measuring the time between the programmed step gradient and the first deflection in the UV chromatogram at the flow-rate of 1 ml min⁻¹.

An ESA Coulochem 5100A electrochemical detector (Bedford, MA, USA) was used with an ESA 5014 flow cell. The flow cell contains two flow-through working electrodes (E1 and E2) made of porous graphite (E1: 4–5 cm²; E2: 1 cm²; total cell volume: 4 μ l). The ESA 5014 flow cell was connected after the UV detector and induced 20 bar back-pressure at 1 ml min⁻¹. In addition, an ESA Model 5020 guard cell was connected between the

pulse damper and the autosampler to pre-electrolyze the mobile phase. Both cells were equipped with ESA graphite in-line filters (0.2 μm). The ESA cells contain a solid-state palladium reference electrode and the operating potentials are typically 0.3 V lower than with a Ag/AgCl reference electrode.

Signals from the detectors were collected at 1 Hz by BORWIN Chromatography Software (Le Fontanil, France). The data system was calibrated by applying constant potential pulses from the check and diagnostic functions of the ED and UV detectors, and the amplitudes of these pulses were checked with a voltmeter.

2.4. Chromatographic conditions

Reversed-phase HPLC was performed mainly on a single Kromasil C₈ (5 μm , 100 Å, 150×4.6 mm) column (Eka Nobel, Bohus, Sweden). A similar column packed by Higgins Analytical (Mountain View, CA, USA) was used in few experiments and is referred in the text as another Kromasil C₈ column. Solution A was 35 mM phosphate buffer (pH 2.1), and solution B was 59 mM phosphate buffer (pH 2.1)–acetonitrile (60:40, v/v). Solution A was prepared by mixing 35 mM H₃PO₄ and 35 mM NaH₂PO₄ solutions until pH 2.1 was obtained. The phosphate buffer in solution B was prepared in the similar manner using 59 mM solutions, and finally acetonitrile (ACN) was added (final concentration of phosphate buffer is 35 mM). In the isocratic elution of most polar compounds, solution B was 35 mM phosphate buffer (pH 2.1)–ACN (93:7, v/v). Solutions A and B were filtered (Durapore 0.22 μm , Millipore, Bedford, MA, USA), and degassed by sparging with helium. Separations were performed at ambient temperature at the flow-rate of 1.0 ml min⁻¹.

In the gradient elution of MEA, its metabolites, and enzyme inhibitors bestatin and SCH 39370, the ACN content of the mobile phase was increased linearly from 4 to 5% during the first 9 min, then to 15% during 5 min, and finally to 30% during 21 min. After the 35-min run, the ACN content was returned to 4% during 3 min, and the column was re-equilibrated for 17 min before the next injection.

After optimization, UV detector was set to 205 nm (filter time 0.5 s). The first (screen) electrode of the

ESA 5014 flow cell was set to 0.25 V, the second (analytical) electrode to 0.60 V (response time 2 s), and the ESA 5020 guard cell to 0.62 V. The electrodes were equilibrated for 24 h before the analysis.

In isocratic studies, the retention time of unrestrained compound (t_0) was determined by injecting GBR solution and measuring the retention time of the first peak in the UV chromatogram. Number of plates (N) was determined using the peak width at half height, and peak asymmetry factor (A_s) was measured at 10% of the peak height. Both parameters were determined using UV detection.

2.5. Hydrodynamic voltammograms and efficiency of the analytical electrode

Hydrodynamic voltammograms of MEA, its metabolites, and enzyme inhibitors bestatin and SCH 39370 were determined by measuring their responses at 13 different potentials between 0.30 V and 0.75 V. Gradient elution was used, and measurements were started from the highest potential. The first electrode was kept at 0.25 V where none of the compounds was oxidized, whereas the guard cell was set to a 0.05 V higher potential than the analytical electrode. The following samples were analysed at each potential: blank gradient, GBR solution, 2 μM (100 pmol/50 μl) and 40 nM (2 pmol/50 μl) mixtures of the compounds in GBR solution, both analyte containing mixtures as duplicates.

The number of electrons released from the compounds was determined by comparing their responses (peak areas) to the response of hydroquinone that is known to donate two electrons [25]. Hydroquinone was analysed at 0.60 V where maximum response was obtained. The first electrode was set to -0.2 V to avoid the oxidation of hydroquinone at this electrode. The retention time of hydroquinone was 5.5 min with phosphate buffer–ACN (96:4, v/v). Two separate hydroquinone solutions (2 μM , 100 pmol/50 μl) were prepared in the mobile phase and analysed as duplicates.

The electrolytic efficiency of the analytical electrode was determined by reversing the flow direction through the ESA 5014 cell, setting both electrodes to 0.60 V, and injecting several samples of 2 μM hydroquinone solution. The efficiency was 100% at

this analyte concentration, since hydroquinone was completely oxidized at the analytical electrode (the up-stream electrode in this experiment).

