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Gradient liquid chromatography of leucine-enkephalin peptide and its metabolites with electrochemical detection using highly boron-doped diamond electrode

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Abstract

Boron-doped diamond thin film (BDD) electrodes have been used to study the oxidation reactions and to detect leucine-enkephalinamide (LEA) and its metabolites, tyrosine (T), tyrosyl-alanine (TA), tyrosyl-alanine-glycine (TAG) and leucine-enkephalin (LE) using cyclic voltammetry (CV), flow-injection analysis (FIA), and gradient liquid chromatography (LC) with amperometric detection. At diamond electrodes, well-defined and highly reproducible cyclic voltammograms were obtained with signal-to-background (S/B) ratios 5–10 times higher than those observed for glassy carbon (GC) electrodes. The analytical peaks of LC for LEA and its metabolites were well resolved. No deactivation of BDD electrodes was found after several experiments with standard as well as plasma samples, indicating high stability of the electrode. Calibration curves were linear over a wide range from 0.06 to 30 μM with regression coefficients of 0.999 for all compounds. The limits of detection obtained based on a signal-to-noise ratio of 3:1 were 3, 2.2, 2.7, 20 and 11 nM for T, TA, TAG, LE and LEA, respectively. These values were at least one order lower than those obtained at GC electrodes, which has given limits of detection of 22.88, 20.64, 89.57, 116.04 and 75.67 for T, TA, TAG, LE and LEA, respectively. Application of this method to real samples was demonstrated and validated using rabbit serum samples. This work shows the promising use of conducting diamond as an amperometric detector in gradient LC, especially for the analysis of enkephalinamide and its metabolites.

Keywords: Electrochemical detection; Boron-doped diamond electrode; Leucine-enkaphalin; Peptides; Enkephalins

1. Introduction

Enkephaline is the small five amino acid chain belonging to the peptide family. It is known as the body's own natural pain killer and regulates many immune functions, including stress and pain. In recent years, there has been growing interest in using peptides as analgesic agents. Leucine-enkephalin (LE, Tyr-D-Ala-Gly-Phe-Leu) is a naturally occurring opiate-like peptide that might be useful as an analgesic agent [1]. It has been found to act as an in vivo neurotransmitter via interaction with opiate receptor [2]. However it is still poorly understood

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because the function is bidirectional, which means that the peptides can have inhibitory and/or stimulatory effects. For example, the biomedical significance of enkephalin and related peptides is also due to their regulation in neurological disorders such as Huntington's disease and Parkinson's disease, as well as their involvement in regulation of several important physiological functions such as pain transmission and prolactin secretion [3,4]. LE is also widely used as a model compound to study peptide metabolism, as it is known to have three possible degradation pathways and several possible metabolites in the human body [5–7]. The development of a simple and sensitive detection method is required to understand the role of these compounds in biological signaling and physiological processes. Any method developed to replace the conventional methods of analysis must be able to separate and detect all possible metabolites. Therefore, the detection method must be both mass- and concentration-sensitive to be able to detect the small amount of analyte eluting during the separation.

Several detection methods have been developed for the determination of enkephalin in biological samples, such as bioassay, radio or enzyme immunoassay (RIA or ELISA), radioreceptor-assay and mass spectrometry, either alone or in combination with liquid chromatography (LC) [2,8,9]. However, the bioassay, immunoassay and receptor-assay although very selective, involve complicated procedures and time-consuming sample preparation steps. The mass spectrometric method, though sensitive, cannot detect all the peptides simultaneously. UV detection with gradient LC is commonly used for the detection of enkephalin and its metabolites. However, low sensitivity (10–100 pmol per injection) due to low absorptivity of most peptides makes it inappropriate [1]. Fluorescence detection is a promising method, but only specific wavelengths are available for excitation, therefore not all peptides can be detected by this method, and pre-column or post-column derivatization is required to solve this limitation [10,11]. Electrochemical detection is a simple, selective, sensitive and economical method for the detection of enkephalin and its tyrosine metabolites. It does not require any derivatization, since the phenolic group of tyrosine residue is electroactive. However, due to the large difference in the polarities between the parent peptide and the shortest metabolite, gradient elution method is required to detect enkephalins and their metabolites simultaneously. Gradient LC combined with electrochemical detection (ED) is not popular because of the large baseline drift with increased noise, resulting in reduced sensitivity. Some gradient LC-ED methods have also been developed for peptides and used for the purpose of qualitative mapping [12] and quantitative determination [13] but the sensitivity is much lower. Other reports using dual graphite electrodes with the coulometric method gave detection limits in the picomole range [1,14]. These electrodes are less stable due to the adsorption of the peptides or their oxidation products on their surface requiring regular surface reactivation.

