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Detection of the oxidative products of 3-hydroxykynurenine using high-performance liquid chromatography–electrochemical detection–ultraviolet absorption detection–electron spin resonance spectrometry and high-performance liquid chromatography–electrochemical detection–ultraviolet absorption detection–mass spectrometry

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Abstract

Several oxidative products of 3-hydroxy-DL-kynurenine (3-DL-HKY) were detected using high-performance liquid chromatography–electrochemical detection–ultraviolet absorption detection–electron spin resonance spectrometry (HPLC–ED–UV–ESR) and high-performance liquid chromatography–electrochemical detection–ultraviolet absorption detection–mass spectrometry (HPLC–ED–UV–MS). In the HPLC–ED–UV–ESR and the HPLC–ED–UV–MS systems, the ED electrode is placed between an HPLC injector and an HPLC column, and is used as a reactor. 3-DL-HKY was oxidized by the ED electrode with various applied potentials (0–1.0 V). The oxidative products formed were separated by the HPLC column and then detected by the ultraviolet absorption detector, the electron spin resonance spectrometer and the mass spectrometer, respectively. Thus, the HPLC–ED–UV–ESR and the HPLC–ED–UV–MS systems allow us to separate and identify some of the relatively unstable products, including free radical species that form in the redox reactions of 3-DL-HKY. © 1997 Elsevier Science B.V.

Keywords: Electrochemical detection; Detection, LC; 3-Hydroxykynurenine; Xanthommatin; Hydroxanthommatin

1. Introduction

High-performance liquid chromatography–electron spin resonance spectrometry (HPLC–ESR) is a means of spectrometry where an electron spin resonance spectrometer is used as an HPLC detector [1–4]. HPLC–ESR has allowed us to separate the radical adducts that form in the reaction of free radical with spin trap reagent.

HPLC–mass spectrometry (HPLC–MS) has been applied recently to free radical chemistry. The molecular ions of the radical adducts have been successfully detected using HPLC–MS because soft ionization methods, such as thermospray ionization, electrospray ionization and continuous flow fast atom bombardment ionization, are employed for the HPLC–MS.

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The combination of HPLC–ESR and HPLC–MS has been successfully used to separate and identify various kinds of radical adducts [5–10].

An HPLC–ED–ultraviolet absorption detection (HPLC–ED–UV) was recently applied to the elucidation of the redox reactions of radical adducts [11]. The ED electrode is placed between an HPLC injector and an HPLC column, and used as a reactor in HPLC–ED–UV. The ED electrode reduces the radical adducts to their corresponding reduced forms and it also oxidizes radical adducts to their corresponding oxidized forms. Thus, the respective peaks of the reduced, radical and oxidized forms of the radical adducts have been clearly assigned on the HPLC–ED–UV elution profile.

In this paper, HPLC–ED–UV–ESR, where an ESR spectrometer is connected to the HPLC–ED–UV system, and HPLC–ED–UV–MS, where a mass spectrometer is connected to the HPLC–ED–UV system, were employed to clarify the details of the oxidation of 3-hydroxy-DL-kynurenine (3-DL-HKY). The ultraviolet absorption detector (UV), the electron spin resonance spectrometer (ESR), and the mass spectrometer (MS) are used as detectors in HPLC–ED–UV–ESR and HPLC–ED–UV–MS. On the other hand, the ED electrode is placed between the HPLC injector and the HPLC column, and used as a reactor. 3-Hydroxy-DL-kynurenine is oxidized by the ED electrode with various oxidative potentials (0–1.0 V). The oxidative products formed are separated by the HPLC column and detected by the UV, the ESR and the MS.

3-Hydroxy-L-kynurenine (3-L-HKY) is a tryptophan metabolite. The 3-DL-HKY is oxidized to xanthommatin by ferricyanide [12,13]. Xanthommatin is a yellow pigment and was found in insects, especially in the eyes [14–16]. It was also detected in human eyes that had a cataract [17]. On the other hand, a xanthommatin-derived radical was recently detected in a reaction mixture of 3-DL-HKY–H_2O_2–horseradish peroxidase [18].

3-DL-HKY is known to be carcinogenic and neurotoxic [19,20]. In contrast to these deleterious effects, 3-DL-HKY has been reported to have antioxidative activity [21]. Since the oxidation of 3-DL-HKY is supposed to be involved in these toxic effects and antioxidative activity, it is important to clarify the details of the oxidation of 3-DL-HKY. HPLC–ED–UV–ESR and HPLC–ED–UV–MS are employed to investigate the 3-DL-HKY-derived oxidative products, including a free radical species.