2.6. UV spectrum of the mobile phase

The UV spectrum of 35 mM phosphate buffer (pH 2.1)–ACN (96:4, v/v) eluent was measured with and without the guard cell using a Merck-Hitachi L-4500 diode array detector (Tokyo, Japan) that was connected either to the pulse damper or to the guard cell outlet tube. Flow-rate was 1.0 ml min⁻¹.

3. Results

3.1. Chromatography

3.1.1. Isocratic elution

The chromatographic behaviour of enkephalin peptides, their metabolites, and enzyme inhibitors was initially studied by using isocratic elution with different phosphate buffer (pH 2.1)–ACN compositions. The most polar metabolites of enkephalin peptides, namely amino acids and Tyr-containing di- and tripeptides, were eluted from the column with less than 6% of ACN in the mobile phase (Fig. 3). A small amount of ACN in the eluent was necessary to maintain the resolving power of the column. The amino-terminal metabolites of MEA and ME with a D-Ala residue, Tyr-D-Ala and Tyr-D-Ala-Gly, were retained more strongly than the corresponding metabolites of native enkephalins, Tyr-Gly and Tyr-Gly-Gly. This difference was not solely due to the greater lipophilicity of the Ala residue compared to Gly residue, since Tyr-L-Ala, a diastereomer of Tyr-D-Ala, was eluted slightly before Tyr-Gly (data not shown) suggesting that the change in stereochemical configuration was also an important factor.

Amino acids Tyr and Phe differed from the Tyr-containing di- and tripeptides in the respect that their retention factors decreased more slowly when increasing the concentration of ACN (Fig. 3). On the other hand, Met and its amide Met-NH₂ were only weakly retained with *k* values of 1.8 and 1.2 at 1% of ACN, respectively. Oxidized glutathione, a component in GBR solution, was eluted well before

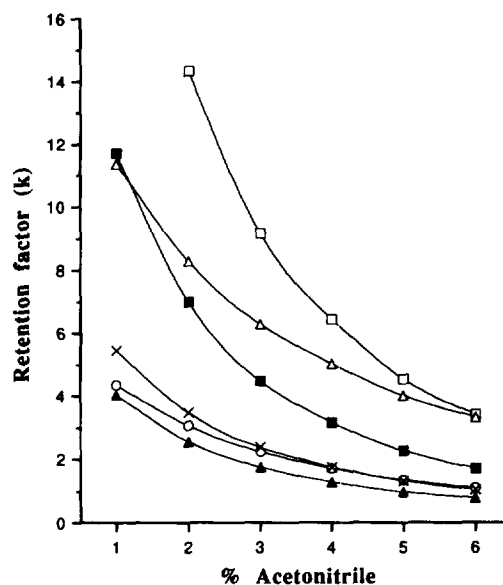


Fig. 3. Effect of acetonitrile concentration on retention of enkephalin metabolites: ○=Tyr; ×=Tyr-Gly; ▲=Tyr-Gly-Gly; □=Tyr-D-Ala; ■=Tyr-D-Ala-Gly; △=Phe. Column: Kromasil C₈ (150×4.6 mm). Mobile phase: 35 mM phosphate buffer (pH 2.1)–acetonitrile. Flow-rate: 1.0 ml min⁻¹.

Tyr-containing compounds with *k* values of 3.3 and 0.8 at 1% and 4% of ACN, respectively.

Slight changes in the structure of MEK had significant effects on the retention (Fig. 4). ME with D-Ala residue instead of Gly residue in position 2 was retained more strongly than MEK as expected from the behaviour of their Tyr-containing metabolites. On the other hand, MEA with D-Ala residue and carboxy-terminal amide group had shorter retention times than MEK. LEK contains a lipophilic Leu residue instead of Met residue, and consequently LEK was retained more strongly than MEK. In addition, LEA with D-Ala residue and carboxy-terminal amide group was eluted before LEK. It appears that amide group in the carboxy-terminal decreases the retention time of the peptide. The retention factors of all these peptides decreased rapidly when increasing the concentration of ACN which is typical for peptides.

Fig. 5 shows that the chromatographic behaviour of enzyme inhibitors differed from that of MEA and its largest metabolites. Retention times of the inhibitors decreased more slowly when increasing the

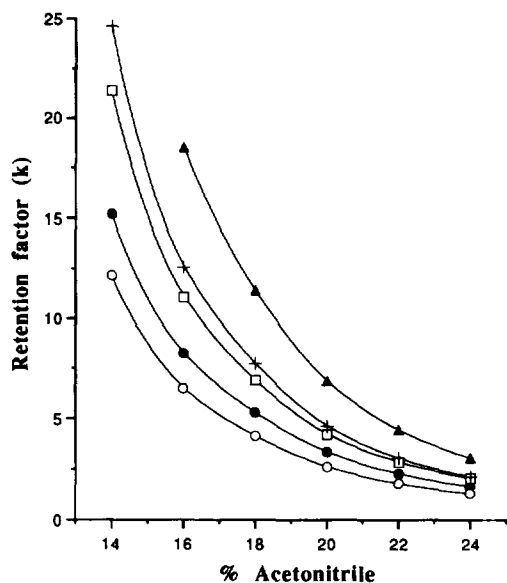


Fig. 4. Effect of acetonitrile concentration on retention of enkephalin peptides: ●=MEK; □=ME; ○=MEA; ▲=LEK; +=LEA. Conditions as in Fig. 3.

