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Quantitative detection of reduced, radical and oxidized forms of α-(4-pyridyl-1-oxide)-*N-tert.*-butylnitrone radical adduct using high-performance liquid chromatography with electrochemical detection

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Abstract

Reduced, radical and oxidized forms of α-(4-pyridyl-1-oxide)-*N-tert.*-butylnitrone (4-POBN)-phenyl radical adduct were detected as separated peaks on the HPLC elution profile using either a high-performance liquid chromatography–ultraviolet absorption detector–electrochemical detector (HPLC–UV–ED) technique or a high-performance liquid chromatography–electrochemical detector–ultraviolet absorption (HPLC–ED–UV) technique. In the HPLC–ED–UV, the Coulochem ED electrode is placed between an injector and an HPLC column, and used as a reactor which oxidizes the reduced form of the 4-POBN-phenyl radical adduct to the corresponding radical and oxidized forms, respectively. Thus, the reduced, radical and oxidized forms of the 4-POBN-phenyl radical adduct are clearly identified. Furthermore, the concentration of the reduced and radical forms of the 4-POBN-phenyl radical adduct is quantitatively determined by the Coulochem ED. It is possible to determine the concentration of the respective forms of the 4-POBN-phenyl radical adduct because Coulochem ED oxidizes all of the radical and reduced forms which flow through the electrode.

Keywords: Radical adducts; α-(4-Pyridyl-1-oxide)-*N-tert.*-butylnitrone

1. Introduction

The spin trapping technique has been developed to detect short-life free radicals [1–3]. Spin trapping is a technique in which a reactive free radical reacts with a double bond of a diamagnetic compound, the spin trap – such as α-(4-pyridyl-1-oxide)-*N-tert.*-butylnitrone (4-POBN), α-phenyl-*N-tert.*-butylnitrone (PBN), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 2-methyl-2-nitrosopropane (NMP) – to form a less reactive free radical, the radical adduct. Many kinds of radical adducts have been successfully detected and identified in various chemical and biological systems using an electron paramagnetic resonance (EPR) spectrometer [4]. Hyperfine coupling constants of EPR have been used to identify the radical adducts. Similar hyperfine coupling constants, however, were often observed for different radical adducts [5], which make the determination of the radical adduct structures difficult. Thus, information other than the EPR hyperfine coupling constants is necessary to identify the radical adducts.

Recently, high-performance liquid chromatography (HPLC) has been employed to detect, isolate and identify the radical adducts. In the HPLC analyses retention times are used to identify the radical adducts. A high-performance liquid chromatography–electron paramagnetic resonance (HPLC–

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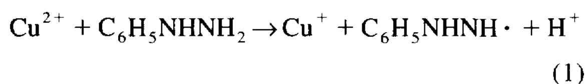
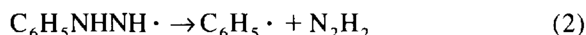
EPR) spectrometry, where an EPR spectrometer is used as a detector, has been utilized to detect, isolate and identify the radical adducts [6–10]. HPLC–EPR spectrometry has been shown to be a powerful method for complicated biological systems because the radical adducts are separated and selectively detected [11]. The EPR detector is, however, limited by its low sensitivity (>0.1 μM) compared with ECD or UV detectors. Furthermore, reduced and oxidized forms of the radical adducts, which often arise from the radical forms of the radical adducts [12], are not detected by the EPR detector. To overcome these weak points of the HPLC–EPR, an HPLC with an electrochemical detector (HPLC–ED) technique has been employed to detect the radical adducts. The HPLC–ED has been applied to the detection of DMPO radical adducts [13,14] and a nitrosobenzene radical adduct [15]. Floyd [13] detected the DMPO-OH radical adduct by HPLC–ED. Addition of ascorbic acid resulted in increase of the intensity of a peak which was assigned to the reduced form of the DMPO-OH radical adduct [13]. On the other hand, Towell and Kalyanaraman used an internal standard (2,2,5,5-tetramethylpyrrolidin-1-yloxy free radical) to determine the concentration of the DMPO radical adduct [14]. They also successfully employed isotope labeled compounds to identify the radical adduct [14].