Highly boron-doped diamond thin film (BDD) electrodes have advantageous features for their application in electroanalysis which include a wide electrochemical potential window [15,16], very low voltammetric background current [17,18], high resistance to deactivation via fouling [19], extreme electrochemical stability [20], and relative insensitivity to dissolved oxygen [21]. Several research groups including our own have previously reported the uniqueness of BDD electrodes for the detection of various environmentally, biologically and clinically important compounds using liquid chromatography, producing sensitive and stable detection [22–24]. However, no reports have been published using BDD electrodes in gradient elution of liquid chromatography. With its unique properties, the BDD electrode is expected to be a more suitable electrode for gradient-LC-ED for peptide detection. In particular, with its low background current, noise can be reduced in order to obtain higher sensitivity. Its chemical resistance may reduce background drift and give a more stable baseline for gradient elution compared to the other electrode materials.

In the present study, we report for the first time, the use of BDD electrodes in gradient LC for amperometric LC-ED determination of LE and its metabolites. In this report, we used leucine enkephalinamide (LEA), since the amide form (in this case in enkephalinamide) is often used for medical preparations due to its higher absorptivity compared to that of enkephalin [1]. BDD electrodes have been shown to achieve high sensitivity, stability, and

reproducibility. Cyclic voltammetry was used to study the electrochemical behavior of these peptides at BDD electrodes.

2. Experimental

2.1. Materials

Tyrosine (T), tyrosyl-alanine (TA), tyrosyl-alanine-glycine (TAG), leucine-enkephalin (LE) and leucine-enkephalinamide were of analytical grade and purchased from Sigma. H_3PO_4, KH_2PO_4, $HClO_4$, NaOH, CH_3COOH and H_3BO_3 were of analytical grade and purchased from Wako and LC grade acetonitrile (ACN) was from Nacalai Tesque. All chemicals were used without further purification. All of these solutions were prepared using ultrapure (18 MΩ) water. Blood samples were collected from a male rabbit (2 months old, 2 kg) in EDTA glass tube and then stored at −20 °C until analysis.

2.2. Preparation of highly boron-doped diamond electrode

The highly boron-doped diamond films were deposited on Si (100) substrate by the microwave plasma chemical vapor deposition (CVD) technique with a commercial microwave plasma reactor (ASTeX, Woburn, MA, USA) at 5 kW for 10 h. The details of the preparation method have been described previously [21,24]. B_2O_3 was dissolved in the solution containing acetone–methanol in the ratio of 9:1 (v/v) at a B/C molar ratio of 10^4 ppm. High purity H_2 was used as the carrier gas. The film quality was confirmed by Raman spectroscopy.

2.3. Voltammetric investigation

Cyclic voltammetric measurements were carried out in a single compartment cell with a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the counter electrode. BDD film was pretreated by ultrasonicating in 2-propanol for about 10 min followed by rinsing with high purity water. The purpose of this pretreatment is to remove the organic impurities that may have remained or formed during the deposition of diamond in the CVD chamber. The GC electrode (GC-20 plate or GC-20 rod, Tokai Carbon Co.) was pretreated by polishing with diamond paste (Fujimi), followed by ultrasonication in pure water. The supporting electrolyte was a mixture of 0.05 *M* H_3PO_4 + 0.05 KH_2PO_4 (H_3PO_4/KH_2PO_4, pH 2.1±0.1). The pH dependence studies were carried out using Britton Robinson buffer, containing 0.04 *M* H_3BO_3, 0.04 *M* H_3PO_4 and 0.04 *M* CH_3COOH. The pH of the buffer was adjusted with NaOH.