The combination of electrochemistry on-line with MS has been extensively investigated [22,23]. On the other hand, this is the first report describing the use of the combination of electrochemistry on-line with ESR spectrometry. In this paper, the combination of HPLC–ED–UV–ESR and HPLC–ED–UV–MS is shown to be a new powerful method for elucidating the redox reactions.

2. Experimental

2.1. Materials

3-Hydroxy-DL-kynurenine and 3-hydroxy-L-kynurenine were obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Horseradish peroxidase was purchased from Oriental Yeast (Osaka, Japan). Potassium ferricyanide was obtained from Wako Pure Chemical Industries, Ltd. Hydrogen peroxide was from Katayama Chemical Industries (Osaka, Japan). All other chemicals used were of analytical grade.

2.2. ESR analysis of the reaction mixture of 3-DL-HKY–hydrogen peroxide–horseradish peroxidase

The reaction mixture contained 0.5 ml of 2 m*M* 3-DL-HKY in sodium phosphate buffer (50 m*M*, pH 7.4), 10 ml of 0.1 *M* hydrogen peroxide in water and 5 μl of 477 U/ml horseradish peroxidase in sodium phosphate buffer (50 m*M*, pH 7.4). The reactions were initiated by adding horseradish peroxidase and were carried out for 5 min at 25°C.

Operating conditions of the ESR spectrometer were: power, 16 mW; modulation width, 0.1 mT; magnetic field, 336.768 mT; sweep time, 4 min; sweep width, 10 mT; amplitude, 1600; time constant, 0.3 s. The magnetic field was fixed at 336.768 mT throughout the experiments.

2.3. Oxidation of 3-DL-HKY by horseradish peroxidase and by potassium ferricyanide

A reaction mixture of 3-DL-HKY with horseradish peroxidase contained 0.5 ml of 2 m*M* 3-DL-HKY in

sodium phosphate buffer (50 m*M*, pH 7.4), 5 μl of 0.1 *M* hydrogen peroxide in water and 10 ml of 477 U/ml horseradish peroxidase in sodium phosphate buffer (50 m*M*, pH 7.4). The reactions were initiated by adding horseradish peroxidase and were carried out for 1 min at 25°C. The reaction mixture was diluted by the addition of 2 ml of 50 m*M* sodium phosphate buffer (pH 7.4). A 1-ml volume of the diluted reaction mixture was applied to the HPLC–UV–ESR system immediately after the dilution.

A reaction mixture of 3-DL-HKY with potassium ferricyanide contained 0.5 ml of 2 m*M* 3-DL-HKY in sodium phosphate buffer (50 m*M*, pH 7.4) and 3 mg of potassium ferricyanide. The reaction was performed for 1 min at 25°C. The reaction mixture was diluted by the addition of 2 ml of 50 m*M* sodium phosphate buffer (pH 7.4). A 1-ml volume of the diluted reaction mixture was applied to the HPLC–UV–ESR system immediately after the dilution.

2.4. Samples for HPLC–ED–UV–ESR and HPLC–ED–UV–MS analyses

A 1-ml volume of 0.4 m*M* 3-DL-HKY (or 3-L-HKY) was applied to the HPLC–ED–UV–ESR and the HPLC–ED–UV–MS systems. The 0.4 m*M* 3-DL-HKY (or 3-L-HKY) solution was dissolved in sodium phosphate buffer (50 m*M*, pH 7.4).