A phenyl radical is produced by a metal-catalysed one-electron oxidation of phenylhydrazine [Eq. (1) and Eq. (2)] [15]

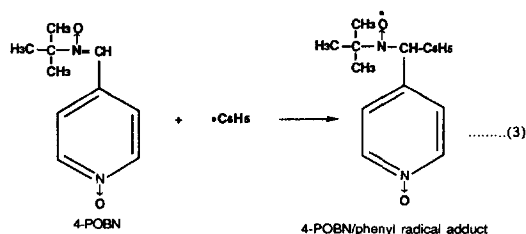


$$Cu^{2+} + C_6H_5NHNH_2 \rightarrow Cu^{+} + C_6H_5NHNH\cdot + H^{+} \quad (1)$$



$$C_6H_5NHNH\cdot \rightarrow C_6H_5\cdot + N_2H_2 \quad (2)$$

which is then trapped by a spin trapping reagent, 4-POBN (Scheme 1): the 4-POBN-phenyl radical adduct is easily reduced and oxidized by oxidizing and reducing agents in the reaction mixture. The Cu^{2+} and Cu^{+} ions are possible candidates for the oxidizing and reducing agents. In this paper we determined the concentrations of the reduced, radical and oxidized forms of the 4-POBN-phenyl radical adduct without an internal standard. The concentrations of the respective forms of the radical adducts were determined using a Model 5100 A Coulochem



Scheme 1.

electrochemical detector with a Model 5010 analytical cell on which 100% of the reduced and radical forms of the 4-POBN-phenyl radical adduct is oxidized. Furthermore, the guard cell is used as a reactor to oxidize the reduced and radical forms of the 4-POBN-phenyl radical adduct. The ED oxidation allows us to correlate the reduced form of the 4-POBN-phenyl radical adduct to the corresponding radical and oxidized forms, respectively. Thus, the HPLC–ED technique is shown to be a powerful method to perform the quantitative study of radical reactions where determinations of the respective concentrations of the reduced, radical and oxidized forms are essential.

2. Experimental

2.1. Materials

Phenylhydrazine oxalate was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). α-(4-Pyridyl-1-oxide)-*N-tert.*-butylnitrone (4-POBN) was obtained from Sigma (St Louis, MO, USA) and Sep-pak C_{18} from Waters (Milford, MA, USA). All other chemicals used were commercial products of the highest grade available.

2.2. Formation of the 4-POBN-phenyl radical adduct

The reaction mixture contained 16 mg of 4-POBN, 7 mg of phenylhydrazine oxalate and 0.5 mM $CuCl_2$ in 1.6 ml 50 mM carbonate buffer (pH 10.0). The reaction mixture without $CuCl_2$ was bubbled with

nitrogen gas for 5 min and then the reaction was started by adding $CuCl_2$. The reaction bottle was capped during the reaction. After 2 h-reaction in the dark, 3.2 ml 0.2 *M* boric acid was added. The reaction mixture was applied to Sep-pak C_{18} cartridges, washed with 3 ml of distilled water and eluted with 2 ml of methanol. The samples were applied to the HPLC–ED–UV and the HPLC–UV–ED.

2.3. HPLC–ED–UV and HPLC–UV–ED

The HPLC used for the HPLC–ED–UV and HPLC–UV–ED consisted of a Rheodyne injector (Reodyne Cotati, CA, USA), a Hitachi 655A-11 pump and a Hitachi L-5000 LC controller. A Hitachi 655 variable UV detector (Hitachi, Tokyo, Japan) was operated at 254 nm for the HPLC–ED–UV and the HPLC–UV–ED. The electrochemical detector employed here was a Model 5100 A Coulochem electrochemical detector with a Model 5020 guard cell or a Model 5010 analytical cell (ESA, Bedford, MA, USA). The analytical cell is a low volume flow-through cell containing a reference electrode (Pd), a counter and two porous graphite electrodes. The guard cell consists of a reference electrode (Pd), a counter and a porous graphite electrode. The column (150×4.6 mm I.D.) packed with TSKgel ODS-120T (5 μm particle size) (Tosoh, Tokyo, Japan) was used at a flow-rate of 1.0 ml/min with 50 m*M* ammonium acetate–32% acetonitrile (v/v) or 50 m*M* phosphoric acid–32% acetonitrile (v/v). The TSKgel ODS-120T is a non-silanol type disposed with an end cap to a remaining silanol. The pore size of the TSKgel ODS-120T is 120 Å. The column was kept at 30°C throughout the analyses.