2.4. LC analysis

The LC system consisted of two micro-LC pumps (GL Sciences, PU-611C), an autosampler (Spark-Holand Triathlon) with an injection loop, an amperometric detector (GL Sciences, ED-623B), and a data acquisition system (EZ Chrom Elite, Scientific Software, Inc.). A reversed-phase C_{18} column (Inertsil ODS-3 150×4.6 mm I.D.; particle size, 5 μm) was used for the chromatographic separation. The flow-rate for the pump was 1 ml min^{-1} and was confirmed before every experiment by measuring the volume of the buffer collected at the outlet for 10 min. The wall-jet type flow cell consisted of the Ag/AgCl/1 *M* LiCl reference electrode and a stainless steel tube as counter electrode, which also served as the tube for the solution outlet. A 0.5-mm-thick silicon rubber gasket was used as a spacer in the cell. Geometric area was estimated to be 0.64 cm^2 and the cell volume was estimated to be 24 μl by assuming a 25% compression of the gasket. Solution A was 35 m*M* phosphate buffer (pH 2.2) and solution B was 59 m*M* phosphate buffer (pH 2.2) with ACN (60:40, v/v) (final concentration of phosphate buffer is 35 m*M*). ACN content in the mobile phase was increased linearly from 2.5 to 5% during the first 9 min, then to 15% during the next 5 min and finally to 30% during the next 21 min. After a 35-min run, the ACN content was decreased to 4% during 3 min, and the column was re-equilibrated for 17 min before the next injection. Hydrodynamic voltammograms were obtained for each compound prior to amperometric determination and the detection potential was chosen in the limiting current range of the voltammogram. The chromatograms were obtained at an applied potential 1.2 V electrode and 0.9 V versus Ag/AgCl/1 *M* LiCl for

BDD and GC electrodes, respectively. The temperature was maintained at 26 °C. Measurements were carried out at 205 nm with a UV detector for comparative studies.

2.5. Preparation of real samples

The extraction procedure of Schreiber-Deturmeny et al. was used for blood sample extraction [25]. A 200-μl aliquot of 20% $HClO_4$ was added to a 200-μl blood sample. After mixing for a few seconds, the sample was centrifuged at 3000 *g* for 5 min. The resultant supernatant was then neutralized using NaOH, while a known amount of working standard solution was spiked into the extract along with its metabolites. Then the sample was injected onto the LC column. The dilution factor for the sample was 4. The final solutions were filtered using a 0.4-μm pore filter.

3. Results and discussion

3.1. Voltammetric studies

Fig. 1 shows cyclic voltammograms obtained for a solution containing 100 μ*M* tyrosine in 0.1 *M* phosphate buffer (pH 2.1) together with the corresponding background voltammograms for BDD and GC electrodes at a potential sweep rate of 100 mV s^{-1}. At the BDD electrode, the oxidation of tyrosine occurred at ~1.181 V versus SCE. It is slightly higher than that observed for the GC electrode at ~0.961 V versus SCE. It is well established that diamond exhibits higher over potential for multistep redox reactions [26]. No cathodic peaks were observed on the reverse scan within the investigated potential range (−0.5 to +1.5 V vs. SCE). At both BDD and GC electrodes, the voltammograms were well defined and exhibited similar features. However, the *S*/*B* ratio for BDD electrodes was found to be 5–10 times higher than that for GC electrodes, which is due to the low background current at the BDD electrode. This indicates the possibility of better sensitivity at BDD electrodes. Table 1 presents the comparison data of *S*/*B* ratios obtained from the cyclic voltammetry data for the oxidation of 10 μ*M* concentrations of LEA and its metabolites in 0.1 *M* phosphate buffer (pH 2.1) at BDD and GC electrodes.

