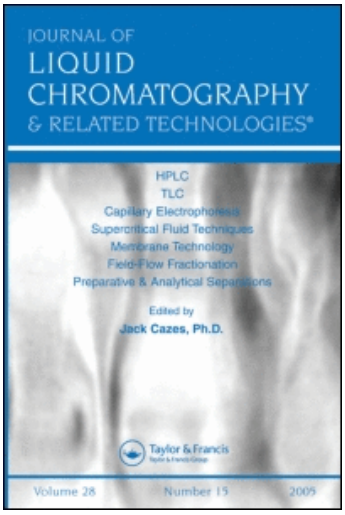

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SIMULTANEOUS DETECTION OF SUPEROXIDE ANION, HYDROXYL RADICAL, AND METHYL RADICAL BY USE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTRON SPIN RESONANCE

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ABSTRACT

Electron spin resonance (ESR) spectroscopy has been used to investigate free radical formation in chemical and biochemical reaction. Spin trapping technique has been used to detect short-lived radicals. The radical chromatography using ESR for HPLC detector was carried out for simultaneous detection of free radicals of similar spin adducts g value. Free radical mixtures, such as O_2^-, •OH and •CH_3, were prepared by mixing DMSO, H_2O_2, NaOH and ferric ions in the presence of 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) and N-t-butyl-phenylnitrone (PBN) as spin trapping agents.

DMPO or PBN adducts of O_2^-, •OH and •CH_3 were well separated on Inertsil ODS-2 column with citrate buffer (pH 7.0) /acetonitrile (80/20) or HEPES buffer (pH 7.0) /acetonitrile (50/50) as a mobile phase.

When the sample solution of mixed free radicals was loaded on the column, three peaks due to the spin adducts appeared in the ESR chromatogram. Corresponding ESR spectra of each peak was recorded. This specific and direct detection method permitted the simultaneous quantitation of mixed free radicals in the sample, and it is applicable for various samples including biological materials.

INTRODUCTION

The roles of free radicals in various diseases have been investigated[1-2] and detection methods are attracting much attention from various medical fields. However, the short life of these species makes the detection difficult, and in order to overcome these difficulties, spin trapping has been developed and widely applied,[3-5] short-lived free radicals could be detected as stable spin adducts with ESR.

Several different free radicals are produced in a given system and it is difficult to identify each spin adduct because each spectra have similar g values.[6] A high performance liquid chromatography-ESR (HPLC-ESR), where an ESR spectroscopy is usual as a detector, has been utilized to detect, isolate, and identify a certain one radical adduct.[7-9]

In this paper, we determined the spin-trapped superoxide anion, hydroxyl radical, and methyl radical simultaneously by using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and N-t-butyl-α-phenylnitrone (PBN) for spin trapping agent.

EXPERIMENTAL

Chemical and Reagents

Hydrogen peroxide (30% w/v), dimethylsulfoxide (DMSO), ammonium iron (II) sulfate hexahydrate, sodium hydroxide, diethylenetriamine-N, N, N', N", N"-pentaacetic acid (DTPA), hypoxanthine (HPX) were obtained from Wako Pure Chemical Industries (Osaka, Japan). 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) and 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were purchased from Dojindo Laboratories (Kumamoto, Japan). N-t-butyl-phenylnitrone (PBN), xanthine oxidase (XOD), and 4-hydoroxyl-6-tetramethylpyperidon-N-oxyl (4-hydroxy TEMPO) were also obtained from Sigma Co. (St.Louis, MO).

Instruments

The HPLC system used for HPLC-ESR consisted of a Rheodyne (Cotati, CA, USA) Model 7025 loop injector for sample injection, and a Shimadzu LC-10AS pump (Shimadzu, Kyoto Japan) for the delivery system of mobile phase. ESR spectroscopy used was a JEOL RE-1X ESR spectroscopy (JEOL, Tokyo, Japan). The HPLC column used was an Inertsil ODS-2 (4.6 mm i.d.×150 mm) (GL Science, Tokyo, Japan). A quartz flow cell (200 μL), fixed in the ESR cavity was connected to the exit of the column with teflon tubing (0.5 mm i.d.).