The guard cell was placed between the injector and the column, and the UV detector was placed after the column in the HPLC–ED–UV where the guard cell was used as a reactor rather than a detector. In the HPLC–UV–ED, the UV detector was placed between the column and the ED analytical cell. The analytical cell employed in the HPLC–UV–ED was used as a detector.

Signals from the UV detector and the ED were monitored using Shimadzu C-R7A Chromatopac (Shimadzu, Kyoto, Japan).

2.4. Mass spectrometry

Measurements of mass spectra were done using a Hitachi M-1200AP LC–MS system (Hitachi, Ibaragi, Japan) with an atmospheric pressure chemical ionization (APCI). Operating conditions were: column, Develosil ODS-HG-5 (150×4.6 mm I.D.); column temperature, 40°C; mobile phases, 50 m*M* ammonium acetate–32% acetonitrile (v/v); flow-rate, 1.0 ml/min; nebulizer temperature, 200°C; desolvator temperature, 400°C; drift voltage, 60 V.

2.5. UV spectra

UV spectra of the 4-POBN-phenyl radical adduct were measured using a Shimadzu UV-160 A spectrophotometer. The reduced, radical and oxidized forms of the 4-POBN-phenyl radical adduct were purified using the HPLC–ED–UV. The HPLC mobile phase [50 m*M* ammonium acetate–32% acetonitrile (v/v)] was placed in the reference cell for the UV measurements. The UV measurements were done at 30°C.

3. Results and discussion

3.1. HPLC–ED–UV analyses of the 4-POBN-phenyl radical adduct

The reaction mixture containing phenylhydrazine, 4-POBN and $CuCl_2$ in carbonate buffer (pH 10.0) was analyzed using the HPLC–ED–UV (Fig. 1). Peaks 1, 2 and 3 were detected in the HPLC–UV elution profile at the retention times of 3.2, 4.1 and 5.4 min, respectively (Fig. 1A). The prominent peak appearing at the retention time of 1.8 min is 4-POBN itself (Fig. 1A). The respective peak fractions were purified. An HPLC–UV rechromatography of the respective purified peak fraction showed a single peak at the corresponding retention time, indicating that UV light of the UV detector hardly performs the photolysis of the respective peak fraction under the conditions employed here. The HPLC–UV elution profile of the purified peak 3 fraction is shown in Fig. 1B. The HPLC–ED–UV elution profiles of the peak 3 fraction are shown where +0.2 V (Fig. 1C) and +0.6 V (Fig. 1D) were applied on the ED guard