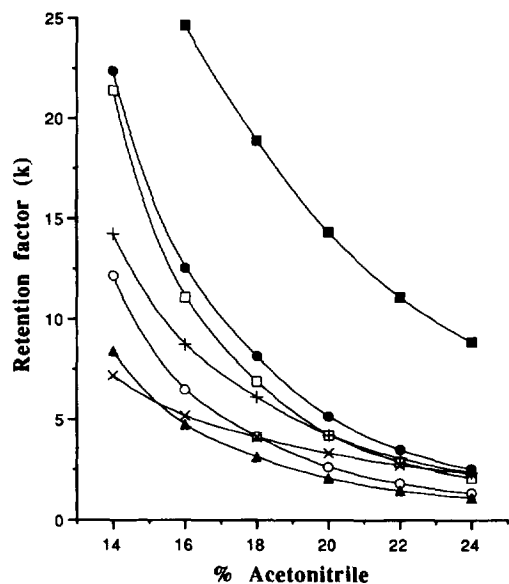


Fig. 5. Effect of acetonitrile concentration on retention of MEA, its metabolites and enzyme inhibitors: ○=MEA; □=ME; ▲=Tyr-D-Ala-Gly-Phe; ●=bestatin; x=captopril; +=SCH 39370; ■=thiorphan. Conditions as in Fig. 3.

concentration of ACN. The retention order of captopril, Tyr-D-Ala-Gly-Phe and MEA changed when the composition of mobile phase was varied, as did the retention order of SCH 39370 and ME.

The compounds were eluted as sharp, symmetric peaks with the exception of captopril that appeared as a broad, fronting peak. The N and A_s values of 2.5 nmol of captopril (k 4.1) were 670 and 0.49, respectively. The anomalous behaviour of captopril has been noticed earlier and has been attributed to the hindered rotation of the peptide bond [26,27]. The peak shape of other compounds was very good, e.g., the N and A_s values of 0.1 nmol of tyrosine (k 4.4) were 7700 and 1.11, and the same parameters of 0.1 nmol of MEA (k 4.2) were 7400 and 1.16, respectively.

3.1.2. Gradient elution

Based on the isocratic studies, a gradient method was developed for MEA, its metabolites and enzyme inhibitors bestatin and SCH 39370 (Fig. 6). These enzyme inhibitors were chosen, since they were chemically stable in GBR solution [28]. In the beginning of the elution, the ACN content was slowly increased from 4% to 5% to separate the most polar metabolites. After the elution of Tyr-D-Ala, the ACN content was increased rapidly to 15%. Thereafter ACN content was increased more slowly, since in isocratic studies MEA, its largest metabolites, and the enzyme inhibitors were best separated with ACN content between 15% and 19%. The concentration of ACN was increased up to 30% to elute possible strongly retaining impurities. All the compounds were completely separated with this method. An identical separation was obtained with another Kromasil C_8 column with slightly longer retention times.

Other peptides and enzyme inhibitors that were used in isocratic studies were also eluted using the same gradient method and most of the compounds were separated from each other with thiorphan as the last compound with t_R of 33.7 min. However, the method needs to be changed for certain compounds using the data from the isocratic studies. For example, to analyse native enkephalins and their metabolites, the ACN content of the mobile phase could be kept at 2% for first 7 min to separate Tyr-Gly-Gly, Tyr, and Tyr-Gly with retention times of 4.9, 5.6