2.4.1. HPLC–ED–UV–ESR (or HPLC–UV–ESR)

The HPLC used in the HPLC–ED–UV–ESR (or HPLC–UV–ESR) set-up consisted of a Rheodyne injector (Rheodyne, Cotati, CA, USA), an Hitachi 655A-11 pump and an Hitachi L-5000 LC controller. An Hitachi 655 variable UV detector (Hitachi, Tokyo, Japan) was operated at 270 nm in the HPLC–ED–UV–ESR (or HPLC–UV–ESR) systems. The electrochemical detector employed was a Model 5100 A Coulochem electrochemical detector with a Model 5020 guard cell (ESA, Bedford, MA, USA). The guard cell consists of a reference electrode (Pd), a counter electrode and a porous graphite electrode. The column (150 mm×4.6 mm I.D.), packed with TSK gel ODS-120T (5 mm particle size) (Tosoh, Tokyo, Japan) was used at a flow-rate of 1.0 ml/min. The pore size of the TSK gel ODS-120T is 120 Å. The column was maintained at 30°C throughout the analyses. For the HPLC–ED–UV–ESR (or HPLC–UV–ESR) analyses, two solvents were used: A, 50 m*M* acetic acid; B, 50 m*M* acetic acid–90% (v/v) methanol. A combination of an isocratic and a linear gradient was used for all of the experiments, except for the HPLC–ED–UV–ESR analysis of 3-L-HKY and the conditions were as follows: 0–2 min, 100% A in B (isocratic); 2–20 min, 100–50% A in B (linear gradient); 20–25 min, 50–0% A in B (linear gradient); 25–30 min, 0% A in B (isocratic). The following conditions were employed for the HPLC–ED–UV–ESR analyses of 3-L-HKY: 0–2 min, 100% A in B (isocratic); 2–40 min, 100–50% A in B (linear gradient); 40–50 min, 50–0% A in B (linear gradient); 50–60 min, 0% A in B (isocratic). The guard cell was placed between the HPLC injector and the HPLC column, where the guard cell was used as a reactor. Signals from the UV detector were monitored using Shimadzu C-R7A Chromatopac (Shimadzu, Kyoto, Japan). The eluent from the UV monitor was introduced into a JEOL JES-FR30 Free Radical Monitor (JEOL, Tokyo, Japan). The ESR spectrometer was connected to the UV monitor using a Teflon tube, which passed through the center of the ESR cavity. The operating conditions of the ESR spectrometer were: power, 16 mW; modulation width, 1 mT; magnetic field, 336.768 mT; time constant, 1 s. The magnetic field was fixed at 336.768 mT throughout the experiments.

2.4.2. HPLC–ED–UV–MS

The HPLC used for the HPLC–ED–UV–MS system consisted of a Rheodyne injector and an Hitachi L-7100 pump. An Hitachi L-7400 UV detector was operated at 270 nm in the HPLC–ED–UV–MS system. The guard cell was placed between the HPLC injector and the HPLC column, and the UV detector was placed after the column, where the guard cell was used as a reactor. The eluent from the UV monitor was introduced into the Hitachi M-1200AP LC–MS system (Hitachi, Ibaragi, Japan) with electrospray ionization (ESI). Operating conditions of the LC–MS system were: nebulizer, 180°C; aperture 1, 120°C; N_2 controller pressure; 2.0 kgf/cm^2; drift voltage, 70 V; multiplier voltage, 1800 V; needle voltage, 3000 V; polarity, positive; resolution, 48. The other conditions were as described for the HPLC–ED–UV–ESR system except that for the HPLC–ED–UV–MS analytical condition of the peaks 2 and 3. The HPLC–ED–UV–MS analysis of peaks 2 and 3 used a moderate gradient

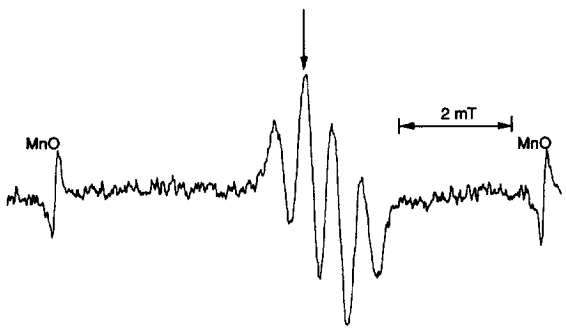

Fig. 1. ESR spectrum of the reaction mixture, 3-DL-HKY–H_2O_2–horseradish peroxidase. The reaction and the ESR conditions were as described in Section 2. The magnetic field of the ESR spectrometer was fixed at the position indicated by an arrow for the analyses by HPLC–ED–UV–ESR (or HPLC–UV–ESR).

elution to separate the peaks: 0–2 min, 100% A in B (isocratic); 2–40 min, 100–50% A in B (linear gradient); 40–50 min, 50–0% A in B (linear gradient); 50–60 min, 0% A in B (isocratic) where A and B are 50 m*M* acetic acid and 50 m*M* acetic acid–90% (v/v) methanol, respectively.

3. Results and discussion

3.1. ESR spectrum of the reaction mixture of 3-DL-HKY–H_2O_2–horseradish peroxidase

ESR measurement of the reaction mixture (3-DL-HKY–H_2O_2–horseradish peroxidase) was performed (Fig. 1). A relatively stable radical was detected. The ESR spectrum is almost the same as the one reported previously [18]. For the HPLC–ED–UV–ESR analyses, the magnetic field of the ESR spectrometer was fixed at the position indicated by the arrow in Fig. 1.

