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DETECTION OF SOME RETINOID RADICALS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTRON SPIN RESONANCE SPECTROSCOPY OR ELECTROCHEMICAL DETECTION

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SUMMARY

Radical species were detected in the incubation mixtures of some retinoids (retinoic acid, retinal, retinol and retinyl acetate) by using the spin-trapping technique. The spin-adducts were resolved by high-performance liquid chromatography on a reversed-phase column with isocratic elution and detected by electron spin resonance spectroscopy and electrochemical detection. The spin-adducts were eluted in the order retinoic acid, retinol, retinyl acetate, in a similar manner to the retinoids themselves. These results suggest that the spin-adducts are products of nitrosobenzene with retinoid radicals in which the retinoid radicals retain their original chemical structures.

INTRODUCTION

Radical species participate in many biological reactions, *e.g.*, lipid peroxidation (enzymatic and non-enzymatic)[1,2], generation of active oxygen[3]. It has been recognized that biological damage mediated by radical species is an important factor in disease and aging[3]. Extensive studies on radical species have been undertaken using various techniques. Electron spin resonance (ESR) spectroscopy is a direct method for detection of radical species. However, it has been difficult to obtain ESR spectra of the free radicals because of their short lifetimes. Recently, spin trapping has been developed to detect and identify the short lifetime free radicals in reacting systems. This technique consists of using a spin trap, *i.e.*, a compound that forms a stable free radical by reacting covalently with an unstable free radical. Indeed, many radical species were detected by using this technique. However, the spin-adducts for many radical species have not been characterized because the complete chemical structure of radical species cannot be determined by analysis of the ESR signals of the spin-adducts alone, particularly in systems containing plural radical species. To overcome these difficulties, separation of the spin-adducts is necessary. Thus, high-performance liquid chromatography in combination with electron spin resonance spectroscopy (HPLC–ESR) has become a new strategy for the elucidation of reactions involving radical species[4]. Recently, we separated retinoic acid radicals by using this tech-

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nique[5], and the mechanism of haemoglobin- (or haematin-) catalyzed retinoic acid 5,6-epoxidation which involves a radical intermediate could be clarified.

In this paper, we describe the application of HPLC–ESR and of HPLC with electrochemical detection (HPLC–ED) to the retinoid radicals (retinoic acid, retinol, retinal and retinyl acetate). The advantages and disadvantages of the two techniques are discussed.

MATERIALS AND METHODS

Materials

all-*trans*-Retinoic acid, all-*trans*-retinal, all-*trans*-retinol and all-*trans*-retinyl acetate were obtained from Sigma (St. Louis, MO, U.S.A.), nitrosobenzene from Nakarai (Kyoto, Japan). All other chemicals used were commercial products of the highest grade available.

Instruments

HPLC was performed on a Jasco Trirotor-V with an electrochemical detector VMD-501 (Yanagimoto, Kyoto, Japan) and an electron spin resonance spectrometer FX2XG (JEOL, Tokyo, Japan).

ESR measurements

The ESR spectra were obtained after incubation for 30 min at 20°C. The standard incubation mixture contained, in a total volume of 0.44 ml, 0.4 ml of buffer (0.1 *M* potassium phosphate, pH 7.5 and 4% Triton X-100), 20 μl of retinoic acid (1 mg/0.1 ml in dimethyl sulphoxide) and 20 μl of nitrosobenzene (0.1 *M* in methanol). The measurements were made at 20°C by using an aqueous solution cell. The microwave power was 5 mW, the modulation frequency was 100 kHz and the amplitude 63 μT. The sweep time was 2.5 mT/min, and the receiver gain was $2.0 \cdot 10^3$ with a response time of 0.3 s.

HPLC–ESR measurements

The HPLC–ESR technique was as described previously[5]. The ESR spectrometer was connected to the HPLC system with a PTFE tube (0.5 mm I.D.) which passed through the ESR cell (Fig. 1). After incubation for 30 min at 20°C, the mixture was subjected to HPLC–ESR. A 2-ml volume was injected into the HPLC–ESR

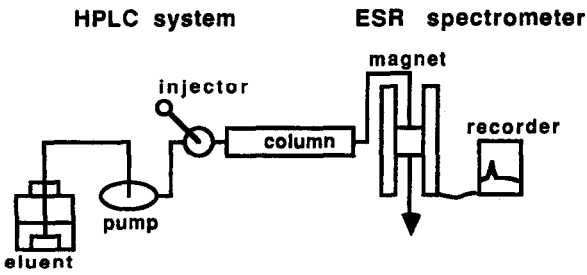

Fig. 1. Diagram of apparatus used for HPLC–ESR.