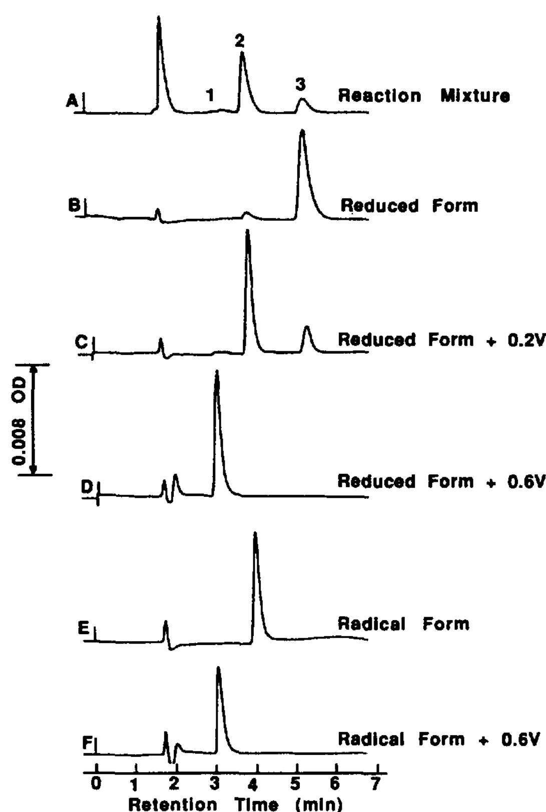


Fig. 1. HPLC–ED–UV analyses of the 4-POBN-phenyl radical adduct. Reaction conditions were as described in Section 2 except for the mobile phase. The mobile phase of the HPLC–ED–UV was 50 m*M* ammonium acetate–32% acetonitrile (v/v). (A) The reaction mixture (5 μl) without the ED guard cell; (B) the purified peak 3 fraction (5 μl) without the ED guard cell; (C) the purified peak 3 fraction (5 μl) using the ED guard cell with a +0.2 V applied potential; (D) the purified peak 3 fraction (5 μl) using the ECD guard cell with a +0.6 V applied potential; (E) the purified peak 2 fraction (5 μl) without the ED guard cell; (F) the purified peak 2 fraction (5 μl) using the ED guard cell with a +0.6 V applied potential.

cell, respectively. Intensities of peak 1 and peak 2 increase respectively, indicating that the reduced form of the 4-POBN-phenyl radical adduct is oxidized to the corresponding radical form (peak 2) and then to the corresponding oxidized form (peak 1) depending on the magnitude of the applied potential on the guard cell. The radical form (peak 2) of the 4-POBN-phenyl radical adduct is also oxidized to the corresponding oxidized form (peak 1) (Fig. 1E and Fig. 1F). Thus, peaks 1, 2 and 3 could be assigned to the oxidized, radical and reduced forms of the 4-POBN-phenyl radical adduct, respectively (Fig. 2).

In order to know whether the electrochemical reactions are reversible or not, the purified radical and oxidized forms of the 4-POBN-phenyl radical adduct were analyzed using the HPLC–ED–UV. Various potentials (from −0.55 V to −1.4 V) were applied on the guard cell. Both the radical and oxidized forms were hardly reduced by the guard cell under the neutral mobile phase [50 m*M* ammonium acetate–32% acetonitrile (v/v)] (data not shown). On the other hand, the HPLC–ED–UV analyses of the 4-POBN-phenyl radical adduct were performed under an acidic mobile phase [50 m*M* phosphoric acid–32% acetonitrile (v/v)] (Fig. 3). The reduced, oxidized and radical forms of the 4-POBN-phenyl radical adduct showed different retention times in the HPLC–UV elution profile with the acidic mobile phase compared with the neutral mobile phase. The retention times for the reduced, oxidized and radical forms of the 4-POBN-phenyl radical adduct are 2.9, 3.7 and 5.2 min, respectively. The radical form of the 4-POBN-phenyl radical adduct was reduced at a −1.0 V applied potential (Fig. 3B). A high concentration of H^+ ions seems to stimulate the reduction of the radical form. The above result is reason-

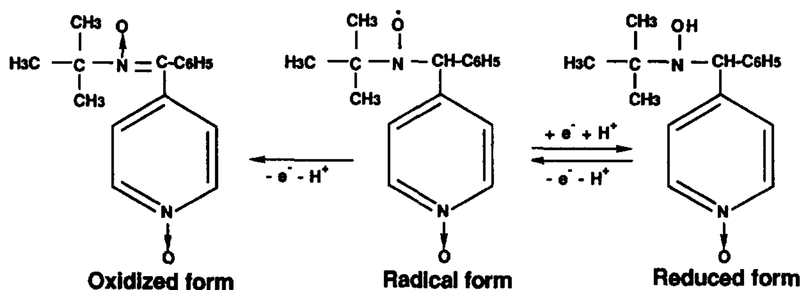


Fig. 2. Structures of the oxidized, radical and reduced forms of the 4-POBN-phenyl radical adduct.