3.2. HPLC–ED–UV–ESR analyses of the 3-DL-HKY

The HPLC–ED–UV–ESR analyses of 3-DL-HKY were performed to identify the free radical formed in the reaction mixture (3-DL-HKY–H_2O_2–horseradish peroxidase). The analyses were performed using 0.0 and 0.5 V applied potentials of the ED, respectively (Fig. 2). A peak (Peak 1) with a retention time of 4.5 min was detected on the UV trace of the HPLC–

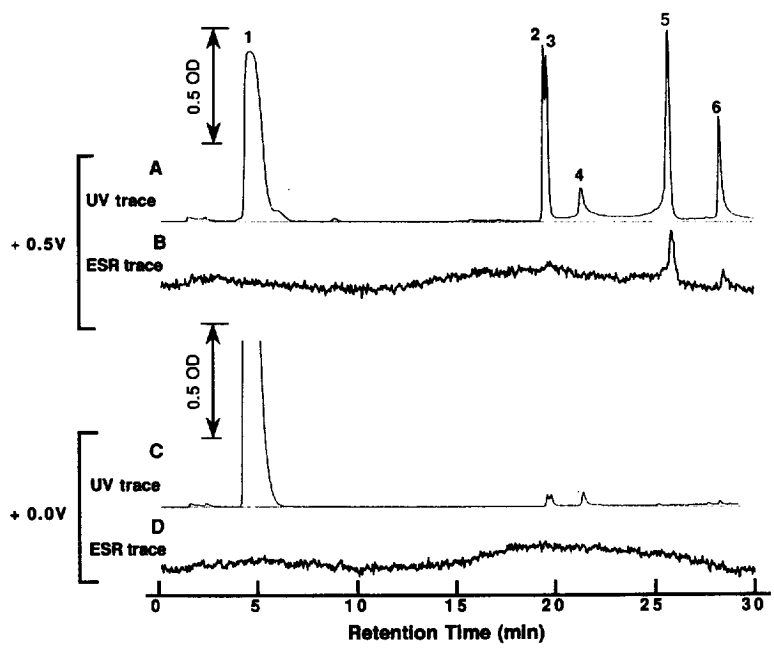

Fig. 2. HPLC–ED–UV–ESR analyses of 3-DL-HKY. The reaction conditions were as described in Section 2. (A) UV trace of the HPLC–ED–UV–ESR analysis of 3-DL-HKY using an applied potential of +0.5 V; (B) ESR trace of the HPLC–ED–UV–ESR analysis of 3-DL-HKY using an applied potential of +0.5 V; (C) UV trace of the HPLC–ED–UV–ESR analysis of 3-DL-HKY using an applied potential of 0.0 V; (D) ESR trace of the HPLC–ED–UV–ESR analysis of 3-DL-HKY using an applied potential of 0.0 V.

ED–UV–ESR elution profiles at the 0.0 and 0.5 V applied potentials of the ED (Fig. 2A,C). Peak 1 is 3-DL-HKY itself. On the other hand, five prominent peaks (peaks 2–6) were detected on the UV trace of the HPLC–ED–UV–ESR elution profile at an ED applied potential of 0.5 V (Fig. 2A). The peak height of peak 1 decreased at an ED applied potential of 0.5 V, indicating that 3-DL-HKY was oxidized by the electrode of the ED during the HPLC–ED–UV–ESR analysis. The retention times of peaks 2, 3, 4, 5 and 6