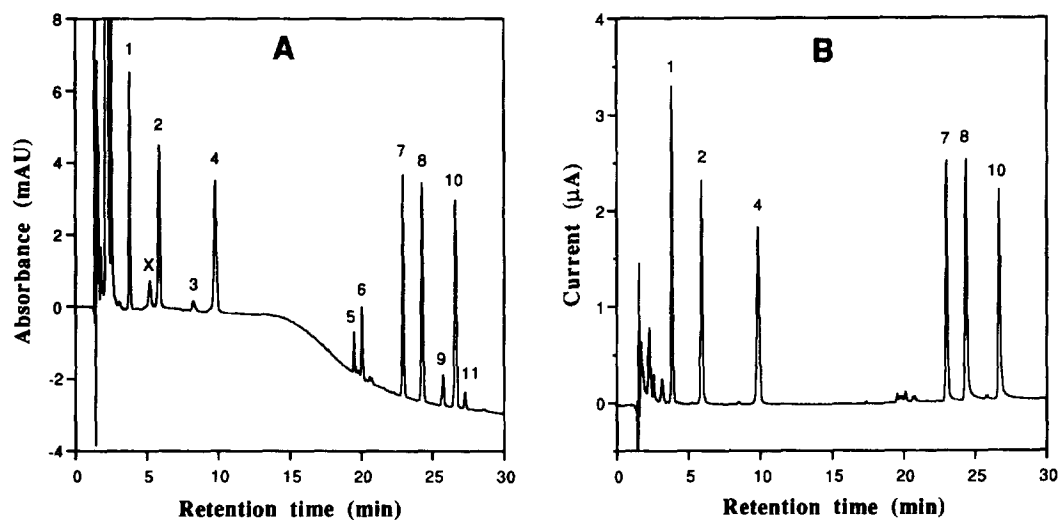


Fig. 6. Gradient elution of a mixture of MEA, its metabolites, and enzyme inhibitors ($2 \mu\text{M}$, 100 pmol of each) in GBR solution. (A) UV detection at 205 nm, (B) ED detection at 0.60 V. Peaks: 1=Tyr; 2=Tyr-D-Ala-Gly; 3=Phe; 4=Tyr-D-Ala; 5=Phe-Met; 6=D-Ala-Gly-Phe-Met-NH₂; 7=Tyr-D-Ala-Gly-Phe; 8=MEA; 9=SCH 39370; 10=ME; 11=bestatin; X=impurity in GBR solution. Column: Kromasil C₈ (150×4.6 mm). Mobile phase: Solution A is 35 mM phosphate buffer (pH 2.1), and solution B is 59 mM phosphate buffer (pH 2.1)-acetonitrile (60:40, v/v). Acetonitrile concentration was increased from 4 to 5% in 9 min, then to 15% in 5 min and finally to 30% in 21 min. Flow-rate: 1.0 ml min⁻¹. Guard cell potential: 0.62 V.

and 6.1 min, respectively (see Fig. 3). Further, the separation of MEK (t_{R} 25.4 min) and SCH 39370 (t_{R} 25.8 min) was improved by increasing the ACN content more rapidly than in the original method, since in isocratic studies MEK was eluted before SCH 39370 only when the ACN content was over 16%. When the original method was modified so that at 9 min after injection the ACN content was increased from 5% to 18% during 2 min and thereafter at the rate of 1% min⁻¹, MEK and SCH 39370 were eluted with retention times of 19.4 and 20.0 min, respectively. The peaks were narrower than with the original method and they were completely separated. Similar improvement was obtained with another Kromasil C₈ column.

3.2. UV detection

The wavelength of UV detection for the gradient HPLC analysis of MEA, its metabolites and enzyme inhibitors bestatin and SCH 39370 was selected between 205 nm and 214 nm. The peaks at 205 nm were 2.4–6.2 times higher than at 214 nm with largest improvements for those compounds that do not contain a phenolic group and are therefore not

well suited for ED detection. On the other hand, baseline noise and baseline drift at 205 nm were only 1.1 and 1.7 times higher than at 214 nm, respectively. These results were obtained without the guard cell. When the guard cell was used, the background absorbance increased and the baseline drift changed from upward drift to downward drift (see Section 3.4). With the guard cell, the baseline noise and drift were equal at 205 and 214 nm. Since 205 nm offered better detection limits with and without the guard cell, it was chosen for the analysis.

Fig. 6A shows that the Tyr-containing peptides gave higher responses than other compounds. The detection limits (S/N 3) of the compounds in Fig. 6A ranged from 1.5 pmol of MEA to 30.0 pmol of Phe per 50 µl injection (see Fig. 8 for the response of 2 pmol of MEA). The detection limit of captopril (t_{R} 22.6 min) was 100–200 pmol due to its poor peak shape, whereas the detection limit of thiorphan (t_{R} 33.7 min) was 20 pmol. UV detection was very reproducible. For example, the R.S.D. of peak height of 10 pmol of MEA within-day and within 3-day batch were 1.28% and 2.87% ($n=6$), and the corresponding values of 15 pmol of Tyr were 1.48% and 2.88% ($n=6$), respectively. The retention times of

the compounds remained within 0.3 min range during the 3-day period.

3.3. ED detection

3.3.1. Hydrodynamic voltammograms

The hydrodynamic voltammograms of MEA, its metabolites and SCH 39370 are shown in Fig. 7 according to peak area (charge). The peak areas are useful in the comparison of electroactivity, since they are not affected by the band broadening that occurs during chromatographic elution [21]. Fig. 7 shows that Tyr was oxidized at lowest potential and reached a plateau at 0.53 V. Tyr-D-Ala-Gly and Tyr-D-Ala-Gly-Phe were oxidized at 50 mV higher potential than Tyr, but their plateau level was slightly higher. On the other hand, the responses of MEA and ME increased up to 0.75 V that was the highest potential used. The increase of their response above 0.55 V was due to the oxidation of carboxy-terminal part of the molecule as seen from the