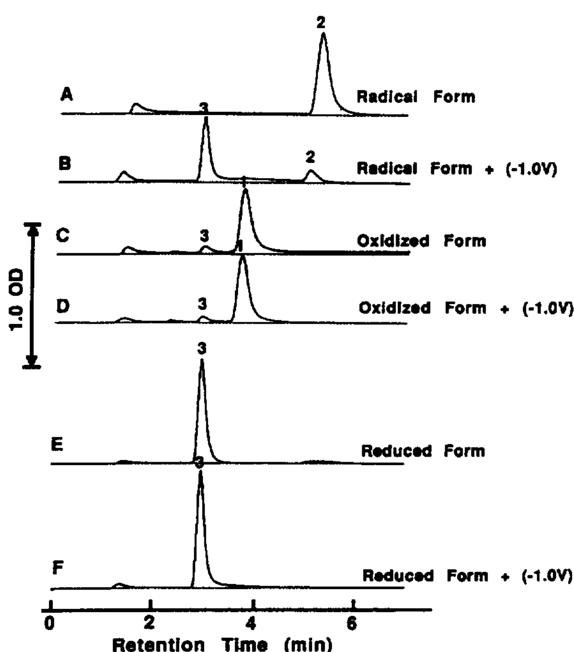


Fig. 3. Reduction of the 4-POBN-phenyl radical adduct. Analytical conditions were as described in Section 2 except for the mobile phase. The mobile phase of the HPLC–ED–UV was 50 m*M* phosphoric acid–32% acetonitrile (v/v). (A) The purified radical form fraction (10 μl) of the 4-POBN-phenyl radical adduct without the ED guard cell; (B) the purified radical form fraction (10 μl) of the 4-POBN-phenyl radical adduct using the ED guard cell with a −1.0 V applied potential; (C) the purified oxidized form fraction (10 μl) without the ED guard cell; (D) the purified oxidized form fraction (10 μl) using the ED guard cell with a −1.0 V applied potential; (E) the purified reduced form fraction (10 μl) without the ED guard cell; (F) the purified reduced form fraction (10 μl) using the ED guard cell with a −1.0 V applied potential.

able because the radical form is reduced by receiving both an electron and a H^+ ion (Fig. 2). On the other hand, the oxidized form of the 4-POBN-phenyl radical adduct could not be reduced even under acidic conditions (Fig. 3D).

In order to confirm the above assignment of the three peaks, mass spectra were measured using a LC–MS with an APCI. Peaks 1, 2 and 3 gave the molecular ions m/z 271, m/z 271 and m/z 273 which correspond to the $[M+H]^+$ of the oxidized form, M^+ of the radical form and $[M+H]^+$ of the reduced form, respectively. Mass spectra of the respective peaks have already been obtained by using an on-line HPLC–EPR–MS with a Vestec electrospray source [12]. Thus the above assignment was confirmed showing that the HPLC–ED–UV technique is a powerful tool to identify the respective forms of the radical adduct.

Applied potential (guard cell) dependence of the peak height (UV detector) was measured under neutral mobile phase [50 m*M* ammonium acetate–32% acetonitrile (v/v)] using the HPLC–ED–UV for the peak 3 (reduced form) and peak 2 (radical form) fractions of the 4-POBN-phenyl radical adduct (Fig. 4), respectively. The peak heights of the reduced and radical forms decreased to half the maximum peak heights at +0.18 V and +0.40 V applied potentials, respectively, indicating that the reduced form is more easily oxidized than the radical form under the conditions employed here. If the reduced form was analyzed using the HPLC–ED–UV with a +0.2 V applied potential, the radical form peak would be predominant. The peak heights of the oxidized and radical forms were changing in a symmetric manner when the purified radical form fraction was analyzed using the HPLC–ED–UV with various applied potentials on the guard cell (Fig. 4). This result indicates that the oxidized form is relatively stable and does not turn to further oxidized forms under the conditions employed here.

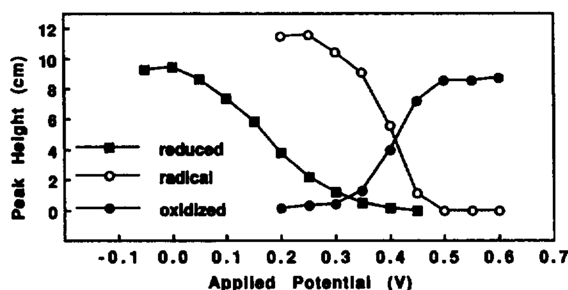


Fig. 4. Applied potential dependence of the peak height of 4-POBN-phenyl radical adduct. Ten μl of the purified peak 2 fraction (radical form) or peak 3 fraction (reduced form) was applied to the HPLC–ED–UV analyses. The peak heights were monitored on the HPLC–ED–UV with different applied potentials (+0.45 V to −0.05 V for the reduced form, +0.6 V to +0.2 V for the radical form). The HPLC conditions were as described in Section 2 except for the mobile phase. The mobile phase of the HPLC–ED–UV was 50 m*M* ammonium acetate–32% acetonitrile (v/v).