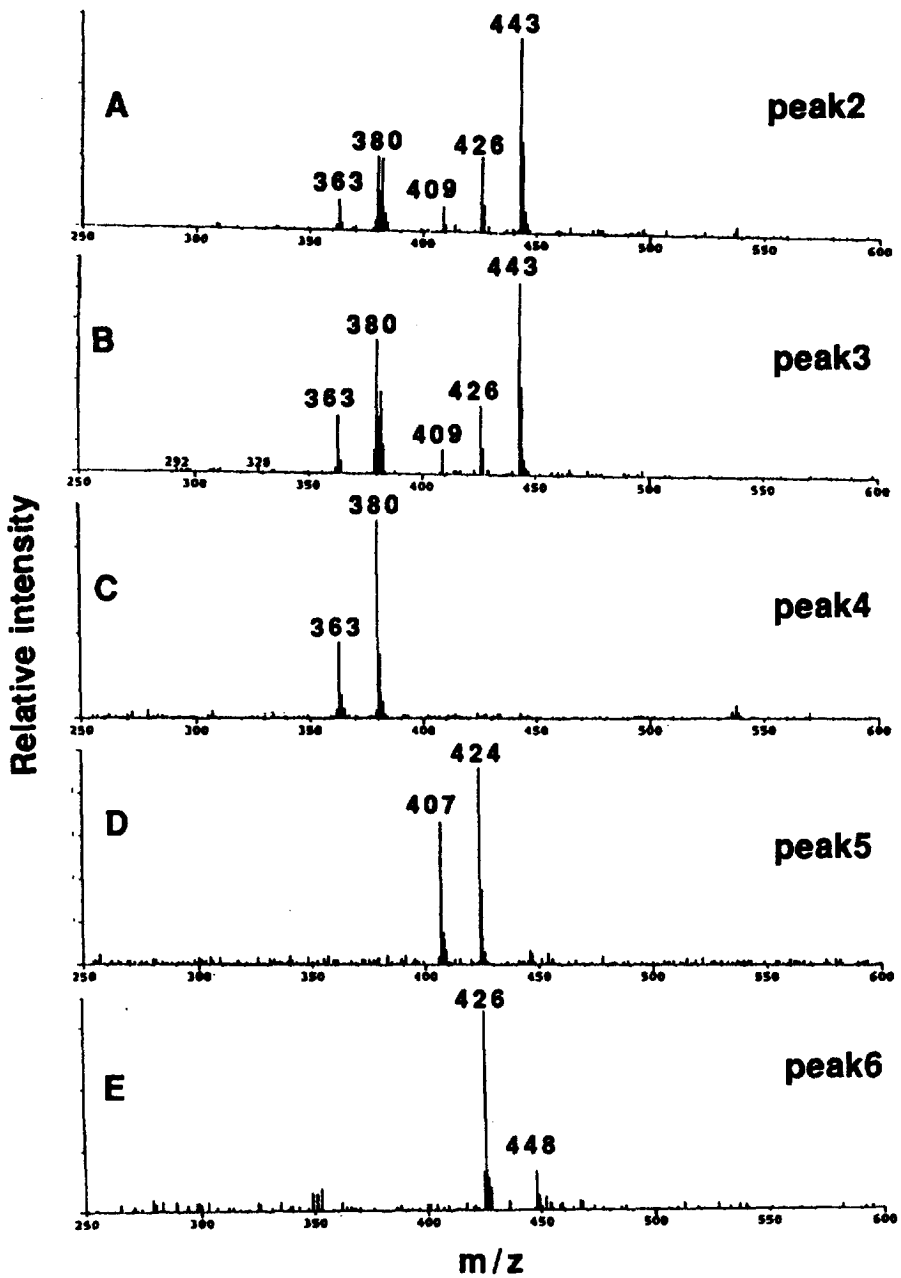

Fig. 3. HPLC–ED–UV–MS analyses of peaks 2, 3, 4, 5 and 6. The reaction and the HPLC–ED–UV–MS conditions were as described in Section 2. (A) peak 2; (B) peak 3; (C) peak 4; (D) peak 5 and (E) peak 6.

are 19.3, 19.5, 21.2, 25.5 and 28.0 min, respectively. Of the five peaks, peaks 5 and 6 were also detected on the ESR trace of the HPLC–ED–UV–ESR elution profile (Fig. 2B). The results indicate that the fractions giving rise to peaks 5 and 6 contain radical species.