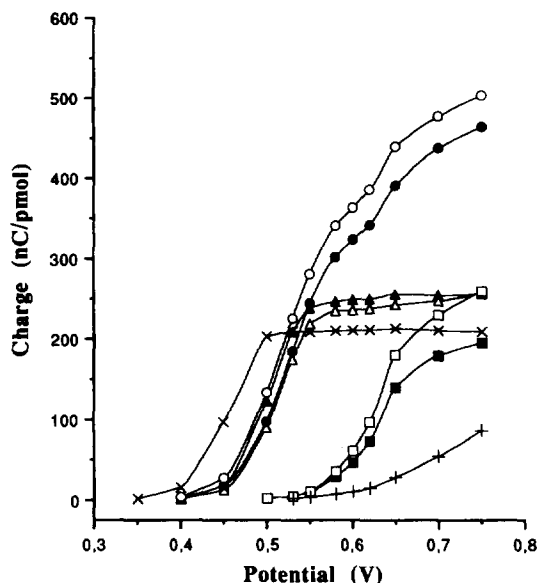


Fig. 7. Hydrodynamic voltammograms of MEA, its metabolites, and enzyme inhibitor SCH 39370 according to charge (peak area): x = Tyr; ▲ = Tyr-D-Ala-Gly; △ = Tyr-D-Ala-Gly-Phe; ● = ME; ○ = MEA; ■ = Phe-Met; □ = D-Ala-Gly-Phe-Met-NH₂; + = SCH 39370. Mixture of 100 pmol of each compound in GBR solution was analysed at each potential as a duplicate using the same gradient method as in Fig. 6.

voltammograms of D-Ala-Gly-Phe-Met-NH₂ and Phe-Met. In fact, the sum of the responses of Tyr-D-Ala-Gly and Phe-Met is very close to the response of ME that contains these sequences.

The response of D-Ala-Gly-Phe-Met-NH₂ and Phe-Met was thought to be due to the oxidation of the carboxy-terminal Met-NH₂ and Met residues that contain an electroactive sulfur atom. This was verified by determining the responses of Met-NH₂ and Met (dissolved in phosphate buffer) at 0.75 V. The peak area of Met-NH₂ was equal to D-Ala-Gly-Phe-Met-NH₂, whereas the peak area of Met was close to that of Phe-Met.

Enzyme inhibitor SCH 39370 started to oxidize at 0.55 V and gave only a weak response. However, its response was still increasing at 0.75 V. On the other hand, bestatin and Phe were not oxidized even at 0.75 V.

The number of electrons transferred in the oxidation reaction was determined by comparing the peak areas of the compounds (Fig. 7) with the peak area of hydroquinone that is known to donate two electrons [25]. Since the electrolytic efficiency of the analytical electrode was 100%, the theoretical response of hydroquinone is 193.0 nC pmol⁻¹ (2 × 96.5 nC pmol⁻¹) [21]. The measured response of hydroquinone was 202.5 ± 4.2 nC pmol⁻¹ (n = 4). This small difference may be due to the fact that the injection loop is slightly larger than the nominal value.

Since the maximum response of Tyr was nearly equal to hydroquinone, each Tyr molecule donated 2 electrons. In the oxidation of Tyr-D-Ala-Gly and Tyr-D-Ala-Gly-Phe, an average of 2.5 electrons were transferred at 0.75 V. Sulfur-containing Phe-Met and D-Ala-Gly-Phe-Met-NH₂ donated 2.0–2.5 electrons at the highest potential, whereas ME and MEA with two electroactive residues donated 4.5–5.0 electrons. On the other hand, only 1 electron (average 0.9) was released from SCH 39370, but its response was still increasing at 0.75 V.

3.3.2. Selection of the operating potential

The major criteria in the selection of operating potential was the suitability of the gradient LC-ED method for trace analysis of MEA and its Tyr-containing metabolites. This was studied by analysing a mixture of 2 pmol of each compound in GBR

solution at different potentials. The responses of the compounds closely followed the hydrodynamic voltammograms in Fig. 7. However, the responses of oxidized glutathione and its impurities in GBR solution started to increase rapidly above 0.60 V and interfered with the determination of Tyr and Tyr-D-Ala-Gly. Thus, the selectivity of the method decreased significantly above 0.60 V.

In addition, the background current and baseline drift started to increase markedly above 0.60 V. The background current at the initial conditions of the gradient elution (4% of ACN) was about 250 nA at 0.60 V, but much higher, about 520 nA and 1.9 μ A at 0.65 and 0.75 V, respectively. The magnitude of background current affected the stability of baseline during gradient elution. The baseline drifted downward at 0.60 V, but the magnitude of the drift was only -10 nA during the 35-min run. The negative baseline drift was much larger at higher potentials, about -50 and -380 nA at 0.65 and 0.75 V, respectively. On the other hand, the baseline noise was practically independent of potential.