3.2. Quantification of the reduced and radical forms of the 4-POBN-phenyl radical adduct

The reduced and radical forms of the 4-POBN-phenyl radical adduct were purified by the HPLC–ED–UV. The concentrations of the reduced and radical forms of the 4-POBN-phenyl radical adduct were estimated using the HPLC–UV–ED. Because 100% of the reduced and radical forms of the 4-POBN-phenyl radical adduct, which flow through the analytical cell, are oxidized on the Model 5010 analytical cell of Coulochem electrochemical detector, the concentrations of the reduced and radical forms of the 4-POBN-phenyl radical adduct can be estimated using the following formula:

$$Q = n \times F \times \text{Eq} \quad (3)$$

The n in the Eq. (3) is the number of electrons which one molecule donates or accepts during the oxidation or reduction process on the analytical cell. When the reduced form of the 4-POBN-phenyl radical adduct is oxidized at +0.55 V, $n=2$ because the reduced form of the 4-POBN-phenyl radical adduct is directly oxidized to the oxidized form under the applied potential (see Fig. 2 and Fig. 4). On the other hand, $n=1$ when the radical form is oxidized to the oxidized form (see Fig. 2). In Eq. (3) F represents 1 Faraday of charge (96 493 coulombs), the charge of an Avogadro's number of electrons. Eq in Eq. (3) is the amount (mol) of the reduced or radical forms of the 4-POBN-phenyl radical adduct which are oxidized on the analytical cell. Q in Eq. (3) is the total current (coulombs) flowing on the electrode, and can be estimated from the peak area of the electrochemical detector, i.e., $Q=\int i \, dt$=peak area. The peak areas (mV×s for UV and μC for ED) of 5 different injection volumes of the purified reduced and radical forms are presented in Table 1. Thus the concentrations of the reduced and radical forms of the 4-POBN-phenyl radical adduct were determined using the HPLC–UV–ED without an internal standard.

3.2.1. Quantification of the oxidized form of the 4-POBN-phenyl radical adduct

The oxidized form of the 4-POBN-phenyl radical adduct was purified by the HPLC–ED–UV. It is impossible to determine the concentration of the oxidized form of the 4-POBN-phenyl radical adduct using the method described above for the reduced and radical forms since the oxidized form of the 4-POBN-phenyl radical adduct is inactive on the HPLC–ED analyses. In order to estimate the con-

Table 1
HPLC–UV–ED peak areas and concentrations of the purified respective forms of the 4-POBN-phenyl radical adduct

Volume (μl)	Peak area							
	Reduced form			Radical form			Oxidized form	
	UV (mV×s)	ED (μC)	Conc. (μM)	UV (mV×s)	ED (μC)	Conc. (μM)	V (mV×s)	Conc.[a] (μM)
10	66	97	50	47	51	63	99	70
20	135	189	49	96	104	65	174	67
30	198	276	48	145	157	65	260	67
40	265	365	47	194	211	66	350	68
50	357	482	50	236	256	65	432	67
Mean			49±1.3			65±1.1		68±1.3

The purified respective forms of the 4-POBN-phenyl radical adduct were analyzed. Reaction conditions were as described in Section 2 except for the mobile phase. A mobile phase of the HPLC–UV–ED and the HPLC–ED–UV was 50 mM ammonium acetate–32% acetonitrile (v/v). The applied potential of the ED analytical cell is +0.55V.

[a] In order to estimate the concentration of the oxidized form, the standard UV peak area of the oxidized form, which formed from 485 pmol of the reduced form using HPLC–ED–UV, was used. The standard peak area of the oxidized form contains 485 pmol of the oxidized form because all molecules of the reduced form stoichiometrically turn to the oxidized form by the guard cell. The concentrations of the oxidized form in the samples were determined comparing the UV peak areas of the oxidized form of the samples with the standard UV peak area (52.5 mV×s).

centration of the oxidized form, a standard UV peak area of the oxidized form, which formed from 485 pmol of the reduced form using the HPLC–ED–UV, was used. The concentration of the oxidized form of the 4-POBN-phenyl radical adduct was estimated using the HPLC–UV–ED. The standard UV peak area of the oxidized form contains 485 pmol of the oxidized form because all molecules of the reduced form stoichiometrically turn to the oxidized form by the guard cell. The peak areas (mV×s for UV and μC for ED) of 5 different injection volumes of the oxidized form of the 4-POBN-phenyl radical adduct are presented in Table 1. Thus the concentrations of the oxidized form in the samples were determined comparing the peak areas of the oxidized form with the standard UV peak area (52.5 mV×s).