apparatus, because of the low sensitivity of ESR compared with other HPLC detectors, *e.g.*, UV and ED. A column (250 mm × 4.6 mm I.D.) packed with TSK ODS gel (5 μm) was used at a flow-rate of 2.0 ml/min with 10 m*M* ammonium acetate and water–methanol (15:85) as the mobile phase. The column was kept at 40°C throughout. The magnetic field of the ESR spectrometer was fixed at the position ($g = 2.017$) indicated by the bold arrow in Fig. 2. The microwave power was 5 mW, and the modulation frequency was 100 kHz with an amplitude of 0.63 mT. The receiver gains were $5 \cdot 10^3$ (retinoic acid) and $10 \cdot 10^3$ (retinol, retinyl acetate and retinal) with a response time of 1.0 s.

HPLC–ED measurements

The standard incubation mixtures were incubated for 10 min at 20°C. A 10-μl portion was subjected to HPLC–ED analysis. The HPLC conditions were as described above except for the mobile phase which was methanol–water (70:30) containing 10 m*M* ammonium acetate. Applied potential: +0.7 V.

RESULTS

ESR spectrum of the spin-adduct of nitrosobenzene with the radical formed in the incubation mixture of retinoic acid

Retinoic acid and nitrosobenzene were incubated at 20°C for 30 min. An ESR signal was detected in the reaction mixture (Fig. 2)[5–6]. The hyperfine coupling constant (hfc) (1.5 mT) of the ESR signal appears to originate from the interaction of an unpaired electron with the nitrogen atom of nitrosobenzene. The broad and distorted signal could be attributable to the interaction between the spin-adduct and a Triton X-100 micelle, since the interaction disturbs the isotropic rotation of the spin-adduct. The signal was not detected in the absence of nitrosobenzene or retinoic acid. The spectrum of Mn^{2+} in manganese(II) oxide was used as a reference, for which the splitting between the two central peaks is 86.9 G. The phases of the oxide peaks and the sample peaks are different, because of their different positions in a microwave cavity, *i.e.*, the directions of the modulation magnetic field are opposite. Similar spectra were also recorded for reaction mixtures of nitrosobenzene with some other retinoids (retinal, retinol and retinyl acetate)[7].

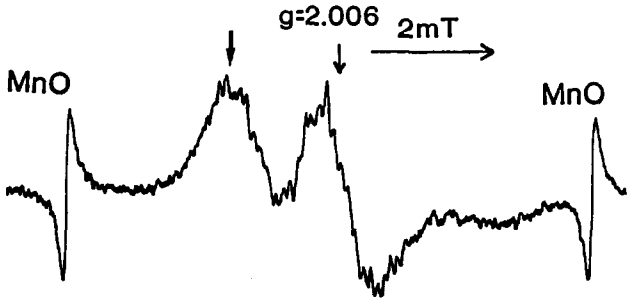

Fig. 2. ESR spectrum of the spin-adducts of nitrosobenzene with radicals formed in an incubation mixture of retinoic acid. The ESR and the incubation conditions were as described in the text.

HPLC–ESR analyses of the spin-adducts of nitrosobenzene with radicals formed in the incubation mixtures of retinoids

The spin-adducts detected in the incubation mixtures of nitrosobenzene with the retinoids were analyzed by using HPLC–ESR (Fig. 3). Portions (2 ml) of the mixtures were employed. Some peaks due to radical species were detected in all the HPLC–ESR elution profiles of the incubation mixtures of the retinoids except for that of retinal. The absence of a peak in the case of retinal seems to be due to the low concentration of the radical formed. The retention times of the peaks were all different. The spin-adducts were eluted from the reversed-phase column in the order retinoic acid, retinol, retinyl acetate, similar to that of the retinoids themselves.