HPLC Conditions

The mobile phases for separation of DMPO and PBN adducts are citrate buffer (pH 7.0) /acetonitrile (80:20), HEPES buffer (pH 7.0) / acetonitrile (50:50) spiked with 0.5 mM of DTPA, respectively. The flow rate of mobile phase was set at 0.4 mL/min for the isolation of DMPO adducts, and 0.5 mL/min for the isolation of PBN adducts.

ESR Conditions

The ESR conditions are as follows: magnetic field, 336.5mT; microwave power, 8 mW; modulation amplitude, 0.063 mT; frequency, 100 kHz; time constant, 0.03 sec; scan range, 10 mT; scan time, 1 min; receiver gain, ×500.

Generation of Superoxide Anion by HPX-XOD System

Superoxide anion (O_2^-) was generated by mixing 2.0 mM HPX with 0.4 unit/mL XOD. Fifty micro liters of HPX, 50 μL of 0.1 M HEPES buffer (pH 7.4) and 5 μL of DMPO was put into a test tube. After addition of 50 μL of XOD to the solution and stirring well, the mixed solution was taken into an aqueous flat ESR cell.

Generation of Hydroxyl Radical by Fenton's Reaction

Hydroxyl radical (•OH) was generated by mixing 0.1 mM $FeSO_4$ with 0.1% H_2O_2 (Fenton's reaction). A mixture of 50 μL of $FeSO_4$ solution, 100 μL of 0.05 M HEPES buffer (pH 7.4), 5 μL of DMPO and 50 μL of H_2O_2 were put into a test tube successively. After stirring, the mixed solution was placed in an aqueous ESR flat cell.

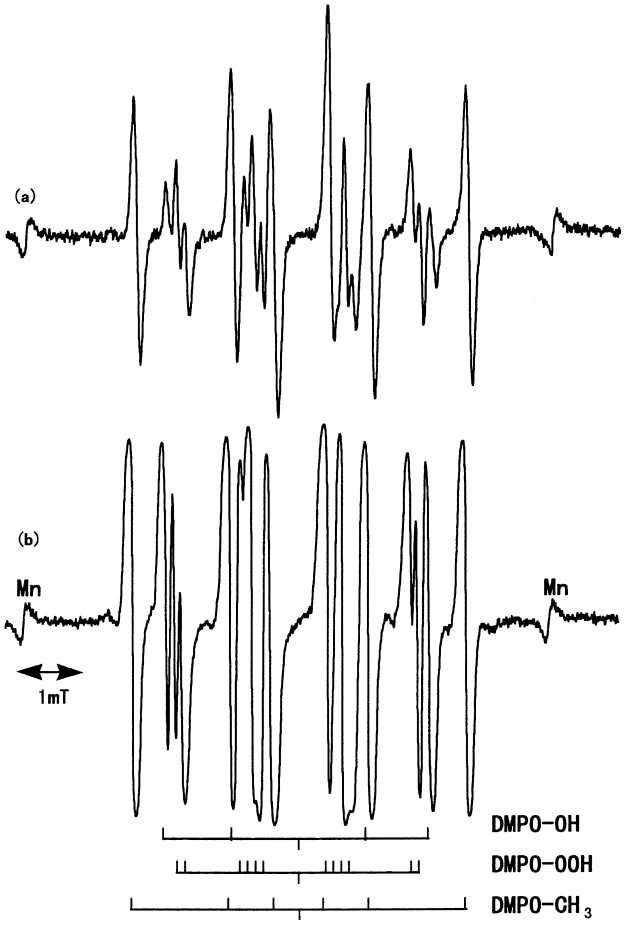

Figure 1. ESR spectra of DMPO adducts obtained from (a) DMSO/H_2O_2/ NaOH system and (b) in the presence of ferric ions.

RESULTS AND DISCUSSION

Non-Enzymatic Generation of Free Radicals

It is well known that enzymatic generation as xanthine/xanthine oxidase be used for the generation of superoxide anion radical. However, if the enzyme is directly applied to HPLC, the column in the HPLC is deteriorated.

Figure 2. ESR spectra of PBN adducts obtained from the DMSO/H_2O_2/NaOH system.