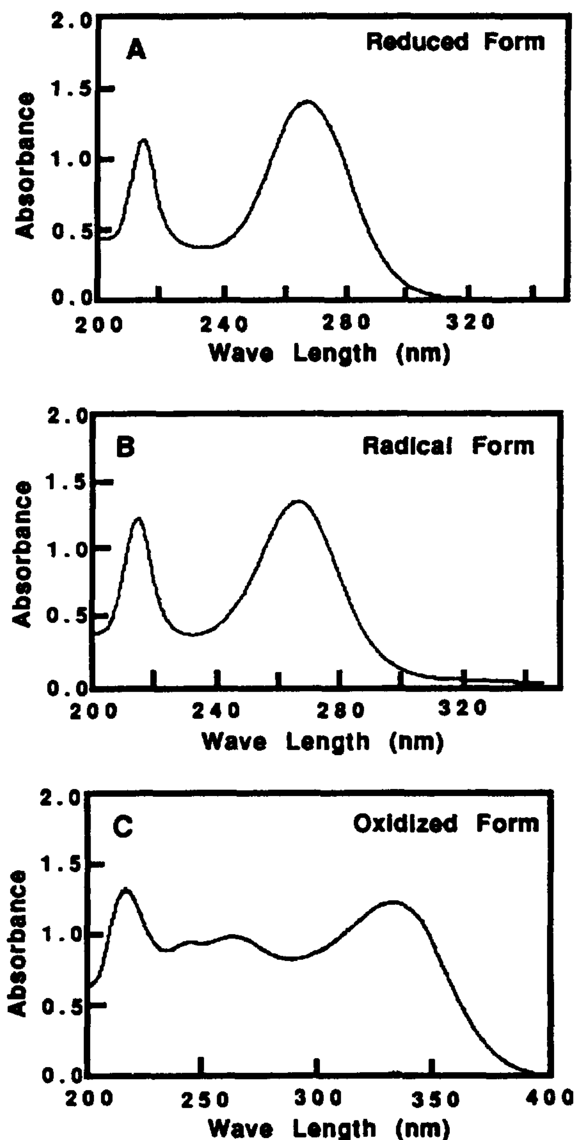


Fig. 5. UV spectra of the purified fractions of respective forms of the 4-POBN-phenyl radical adduct. The conditions of the UV measurement were as described in Section 2. (A) Reduced form; (B) radical form; (C) oxidized form.

3.2.2. *UV spectral analyses*

The UV spectra were measured for the purified peak 1 (reduced form), peak 2 (radical form) and peak 3 (oxidized form) fractions (Fig. 5). The λ_{max} and ε_{max} of the respective forms are as follows: reduced form [λ_{max} = 214 nm, ε_{max} = 2.34×10^4 (cm·l/mol); λ_{max} = 266 nm, ε_{max} = 2.91×10^4 (cm·l/mol)], radical form [λ_{max} = 215 nm, ε_{max} = 1.88×10^4 (cm·l/mol); λ_{max} = 265 nm, ε_{max} = 1.74×10^4 (cm·l/mol)], and oxidized form [λ_{max} = 215 nm, ε_{max} = 1.85×10^4 (Cm·l/mol); λ_{max} = 243 nm, ε_{max} = 1.35×10^4 (cm·l/mol); λ_{max} = 263 nm, ε_{max} = 1.40×10^4 (cm·l/mol); λ_{max} = 330 nm, ε_{max} = 1.76×10^4 (cm·l/mol)]. Molar absorbance coefficients obtained above were estimated by using the respective concentrations which were determined by the above HPLC–UV–ED analyses.

Acknowledgments

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