3.3. HPLC–ED–UV–MS analyses of 3-DL-HKY

The HPLC–ED–UV–MS analyses of 3-DL-HKY were performed to identify the respective peak fractions. Mass spectra of peaks 2, 3, 4, 5 and 6 are shown in Fig. 3. The fractions giving rise to peaks 2 and 3 showed almost identical mass spectra, in which m/z 443, 426, 409, 380 and 363 ions were detected (Fig. 3A,B). The ion m/z 443 corresponds to the $[M+H]^+$ ion of compound II (Fig. 4). The ions m/z 426, 409, 380 and 363 correspond to the $[M-NH_3+H]^+$, $[M-NH_3-OH+H]^+$, $[M-NH_3-OH-CO_2+H]^+$ and $[M-NH_3-OH-CO_2-OH+H]^+$ ions, respectively. The possible structures of peaks 2 and 3 are shown in Fig. 4. Peaks 2 and 3 could be diastereomers, since 3-DL-HKY was used for this experiment. The mass spectrum of the peak 4 fraction showed prominent peaks of m/z 380 and 363 ions (Fig. 3C). The ion m/z 380 corresponds to the $[M+H]^+$ ion of decarboxylated products of xanthommatin, compound V (Fig. 4). The ion m/z 363 is the $[M-OH+H]^+$ ion of the decarboxylated product. The mass spectrum of peak 5 showed m/z 424 and 407 ions (Fig. 3D). The ion m/z 424 corresponds to the $[M+H]^+$ ion of xanthommatin, compound IV (Fig. 4). The m/z 407 ion corresponds to the $[M-OH+H]^+$ ion of xanthommatin. The mass spectrum of peak 6 showed m/z 448 and 426 ions (Fig. 3E). The m/z 426 ion corresponds to the $[M+H]^+$ ion of hydroxanthommatin, compound III (Fig. 4). The m/z 448 ion corresponds to the $[M+Na]^+$ ion of hydroxanthommatin. The structure of the free radical species could be compound VI, which is formed through the one electron reduction of xanthommatin or through the one electron oxida-

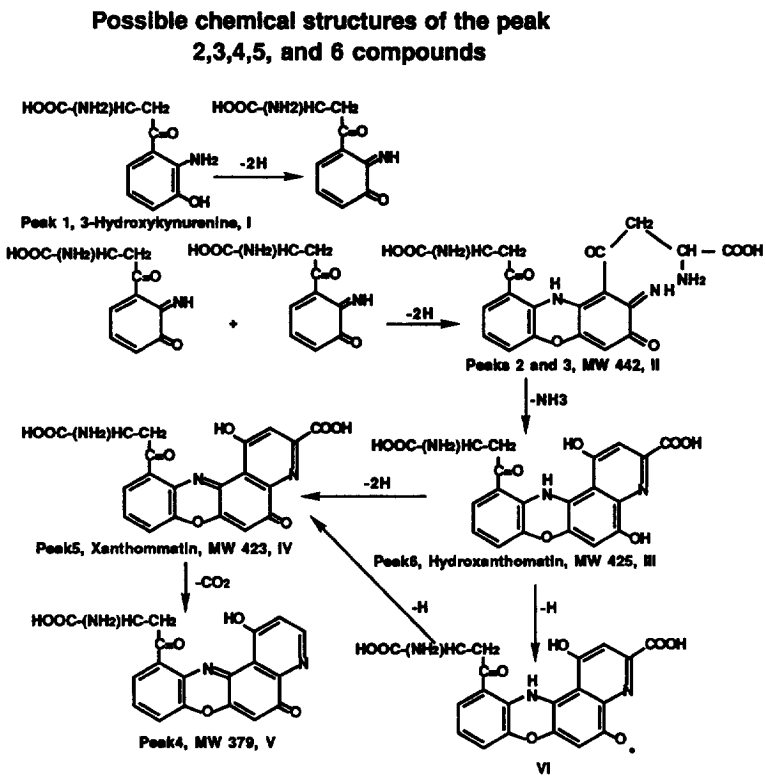

Fig. 4. Possible chemical structures of the compounds that give rise to peaks 2, 3, 4, 5 and 6.

tion of hydroxanthommatin, since the HPLC–ED–UV–ESR retention times of the radical species are the same as those of xanthommatin and hydroxanthommatin, respectively.

3.4. Comparison of the electrochemical oxidation of 3-DL-HKY with the oxidation of 3-DL-HKY by horseradish peroxidase (or potassium ferricyanide)

The elution profile of the HPLC–ED–UV–ESR analysis of 3-DL-HKY was compared with the HPLC–UV–ESR elution profile of the reaction mixtures of 3-DL-HKY–H_2O_2–horseradish peroxidase and 3-DL-HKY–potassium ferricyanide (Fig. 5). The five 3-DL-HKY-derived peaks were detected on all of the elution profiles, although the relative intensities of the five peaks differed, indicating that HPLC–ED–UV–ESR is a powerful technique for examining the reactions catalyzed by horseradish peroxidase. The relative intensity of peaks 2 and 3 is high on the elution profile of the HPLC–ED–UV–ESR analysis of 3-DL-HKY (Fig. 5A). On the other hand, peak 4 was found to be relatively higher on the HPLC–ED–UV–ESR elution profiles of the reaction mixtures of 3-DL-HKY–H_2O_2–horseradish peroxidase and 3-DL-HKY–potassium ferricyanide.