Table 1

Effect of Ferric Ion on ESR Signal Intensity

DMPO Adduct	None S/M	5mM $Fe(NH_4)_2SO_4)_2$ S/M
DMPO-CH_3	6.76	8.92
DMPO-OH	1.33	7.25
DMPO-OOH	2.73	5.79

In this study, a non enzyamtic generation system was examined for generating some free radical materials. The mixture of methyl radical ($\bullet CH_3$), $\bullet$OH and O_2^- was produced chemically using DMSO and H_2O_2 in alkaline condition. The reaction mixture containing 100 μL of DMSO, 5 μL of DMPO, or 50μL of 10% PBN, 50 μL of 30% H_2O_2 and 50 μL of 25 mM NaOH were stirred together, and was placed in ESR flat cell. The ESR spectrum in Figure.1 (a) seem to be mixture of three types of DMPO spin adducts, such as DMPO-O_2, DMPO-OH and DMPO-CH_3. from hyperfine splitting value.[10]

After the addition of 5 μL of $FeSO_4$, signal intensity was remarkably increased as shown in Fig.1 (b). Effect of ferric ion on ESR signal intensity is shown in Table 1. The ratio of signal intensity against Mn^{2+} as a reference is represented by S/M. By addition of Fe^{2+}, signal intensity of DMPO-CH_3, DMPO-OH and DMPO-OOH were increased by a factor of 1.3, 5.5, and 2.1 respectively. Figure 2 shows ESR spectrum of PBN adducts. PBN, a spin trapping reagent, provides little information because its adducts have similar hyper fine splitting constants. Thus it is necessary to isolate these adducts.