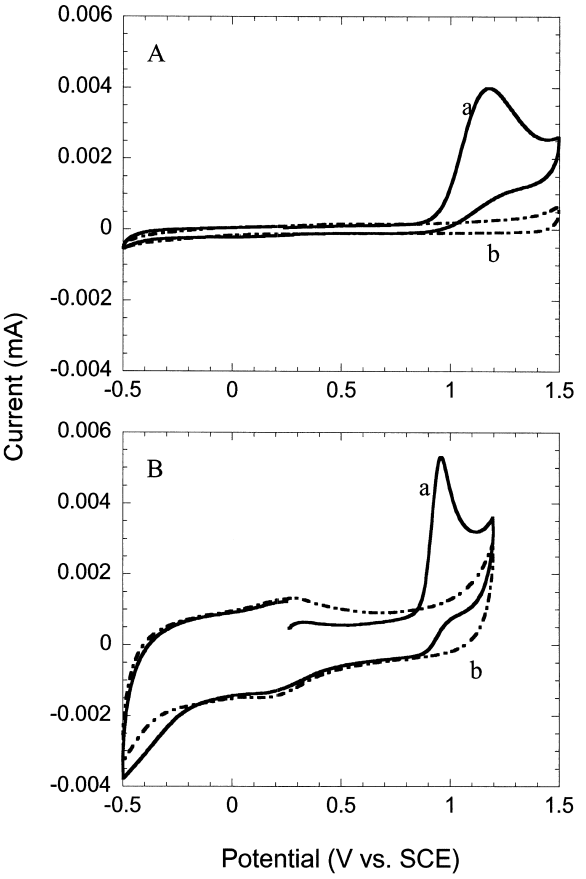

Fig. 1. Cyclic voltammograms at (A) BDD and (B) GC electrode for (a) 10 μ*M* tyrosine in 0.1 *M* phosphate buffer (pH 2.1) (solid line) and (b) only 0.1 *M* phosphate buffer (dotted line). Sweep rate was 100 mV s^{-1}.

At BDD electrodes, the oxidation peak currents of tyrosine decreased after the first cycle but reverted back to the original current after a time delay or with stirring indicating that there is no fouling of the electrode surface. The voltammetric peak currents increased linearly with the square root of the scan rate (regression coefficients = 0.999) from 0.01 to 0.30 V s^{-1} for all of the peptides, suggesting that the reaction is diffusion controlled.

Although the electroactivity of tyrosine has been studied [27–29] earlier, to our knowledge, there has been no report concerning tyrosine oxidation at the BDD electrode. However, Reynolds et al. [27]

Table 1
Comparison of S/B ratios obtained from the cyclic voltammetric data for the oxidation of leucine-enkephalinamide and its metabolites in 0.1 *M* phosphate buffer (pH 2.11), for diamond and GC electrodes

Compound	Diamond electrode		GC electrode	
	E/V versus SCE	S/B	E/V versus SCE	S/B
Tyrosine	1.177	19.00	0.910	4.33
Tyr-D-Ala	1.227	14.64	0.972	2.76
Tyr-D-Ala-Gly	1.258	13.26	0.984	2.12
Leucine-enkephalinamide	0.783	19.25	0.688	1.94
Leucine-enkephalin	1.288	12.25	0.992	1.98

visualized the molecular structure of tyrosine as a *para*-substituted phenol and Rodrigo et al. have proposed a multistep-oxidation reaction mechanism for 4-chlorophenol in acid medium [28]. In the present study, the voltammograms at a diamond electrode for 100 μ*M* tyrosine at a scan rate of 100 mV s^{-1} displayed a single anodic peak over a pH range of 2–10, indicating an irreversible oxidation reaction. Moreover, almost the same current responses were observed over the same pH range, suggesting that the same number of electrons are involved in the reaction mechanism. The peak potential decreased with the increase in pH with a slope of (dE/dpH)~0.059 V/pH up to pH 9.8 above which it became pH-independent. These results are in agreement with the reaction mechanism proposed for tyrosine at GC electrodes, which suggested an irreversible one electron and one proton process [29,30].

$$\mathrm{TyrH} \rightarrow \mathrm{e}^- + \mathrm{H}^+ + \mathrm{Tyr}$$

In the case of tyrosine containing peptides, much less information has been reported about the reaction mechanism. In the present study, at a scan rate of 100 mV s^{-1}, peak potentials decreased with slopes different from that of tyrosine in the pH range 2–10, suggesting the involvement of more electrons in the reaction mechanism. For example, the slope for leucine enkephalin oxidation in the pH range 2–5 is 0.017 V/pH but above pH 5 it is 0.010 V/pH. Ranta et al. reported that 2–3 electrons were released from each Tyr molecule for tyrosine-containing peptide at a glassy carbon disc electrode [1].

Reynolds et al. suggested that the amino acid backbone could influence the redox properties of the phenolic moiety of the tyrosyl residue [27]. Table 1 shows that the peak potentials change with changes in peptide composition. It is interesting to note that with the increase in the chain length of the peptide, the oxidation peak shifted to more positive potentials and became broader. These results indicate that the length of the peptide also influences the kinetics of oxidation. The existence of amide groups in the peptide chain shifted the oxidation peak to more negative potentials. For example, at the BDD electrode, the oxidation potential of leucine enkephalin was found to be 1.288 V versus SCE, while leucine enkephalinamide oxidized at 0.783 V versus SCE. Similarly, at the GC electrode, oxidation potentials of 0.992 and 0.688 V versus SCE were observed for LE and LEA, respectively.