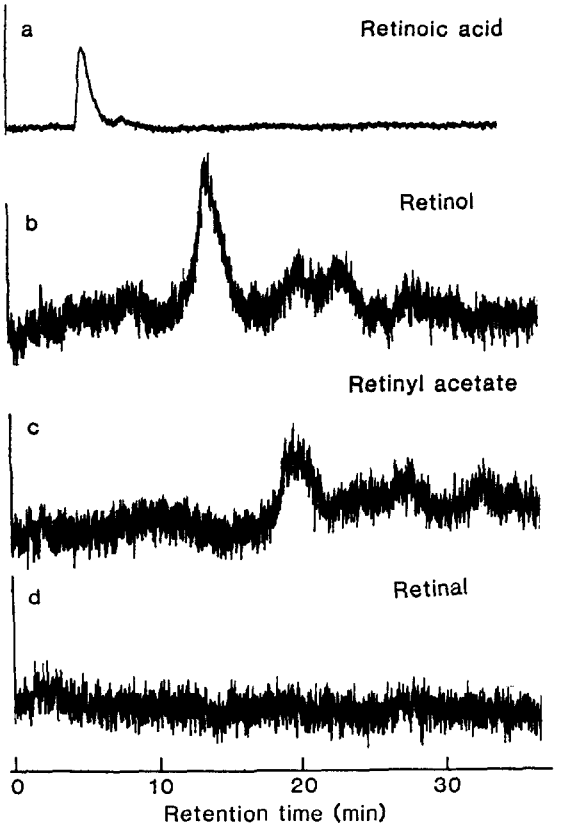

Fig. 3. HPLC–ESR analysis of the spin-adducts of nitrosobenzene with radicals formed in incubation mixtures of retinoids. HPLC, ESR and incubation conditions as described in the text.

HPLC–ED of the spin-adducts of nitrosobenzene with radicals formed in the incubation mixture of retinoic acid

Rather large quantities (2 ml) of the incubation mixtures were subjected to HPLC–ESR because of the low sensitivity of this technique. To overcome this disadvantage, we detected the spin-adducts of nitrosobenzene with the radicals formed

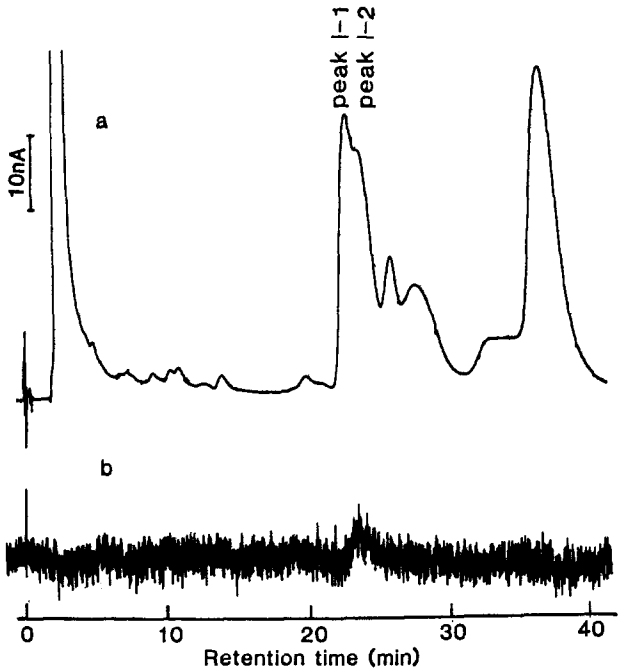

Fig. 4. HPLC–ED analysis of the spin-adducts of nitrosobenzene with radicals formed in an incubation mixture of retinoic acid. HPLC, EC and incubation conditions as described in the text except for the applied volume which was 2 ml. (a) HPLC–ED; (b) HPLC–ESR.

in the incubation mixture of retinoic acid by using the HPLC–ED technique (Fig. 4a). Comparing the elution profile obtained with HPLC–ESR (Fig. 4b) and that with HPLC–ED (Fig. 4a), it is seen that the retention times of peaks I-1 and I-2 in two profiles are identical. These peaks were not detected by HPLC–ED in the absence of nitrosobenzene or retinoic acid (Fig. 5). To confirm that they are due to radical species, HPLC–ED analyses were performed with addition of ascorbic acid to the reaction mixture. This resulted in complete disappearance of the ESR signal (Fig. 6b and c, left). Peaks I-1 and I-2 were not detected in the HPLC–ED elution profiles, suggesting that they are due to radical species.

In the HPLC–ED analysis of the reaction mixture of retinoic acid, the applied potential was varied from +0.4 to +0.9 V *vs.* silver–silver chloride (Fig. 7). The cell current reached a plateau at +0.9 V and half of the maximum cell current was observed at +0.7 V.

DISCUSSION

In this investigation, spin-adducts of nitrosobenzene with some radicals formed in incubation mixtures of retinoids (retinoic acid, retinal, retinol and retinyl acetate) were separated by HPLC–ESR. The retention times of the spin-adducts were all different. The results indicate that the spin-adducts are derived from retinoid radicals,