Another important criteria in the selection of operating potential is the stability of the responses. The hydrodynamic voltammograms of MEA and ME have no plateau, but between 0.58 and 0.62 V their responses change less than at slightly higher or lower potentials (Fig. 7). In addition, Tyr, Tyr-D-Ala-Gly, and Tyr-D-Ala-Gly-Phe have stable responses near 0.60 V. Since 0.60 V also offered a good selectivity and a stable baseline, it was chosen as the operating potential of the analytical (second) electrode. The potential of the first electrode was set to 0.25 V, since Tyr started to oxidize on this electrode at 0.30 V. The components of GBR solution produced large peaks at the first electrode between 1.7 and 2.6 min after injection.

ED detection was more selective for Tyr-containing peptides than UV detection and suffered less from the interference of the GBR solution (Fig. 6). The larger peptides showed some peak tailing and therefore peak heights were used for quantitative measurements. Fig. 8 shows that the gradient LC-ED method can be used in trace analysis. The detection limits (S/N 3) of Tyr and Tyr-containing peptides were 0.4–0.6 pmol. The responses were stable even at picomole level. The R.S.D. of peak height of 1.0 pmol of MEA within-day and within

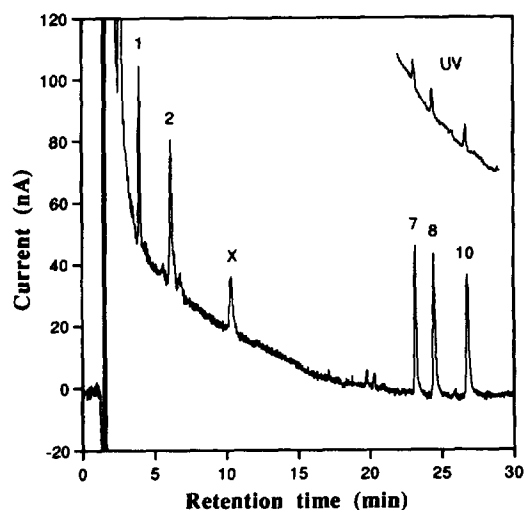


Fig. 8. Gradient HPLC with ED detection of a mixture of MEA and its Tyr-containing metabolites (40 nM, 2 pmol of each) in GBR solution. Potential 0.60 V. Peaks: 1=Tyr; 2=Tyr-D-Ala-Gly; 7=Tyr-D-Ala-Gly-Phe; 8=MEA; 10=ME; X=impurity in GBR solution. UV detection of peaks 7, 8, and 10 at 205 nm is shown in the upper right corner. Chromatographic conditions as in Fig. 6.

3-day batch were 2.68% and 15.9% ($n=6$), and the corresponding values of 1.5 pmol of Tyr were 2.01% and 5.74% ($n=6$), respectively. The method has been applied successfully in the ocular penetration studies of MEA *in vitro* [28].

3.3.3. ED responses of other compounds

The responses of other Tyr-containing peptides were determined at 0.60 V. The peak area of MEK was close to that of ME, whereas the responses of Tyr-Gly, Tyr-Gly-Gly, Tyr-D-Ala, LEK, and LEA were within 10% of the response of Tyr-D-Ala-Gly. Thiol-containing enzyme inhibitors captopril and thiorphan produced a low response at 0.60 V, but their response increased by 5 times when the potential was set to 0.75 V. The response of captopril was 6 and 28 nC pmol^{-1} at 0.60 and 0.75 V, whereas the corresponding responses of thiorphan were 20 and 101 nC pmol^{-1} , respectively. Thus, the potential of 0.60 V is suitable for the detection of other Tyr-containing peptides, but too low for captopril and thiorphan.

3.4. Effect of guard cell on ED and UV baseline drift

The guard cell was connected between the pumps and the autosampler and its function is to purify the mobile phase by oxidizing impurities that would otherwise produce background current and noise at the analytical electrode. The guard cell electrode is usually set to a slightly higher potential than the analytical electrode.

The effect of the guard cell on ED baseline drift was studied with the first electrode at 0.25 V and the analytical electrode at 0.60 V by measuring several blank gradients with the guard cell at 0.62 V and without the guard cell. Fig. 9A presents an example of these measurements. The baseline was more stable with the guard cell, although the background current was higher indicating that an electroactive compound was produced in the guard cell. On the other hand, the guard cell also removed electroactive impurities from the mobile phase, since the background current of the first electrode at 0.25 V was lower with the guard cell than without it (typically 15 nA vs. 80 nA