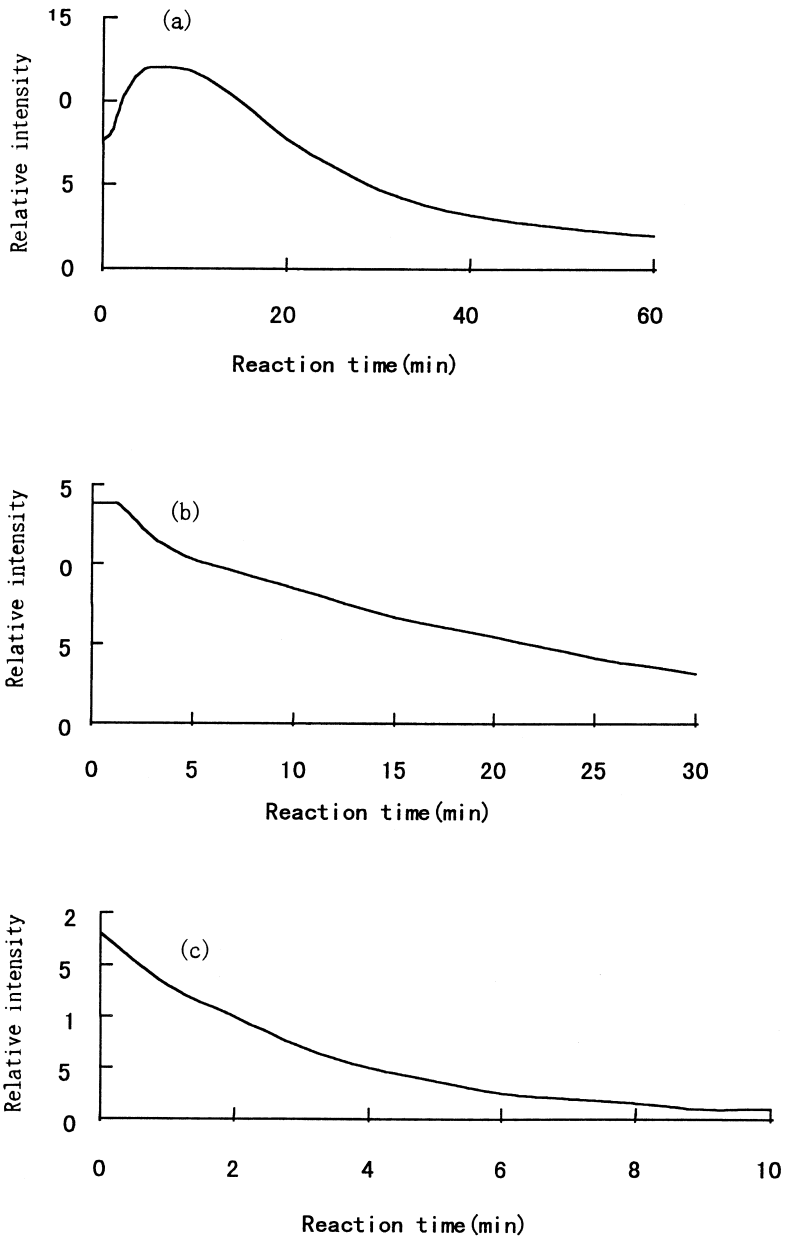

Figure 3. Time course of the ESR signal intensity of (a) DMPO-OOH, (b) DMPO-OH, (c) DMPO-CH_3. Relative intensities (S/M) of DMPO-OOH, DMPO-OH and DMPO-CH_3 adducts against Mn^{2+} was measured after mixing of DMSO,H_2O_2, NaOH and Fe (II) by ESR spectrometry.

Stability of Each Spin Adduct

O_2^- generated by HPX-XOD system was gradually decreased and ESR spectrum disappeared completely within 10 min. As the intensity of spectrum of DMPO-OOH decreased, the intensity of DMPO-OH spectrum increased. The concentration of DMPO-OH produced by Fenton's reaction reached the maximum around 5 min. Signal intensity gradually decreased after 5 min. Relative signal intensities of DMPO-OOH, DMPO-OH and DMPO-CH_3 generated from DMSO/H_2O_2/NaOH/Fe (II) were shown in Fig.3 (a)~(c). The maximum signal intensity of DMPO-OOH was observed at 5 min and signal intensity of DMPO-OOH was gradually decreased. Also signal intensity of DMPO-OH decreased after 2 min, and that of DMPO-CH_3 was gradually decreased.

HPLC-ESR Condition for Separation

In order to determine the free radicals by HPLC-ESR in aqueous solution, TEMPOL was selected as a standard radical among the other radicals for its superior solubility and stability in aqueous condition. The following conditions are set for ESR measurement: Field modulation frequency, 100 kHz; Field modulation width, 0.073 mT; Time constant: 0.1 sec.

Separation of Spin Adducts of Free Radicals

It is difficult to detect three adducts of free radicals by ESR spectrometry because of similar hyper fine splitting constants. In this study, we identified by the use of ESR, separation of three components by HPLC.

In order to determine HPLC conditions for separation of radicals, the optimum eluent was examined. For separation of DMPO adducts, eluent of 20% acetonitrile/citrate buffer (pH 7.0) spiked with DTPA as a chelator was used. As flow cell capacity in the ESR apparatus was 200 μL, it was difficult to detect ESR signal of free radical adducts in high sensitivity at more than 0.5 mL/min. Therefore, an optimum flow rate of 4.0 mL/min. was used to detect in a short time. 50% Acetonitrile/HEPES buffer (pH 7.0) spiked with DTPA was used for separation of PBN adducts. Column temperature was R.T. In this experiment, the magnetic field of ESR was fixed at the position where the ESR signals were expected. The sweep width of 1 mT was applied to cover a wide range. The operation was carried out at 5 sec of sweep time and 10 mW of microwave power. Figure. 4 shows the HPLC/ESR chromatogram obtained from the mixture containing DMPO under these conditions. ESR spectrum from peak (a), (b) and (c) are depicted in Fig.4 (A)~(C), respectively. The spectrum obtained from peak (a)~(c) has hyperfine splitting (HFS).[11] Spectrum (A) has