3.2. Chromatographic detection

Hydrodynamic voltammograms were obtained in order to optimize the detection potential for LC analysis. Fig. 2 shows the hydrodynamic voltammograms obtained at the BDD electrode for 20-μl injections of 10 μ*M* concentrations of each peptide at pH 2.1. The corresponding background voltammogram is also shown. Peak forms were sigmoidal in shape with no interference from oxygen evolution reaction. The highest current was given by tyrosine and varied slightly with the length of peptide. However, currents of LE and LEA decreased a lot compared with those of other shorter peptides. From the data, the potential for amperometric detection was set at 1.2 V versus Ag/AgCl. The detection potential for the GC electrode was set at 0.9 V versus Ag/AgCl (data not shown).

Prior to the chromatographic studies of peptides, the stability of the electrodes was examined under flow conditions for detection of enkephalin peptides.

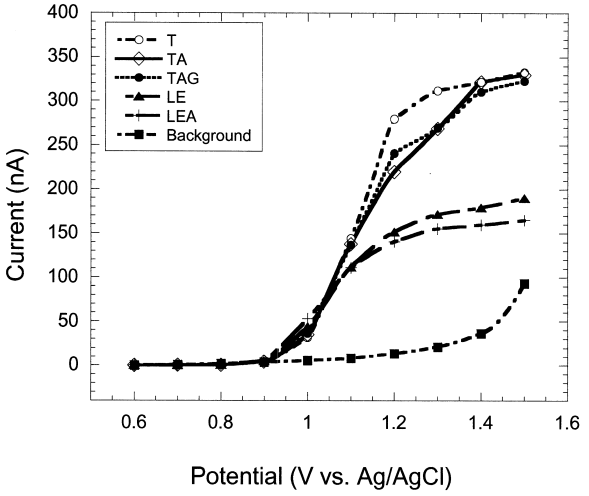

Fig. 2. Hydrodynamic voltammograms for 20-μl injections of 10 μ*M* tyrosine, TA, TAG, LE and LEA in 0.1 *M* phosphate buffer (pH 2.1) at the BDD electrode. The flow-rate was 1 ml min^{-1}.

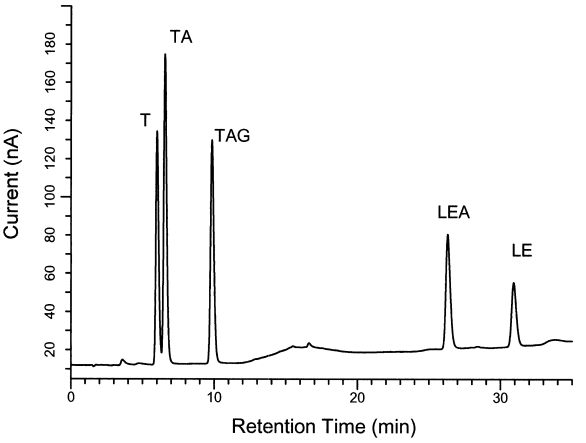

Fig. 3. Chromatogram obtained from gradient LC for 30.0 μ*M* concentrations of LEA and its metabolites at the BDD electrode using an Inertsil ODS-3 column. The flow-rate was 1 ml min^{-1}. Operating potential was 1.2 V versus Ag/AgCl. Acetonitrile content of the mobile phase was increased linearly from 2.5 to 5% during the first 9 min, then to 15% during next 5 min and finally to 30% during next 21 min. Injection volume was 50 μl.

The amperometric response of a BDD electrode for repetitive 50-μl injections of 10 μ*M* enkephalin peptide (28 injections) gave reproducible peak currents, with a peak variability of ~0.81%. These results have shown the absence of electrode fouling under flow conditions, indicating no adsorption of reaction products.