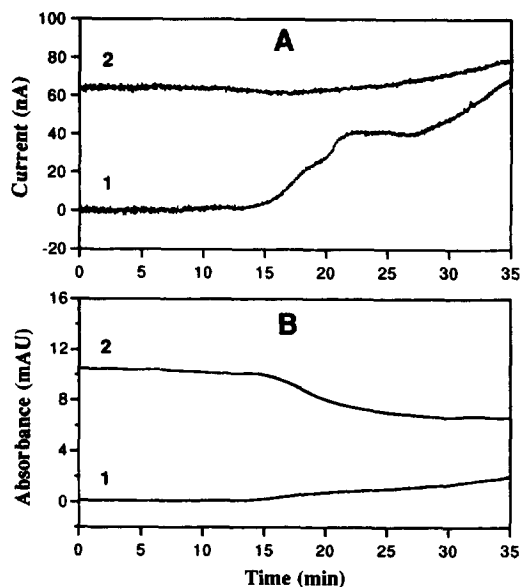


Fig. 9. Effect of guard cell on (A) ED baseline of analytical electrode at 0.60 V and (B) UV baseline at 205 nm during blank gradient: 1=without guard cell; 2=guard cell at 0.62 V. The true background current of the analytical electrode without the guard cell was 70 nA at 0 min. Conditions as in Fig. 6.

at the initial conditions of the elution). Similar studies were performed on three separate days using ACN and phosphate buffer reagents from two different batches. The total magnitude of the baseline drift with the guard cell was 10–60 nA lower than without it. The guard cell did not have any effects on the responses of the compounds.

The guard cell had also a significant effect on UV baseline drift at 205 nm (Fig. 9B). Without the guard cell, UV baseline drifted upward which is expected when the ACN content of the mobile phase increases. However, with the guard cell, the background absorbance at the initial conditions of the gradient was much higher and the baseline drifted downward during the gradient. Similar results were obtained on three different days using ACN and buffer reagents from two different batches. The background current of the guard cell typically decreased from 16 to 6 μ A during the gradient elution. These findings suggested that an UV absorbing compound was generated in the guard cell and its concentration decreased during the gradient elution.

The formation of UV absorbing compound was easily seen from the UV spectrum of the mobile phase (Fig. 10). Without the guard cell, mobile phase had only weak absorbance above 200 nm. However, when the mobile phase had passed through the guard cell, the mobile phase had significant absorbance up to 230 nm. When the guard cell potential was increased from 0.62 to 0.80 V, the background current of the guard cell and UV background ab-

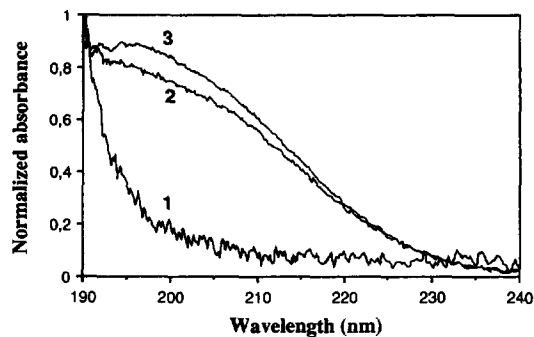


Fig. 10. Effect of guard cell potential on UV spectrum of 35 mM phosphate buffer (pH 2.1)–acetonitrile (96:4, v/v) eluent pumped at the flow-rate of 1.0 ml min⁻¹ without a column: 1=without guard cell; 2=guard cell at 0.62 V; 3=guard cell at 0.80 V. UV spectrum was measured with a diode array detector.

sorbance at 205 nm increased by 2.2 and 1.6 times, respectively, but the shape of the UV spectrum remained the same (Fig. 10). The detection limits of UV detection were slightly higher with the guard cell, since the baseline noise was 1.2–1.3 times higher and the baseline drifted slightly faster during the elution of most important peptides and enzyme inhibitors (20–30 min after injection). However, the guard cell was routinely used due to its advantages in ED detection.

4. Discussion

In this study, enkephalin peptides, their metabolites and several enzyme inhibitors were separated effectively using a reversed-phase octylsilica column and a gradient elution with 35 mM phosphate buffer (pH 2.1) and ACN. Earlier, enkephalin peptides and their metabolites have been separated with reversed-phase HPLC, but enzyme inhibitors were not included and chromatographic behaviour of the compounds was not studied in detail [3,6,7]. In the present study, enkephalin analogs were well resolved from native enkephalins, and the retention order of these peptides was the same as in an earlier isocratic study [29]. The chromatographic behaviour of enzyme inhibitors bestatin, captopril, SCH 39370 and thiorphan differed from the enkephalin peptides in the respect that their retention times decreased more slowly when increasing the ACN content of the mobile phase leading to changes in retention order. Typically, the retention of small organic molecules is less sensitive to the ACN content than the retention of peptides [30]. Due to the differences in chromatographic behaviour, the resolution between closely eluting MEK and SCH 39370 was improved by adjusting the gradient profile.

The electroactivity of Tyr-containing peptides and proteins on carbon electrodes was demonstrated in the beginning of 1980s [31–33]. The oxidation mechanism of Tyr has been studied in detail, but less is known about enkephalin peptides. The oxidation pathways of Tyr have been briefly reviewed [34]. Initially, the phenolic group of Tyr is oxidized with a transfer of one electron. This is followed by chemical reactions that produce electroactive species and depending on the pH of the solution and the time

scale of the experiment these may be oxidized with a release of more electrons. For example, in a hydrodynamic measurement (pH 9.8) with a glassy carbon disc electrode and constant potential, 2–3 electrons were released from each Tyr molecule [34].