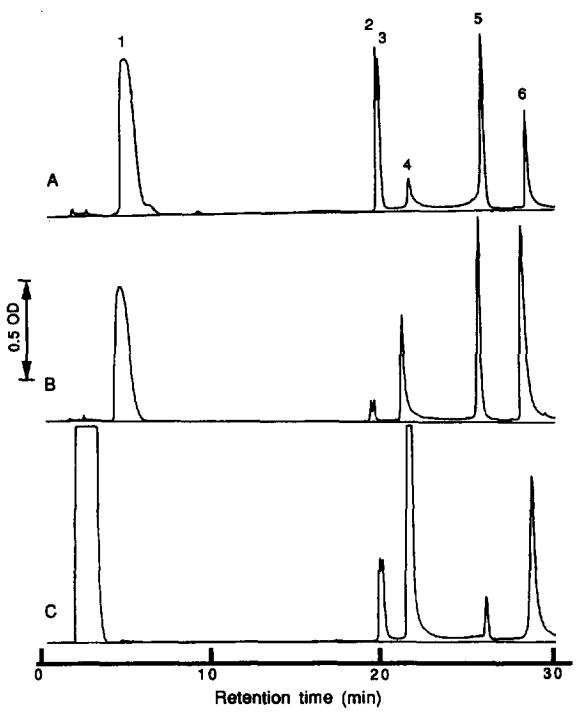

Fig. 5. HPLC–ED–UV–ESR analysis of 3-DL-HKY and the HPLC–UV–ESR analyses of the reaction mixtures containing 3-DL-HKY–H_2O_2–horseradish peroxidase and 3-DL-HKY–potassium ferricyanide. The reaction, the HPLC–ED–UV–ESR and the HPLC–UV–ESR conditions were as described in Section 2. (A) UV trace of the HPLC–ED–UV–ESR analysis of 3-DL-HKY using an applied potential of +0.5 V; (B) UV trace of the HPLC–UV–ESR analysis of the reaction mixture containing 3-DL-HKY–H_2O_2–horseradish peroxidase; (C) UV trace of the HPLC–UV–ESR analysis of the reaction mixture containing 3-DL-HKY–potassium ferricyanide.

3.5. Dependence of the HPLC–ED–UV–ESR analyses of 3-DL-HKY on the applied potential

The dependence of the HPLC–ED–UV–ESR analyses of 3-DL-HKY on the applied potential of the electrochemical detector was analysed (Fig. 6). The UV traces obtained following HPLC–ED–UV–ESR

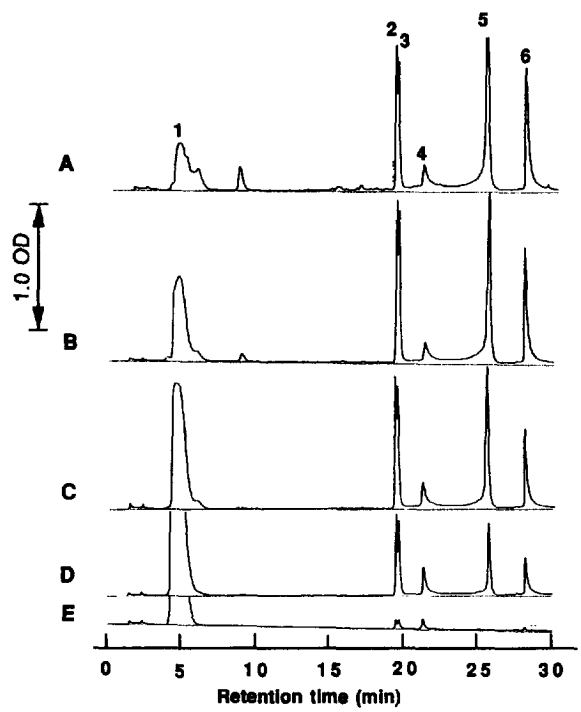

Fig. 6. UV traces of the HPLC–ED–UV–ESR analyses of 3-DL-HKY using various ED applied potentials. The HPLC–ED–UV–ESR conditions were as described in Section 2. The UV traces of the HPLC–ED–UV–ESR analyses with various ED applied potentials are shown: (A) 1.0 V; (B) 0.7 V; (C) 0.5 V; (D) 0.3 V and (E) 0.0 V.

show that the peak height of peaks 2, 3, 4, 5 and 6 increased with increasing ED applied potential. On the other hand, the peak height of peak 1 decreased with increasing applied potential, indicating that the compounds that give rise to peaks 2, 3, 4, 5 and 6 are formed from 3-DL-HKY. The ESR traces of the HPLC–ED–UV–ESR analyses with various ED applied potentials are shown in Fig. 7. The radical peaks increased with increasing applied potentials.