Gradient elution is required for the detection of leu-enkephalinamide and its Tyr-containing metabolites. Generally, baseline drift is the major disadvantage with gradient LC using electrochemical detection. This is based on the fact that the background current depends on solvent and electrolyte composition, and any change in mobile phase composition would shift the baseline. Khaledy et al. reported that several factors contribute to the baseline drift in gradient LC, including pH, change in electrode sensitivity and change in the solvent [31]. It is suggested by Ranta et al. that the baseline drift during gradient elution can be reduced by maintaining the concentration of phosphate buffer constant, to reduce the change in conductivity [1,14]. The sensitivity of the electrode varies mainly due to change in the magnitude of background current as well as the change in the noise level.

Fig. 3 displays the chromatogram for LEA and its Tyr-metabolites at the BDD electrode. Retention times were 5.9, 6.7, 9.8, 26.3 and 30.8 min, for T, TA, TAG, LEA and LE, respectively. This chromatogram was also confirmed using a UV detector. Due to the application of relatively high potential (1.2 V vs. Ag/AgCl), the baseline of the chromatogram using the BDD electrode has drifted after 12 min due to the increase in the concentration of acetonitrile. However, after 15 min, it became relatively stable until 39 min and the returned to the original baseline level when the gradient reversed back to reach the initial condition. The current difference from minimum to maximum baseline is about 13 nA. Fig. 4 shows the baselines of BDD and GC electrodes using the same gradient elution conditions at oxidation potentials for peptide (1.2 and 0.9 V vs. Ag/AgCl for BDD and GC electrodes, respectively). As can be seen, the background current at the GC electrode was larger and relatively noisy. Comparison of baseline drift between the BDD and GC electrodes showed that the difference in values between the minimum and maximum current for the GC electrode at 0.9 V (detection potential) was about three times higher than that for the BDD electrode at 1.2 V. Another notable observation is the large variation in the background current during one cycle for GC and BDD electrodes. At the GC electrode, the current decreased by

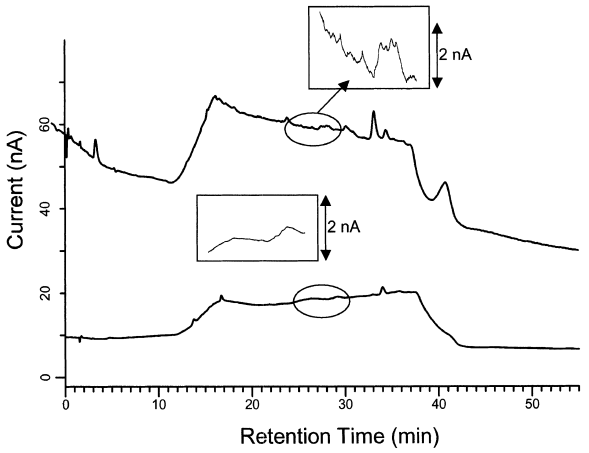

Fig. 4. Baseline obtained at BDD and GC electrodes with gradient elution. Operating potentials were 1.2 and 0.9 V versus Ag/AgCl for BDD and GC electrodes, respectively. Other conditions are the same as for Fig. 3.

about 24 nA during the measurement for 55 min, whereas at the BDD electrode the current decreased by only 1 nA. This may be either due to the adsorption of impurities on the surface of GC electrodes or the oxidation of the electrode surface itself. In addition, GC electrodes required a very long initial stabilization time [24].

After three measurements with a 1 nA decrease in the background current with each measurement, there was about a 3 nA decrease in the baseline. The baseline recovered to the original value by flowing the initial mobile phase (2.5% acetonitrile in phosphate buffer solution) for about 30 min, suggesting that some re-equilibration time is required. In the case of GC electrodes, flowing the mobile phase for 30 min could not recover the baseline to the initial value. Moreover, no significant change in the drift was observed at the BDD electrode while at the GC electrode the drift increased by the cycle, suggesting the GC surface was changed.

As the large baseline drift at the GC electrode was mainly due to the change in the electrode surface, several methods can be used to reduce the drift, for example, using very pure chemicals, using a guard cell as reported by Ranta et al. [14] to purify the mobile phase by oxidizing impurities that might otherwise produce large background current and noise at the electrode and also by avoiding the use of a GC electrode at high potentials. However, in spite of taking the above-mentioned precautions, the GC electrode surface is not stable due to surface oxidation compared to the BDD electrode which is chemically and electrochemically stable for its use at high potentials and plasma sample analysis.