The oxidation of enkephalin peptides on carbon electrodes has been studied using differential pulse voltammetry [31] and constant-potential hydrodynamic measurements [13,29]. The electroactivity has been usually attributed to the oxidation of the Tyr residue, although the oxidation of Met residue in MEK has also been suggested [29]. Recently, the oxidation of Tyr and enkephalin peptides was studied using a glassy carbon electrode with cyclic and linear sweep voltammetry [35]. Measurements were performed in static solution using a wide pH range. A one-electron oxidation of the phenolic group in Tyr residue was suggested for all the compounds including MEK.

In the present study, hydrodynamic voltammograms of MEA and its metabolites were determined at pH 2.1 using a porous graphite electrode that has 100% electrolytic efficiency. The number of electrons released from Tyr was 2 that is within the 2–3 electron range obtained with a glassy carbon disc electrode at pH 9.8 [34]. From Tyr-containing peptides, Tyr-D-Ala-Gly and Tyr-D-Ala-Gly-Phe, about 2.5 electrons were released suggesting that the peptide chain slightly affects the oxidation pathway of the Tyr residue. ME and MEA with both a Tyr and a Met or Met-NH₂ residue donated 4.5–5.0 electrons at the highest potential used. The voltammograms of their metabolites revealed that 2.0–2.5 electrons were transferred from the Met or Met-NH₂ residue that was oxidized at about 130 mV higher potential than the Tyr residue.

The different results on the electroactivity of Met residue in methionine enkephalin and its analogs in the present and earlier studies [29,31,35] suggest that the oxidation potential of Met residue depends significantly on the experimental conditions including the surface properties of carbon electrodes. This is supported by the results from the oxidation of Met amino acid on carbon electrodes. In some studies Met has been oxidized at a considerably higher potential than Tyr [32,33,36], whereas in several other studies Met has not given any response at all [31,35].

In this study, the electroactivity of enkephalin peptides was utilized by developing a gradient LC–ED method for the selective determination of MEA and its Tyr-containing metabolites. At the operating potential of 0.60 V, the response of MEA was largely due to oxidation of the Tyr residue with a small contribution of the Met-NH₂ residue. The responses of Tyr-containing metabolites were close to maximum at this potential, whereas other aromatic or sulphur-containing compounds gave either no or only a weak response. The detection limits of MEA and other Tyr-containing peptides were 0.4–0.6 pmol which are similar or slightly higher than those obtained with isocratic LC–ED methods [10–13]. In addition, the responses were reproducible even at picomole level. To our knowledge, this is the first gradient LC–ED method developed for the quantitative determination of peptides in which carbon electrodes are used without derivatization of the analytes.

A stable ED baseline at the operating potential of 0.60 V was obtained. As a precaution, the concentration of phosphate buffer in the mobile phase was kept constant during the gradient elution to reduce the change in the conductivity of the mobile phase that is known to cause baseline drift [15,37]. In addition, the baseline drift was reduced by on-line pre-electrolysis of the mobile phase with a guard cell. However, contrary to expectations, the baseline current of the analytical electrode was slightly higher with the guard cell than without it meaning that an electroactive compound was formed in the guard cell. On the other hand, the guard cell also removed electroactive impurities from the mobile phase, since the background current of the first electrode at 0.25 V was lower with the guard cell than without it. The guard cell has been used earlier in gradient LC–ED analysis. The guard cell has been reported to reduce background current [15] and stabilize baseline [38], but no details were given in these studies.

In addition, UV detection was combined with ED detection to allow the determination of all the aromatic peptides and enzyme inhibitors many of which are not electroactive. At the wavelength of 205 nm, the detection limits ranged from 1.5 pmol of MEA to 30.0 pmol of Phe. The detection limit of MEA was 7–70 times better than detection limits of enkephalin peptides in earlier studies with UV detection at 214 or 215 nm [3,6,7].

5. Conclusions

Gradient HPLC with combined UV and ED detection is an excellent method for the simultaneous determination of enkephalin peptides, their metabolites and several enzyme inhibitors. ED detection is selective for tyrosine-containing peptides with detection limits below 1 pmol. UV detection at 205 nm allows the determination of all the aromatic peptides and enzyme inhibitors with detection limits in the low picomole range.

6. Abbreviations

Standard 3-letter codes are used for amino acids. In addition, following abbreviations are used for enkephalin peptides:

MEK	Methionine enkephalin (Tyr–Gly–Gly–Phe–Met);
ME	[D-Ala ²]-methionine enkephalin (Tyr–D-Ala–Gly–Phe–Met);
MEA	[D-Ala ²]-methionine enkephalinamide (Tyr–D-Ala–Gly–Phe–Met–NH ₂);
LEK	Leucine enkephalin (Tyr–Gly–Gly–Phe–Leu);
LEA	[D-Ala ²]-leucine enkephalinamide (Tyr–D-Ala–Gly–Phe–Leu–NH ₂).

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