Any oxidative or reductive potentials can be chosen in HPLC–ED–UV–ESR and HPLC–ED–UV–MS. Thus, detailed information cam be obtained from HPLC–ED–UV–ESR and HPLC–ED–UV–MS analyses compared with the oxidation by horseradish peroxidase or potassium ferricyanide.

3.6. HPLC–ED–UV–ESR analyses of 3-L-HKY

To determined whether peaks 2 and 3 are diastereomers or not, 3-L-hydroxykynurenine (3-L-HKY) was analyzed using HPLC–ED–UV–ESR. The UV traces of the HPLC–ED–UV–ESR analyses of 3-L-HKY, 3-DL-HKY and of a mixture of 3-L-HKY and 3-DL-HKY are shown in Fig. 8. Peaks 2 and 3 were detected as separated peaks on the UV trace of the HPLC–ED–UV–ESR analysis of 3-DL-HKY (Fig. 8A). On the other hand, a single peak was detected on the UV trace of the HPLC–ED–UV–ESR analysis of 3-L-HKY, indicating that peaks 2 and 3 are diastereomers of each other (Fig. 8B). On HPLC–ED–UV–ESR analysis of the 1:1 mixture of 3-L-HKY and 3-DL-HKY, peak 3 was observed to be relatively higher (Fig. 8C). The results indicate that the compound in peak 3 is the condensate of two molecules of 3-L-HKY (or 3-hydroxy-D-kynurenine). On the other hand, the compound in peak 2 could be the condensate of 3-L-HKY and 3-hydroxy-D-kynurenine.

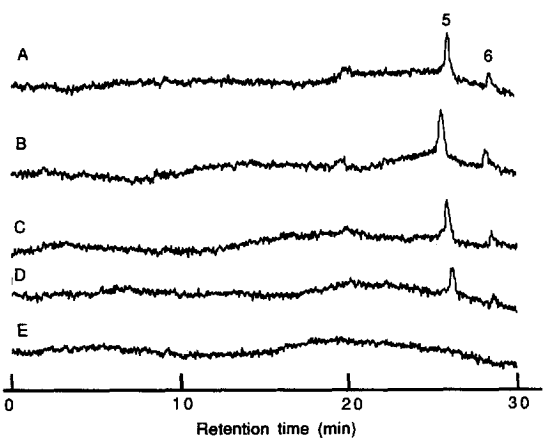

Fig. 7. ESR traces of the HPLC–ED–UV–ESR analysis of 3-DL-HKY using various ED applied potentials. The HPLC–ED–UV–ESR conditions were as described in Section 2. The ESR traces of the HPLC–ED–UV–ESR analyses with various ED applied potentials are shown: (A) +1.0 V; (B) +0.7 V; (C) +0.5 V; (D) +0.3 V and (E) +0.0 V.

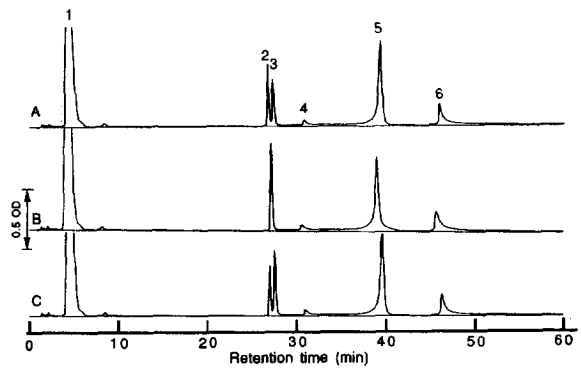

Fig. 8. UV traces of the HPLC–ED–UV–ESR analyses of 3-DL-HKY, 3-L-HKY and of a 1:1 mixture of 3-DL-HKY and 3-L-HKY. The HPLC–ED–UV–ESR conditions were as described in Section 2. (A) UV trace of the HPLC–ED–UV–ESR analysis of 3-DL-HKY; (B) UV trace obtained following the HPLC–ED–UV–ESR analysis of 3-L-HKY and (C) UV trace of the HPLC–ED–UV–ESR analysis of the 1:1 mixture of 3-DL-HKY and 3-L-HKY.

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