Although, UV detection shows good performance using gradient elution, particularly for compounds that are not electrochemically active, in the present study, comparing results obtained with electrochemical detection to that of UV detection have shown that there is a larger drift of baseline (data not shown) and increased noise using UV detection and at 205 nm, where the detection was carried out, acetonitrile absorbs UV light (technical data from Wako Chemical Co., maximum absorption at 204 nm is 0.05). The drift and noise during the change in acetonitrile concentration in the mobile phase were quite large and caused a decrease in sensitivity and thus the measurement of 0.5 μ*M* peptides was unsuccessful.

3.2.1. Linearity and detection limits

The amperometric responses of leucine-enkephalinamide and its metabolites varied linearly with concentrations between 60 n*M* and 30 μ*M* ($n=8$). The BDD electrode, due to its low background current has shown more sensitivity for the detection of peptides. The GC electrode failed to detect enkephalin below 0.1 μ*M* concentration, while at the BDD electrode, analytically useful peaks were observed for 0.06 μ*M*. The correlation coefficients, the regression curves and the detection limits at a signal-to-noise ratio of 3:1 for enkephalin detection at BDD and GC electrodes are shown in Table 2. The detection limits of LEA and its metabolites at the BDD electrode were 2.2–20.1 n*M* (0.1–1.0 pmol) for 50-μl injection, the lowest being observed for Tyr-D-Ala (0.11 pmol). These detection limits are lower than those reported using the coulometric method [14]. In the case of the GC electrode, the detection limits were from 20.64 to 116.04 n*M* (1.03–5.30 pmol).

3.2.2. Recoveries

The recoveries of leucine-enkephalinamide and its metabolites were determined by injecting blank plasma samples spiked with the standard samples. Aliquots of 2 nmol of each of T, TA, TAG, LE and

Table 2
Linear regression and detection limits for the method at BDD and GC electrodes

Compound	At BDD electrode			At GC electrode		
	Linear equation	Regression coefficients	Limits of detection (nM)[a]	Linear equation	Regression coefficient	Limits of detection (nM)[a]
Tyrosine	$y=-14.277+56.174x$	0.9995	3.0	$y=-9.339+5.523x$	0.9992	22.88
Tyr-D-Ala	$y=-10.291+49.566x$	0.9997	2.2	$y=-7.014+40.481x$	0.9989	20.64
Tyr-D-Ala-Gly	$y=-8.779+44.820x$	0.9998	2.7	$y=-7.262+44.977x$	0.9987	89.57
Leucine-enkephalinamide	$y=-4.979+17.928x$	0.9995	11.0	$y=0.426+444.977x$	0.9988	116.04
Leucine-enkephalin	$y=-7.448+16.536x$	0.9991	20.1	$y=-0.303+14.131x$	0.9989	75.67

[a] Based on $S/N=3$.

LEA were spiked into blank rabbit plasma samples. It can be observed from the chromatogram (Fig. 5) that the peaks due to the other blood components did not interfere with those of the peptides. The results are shown in Table 3. The recoveries varied from 96 to 97.83% for all the samples with coefficients of variation (CV) between 2 and 4.86% ($n=3$).

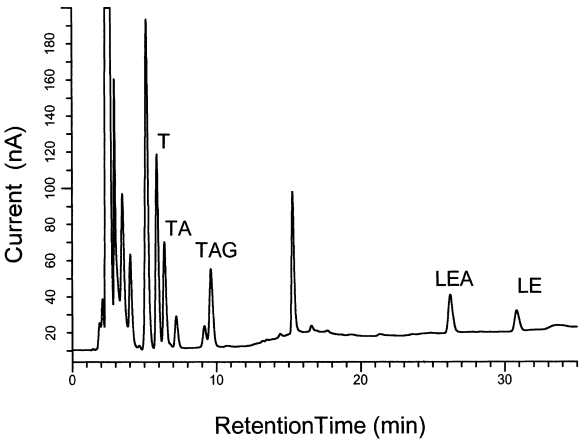

Fig. 5. Chromatogram obtained from gradient LC for the rabbit serum sample spiked with 2 nM each of T, TA, TAG, LE and LEA at the BDD electrode. Other conditions are the same as in Fig. 3.