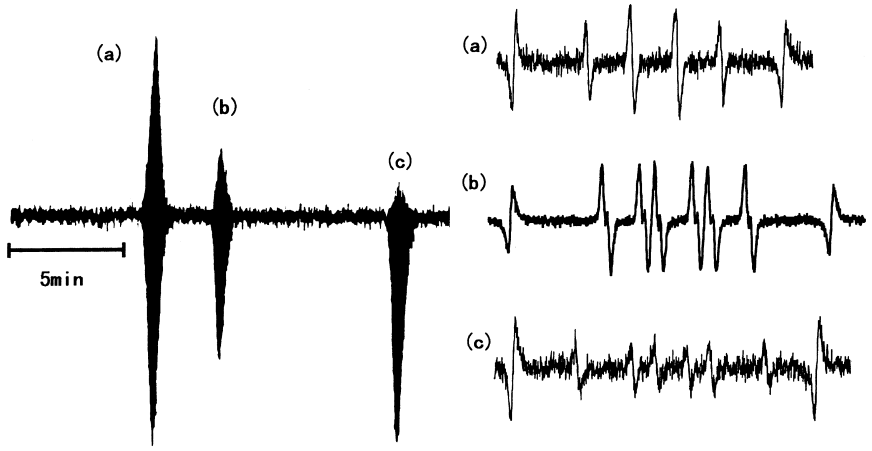

Figure 4. HPLC/ESR chromatogram of DMPO adducts and ESR spectra (a) DMPO-OH, (b) DMPO-OOH, (c) DMPO-CH_3.

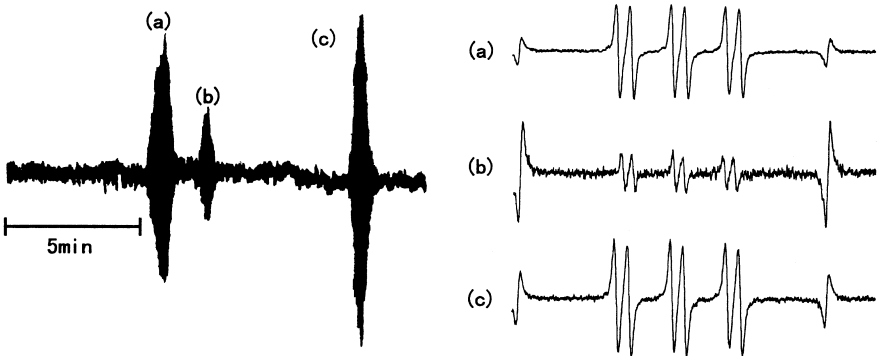

Figure 5. HPLC/ESR chromatogram of PBN adducts and ESR spectra (a) PBN-OH, (b) PBN-OOH, (c) PBN-CH_3.

HFS: a_N = 1.43 mT, $a\beta_H$ = 1.17 mT, $a\gamma_H$ = 0.13 mT. Spectrum (B) has HFS: a_N = $a\beta_H$ = 1.49 mT. Spectrum (C) has HFS: a_N = 1.64 mT, $a\beta_H$ = 2.24 mT. Peak (a), (b), and (c) represented DMPO-OOH, DMPO-OH, and DMPO-CH_3 respectively.

Figure 5 shows the HPLC/ESR chromatogram obtained from the mixture containing PBN. Peak (a), (b), and (c) are obtained from ESR spectrum a, b, and c respectively. Spectrum (A) has HFS: a_N = 15.56 mT, a_H = 3.79 mT.

Spectrum (B) has HFS: a_N = 14.36 mT, a_H = 2.80 mT. Spectrum (C) has HFS: a_N = 15.75 mT, a_H = 3.59 mT. Peak (a), (b), and (c) represent PBN-OH, PBN-OOH, and PBN-CH_2OH respectively.

CONCLUSIONS

Free radical mixtures, such as O_2^-, •OH and •CH_3 were prepared by mixing DMSO, H_2O_2, NaOH and ferric ions in the presence of 5,5-dimethyl-1-pyrrolin-N-oxide (DMPO) or phenyl-tert-butylnitrone (PBN) as spin trapping reagents.

DMPO or PBN adducts of O_2^-, •OH, •CH_3 derived from similar spin adducts of g value were well separated on ODS column with citrate buffer /CH_3CN (80/20) or HEPES buffer (pH 7.0)/CH_3CN (50/50) as a mobile phase.

HPLC-ESR method permitted the simultaneous detection of mixed free radicals in the sample, and it seems to be applicable for various free radical materials generated in biological materials.

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