3.2.3. Reproducibility of electrode performance

The precision of the method was investigated for standard solutions at concentrations of 0.06, 1 and 30 μM. Six measurements were made with each concentration on the same day and the results have shown very good reproducibility with peak variabilities of 1.83–18.4% (Table 4). Current decreased between 1.56 and 7.55% for the five analytes after six measurements. The reproducibility of the BDD electrode surface after plasma analysis was confirmed by comparing the peak currents obtained for 1 μM standard solution before and after the real sample analysis without pretreatment to clean the electrode surface. No significant change in the peak current was found (current varied by ~0.6–2% for all peptides). These results indicate no fouling of the BDD electrode surface after plasma analysis.

During the day-to-day experiments, the BDD electrodes have shown very good reproducibility for

Table 3
Recoveries in blank plasma with 2 nmol of each compound added

Compound	Found amount; mean ($n=3$) (nmol)	Recovery ($n=3$)		
		Mean (%)	CV (%)	SD (nmol)
Tyrosine	0.198	97.83	0.0066	4.86
Tyr-D-Ala	0.194	97.17	0.0055	2.83
Tyr-D-Ala-Gly	0.194	97.16	0.0064	3.30
Leucine-enkephalinamide	0.191	95.33	0.0055	2.88
Leucine-enkephalin	0.192	96.00	0.0040	2.08

Table 4
Precision of the method

	Reproducibility ($n=6$)								
	0.06 μ*M*			10 μ*M*			30 μ*M*		
	Mean (nA)	SD (nA)	CV (%)	Mean (nA)	SD (nA)	CV (%)	Mean (nA)	SD (nA)	CV (%)
Tyrosine	3.04	0.057	1.88	41.24	0.829	2.01	1811.7	36.4	2.00
Tyr-D-Ala	2.41	0.048	2.01	39.06	0.758	1.94	1660.2	35.4	2.13
Tyr-D-Ala-Gly	2.26	0.041	1.83	34.81	0.705	2.03	1459.4	29.6	2.03
Leucine-enkephalinamide	1.97	0.363	18.4	13.08	0.424	3.24	570.7	11.2	1.96
Leucine-enkephalin	1.71	0.335	19.6	13.36	0.152	1.14	528.3	7.2	1.36

the detection of peptides with RSD values below 5%. Current decreased between 2.2 and 7.5% for the five compounds during 3 days of detection. These results show the potential use of the present method with BDD detector with high reproducibility for the rapid analysis of tyr-peptides in blood samples. Diamond outperforms other kinds of electrodes including GC and metal electrodes in terms of stability and reproducibility.

3.3. Comparison with other electrochemical detectors

The results obtained were compared with those for the GC electrode, which is used as an electrochemical detector for gradient LC analysis. The advantages of the BDD electrode are:

(i) The background current noise at the BDD electrode was small, 2–3 times lower than that at the GC electrode. The background current was also low, resulting in a very low limit of detection for LEA and tyr-containing metabolites. LODs for some of the compounds obtained were at the same level as for coulometric detection, i.e. about 2–3 n*M* [14]. It is well established that coulometric detection is more sensitive compared to amperometric detection because measurement of the analyte has 100% efficiency. However, amperometric detection provides simple and rapid detection, due to rapid oxidation reaction and is useful for detection involving low sample volumes such as in capillary electrophoresis. Moreover, amperometric detection provides less fouling of the electrode than coulometric detection with a conventional carbon electrode.

(ii) High stability and no fouling were observed with the BDD electrode during analysis, whereas for the GC electrode, the baseline current continuously decreased during the analysis.

(iii) For the BDD electrode, the stability was very good even after blood sample analysis indicating no adsorption of peptides or blood components on the electrode surface, therefore, no tedious surface cleaning was required.

4. Conclusions

Cyclic voltammetric studies were used to study the mechanism of oxidation reactions. The S/B ratios for the oxidation and scan rate dependence data of tyrosine and related peptides indicated higher sensitivity and a diffusion controlled process at the BDD electrode. The pH dependence studies revealed that one proton and one electron take part in the oxidation mechanism of tyrosine, whereas more electrons and protons are involved in the case of other peptides.

LC with gradient elution is required to separate enkephalin and its metabolites due to their different polarities. The baseline drift during electrochemical detection using the BDD electrode was minimal compared to that observed for GC electrodes. The baseline was relatively stable with very low noise, resulting in low limits of detection of leu-enkephalin and their metabolites (2.2–20.1 n*M*) for 50-μl sample injections. BDD electrodes have also shown high within-day stability during the plasma sample analysis and good day-to-day reproducibility for 3 days.

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