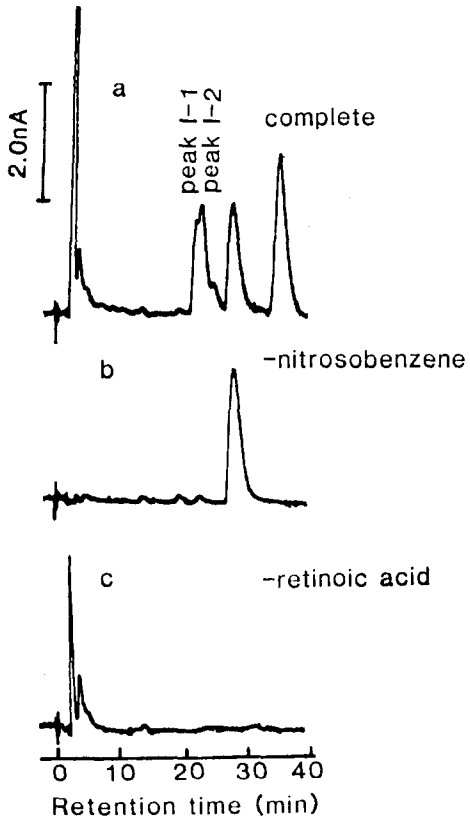

Fig. 5. HPLC–ED analyses of a complete incubation mixture (a) and incubation mixtures in the absence of nitrosobenzene (b) or retinoic acid (c). HPLC, ED and incubation conditions as described in the text.

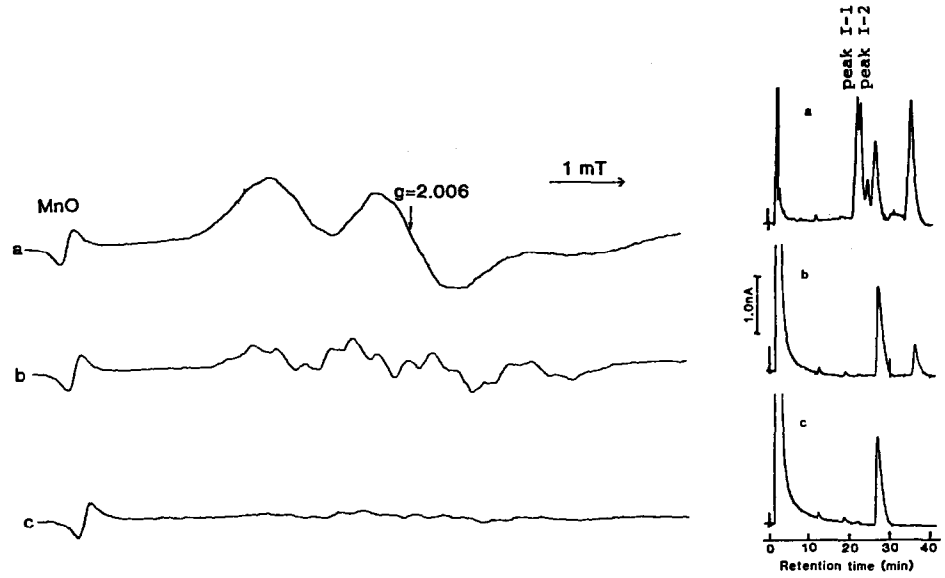

Fig. 6. Addition of ascorbic acid to an incubation mixture of nitrosobenzene with retinoic acid. Left, ESR spectra; right, HPLC–ED analyses. Ascorbic acid was added to the standard reaction mixture. Other ESR, HPLC–ED and incubation conditions as described in the text. (a) Standard incubation mixture without ascorbic acid, (b) standard incubation mixture with 0.2 mg ascorbic acid; (c) standard incubation mixture with 0.5 mg ascorbic acid.

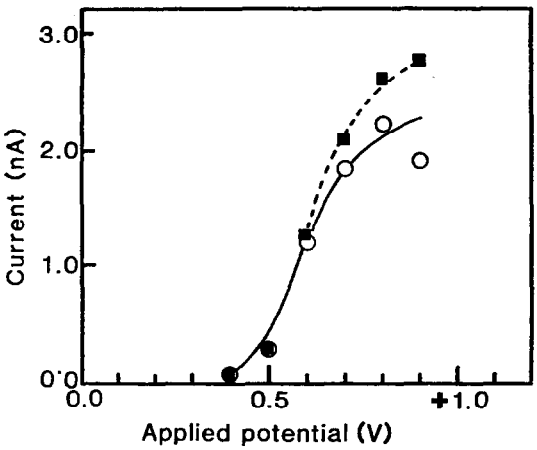

Fig. 7. Peak heights of the peaks I-1 (○) and I-2 (■) at various applied potentials of the electrochemical detector. Incubation and HPLC–ED conditions as described in the text.

not from oxygen radicals. It seems that the chemical structures of the retinoid radicals are not so different from those of the original retinoids, because the retention times of the spin-adducts increase in a similar order to those of the original retinoids.

On the other hand, radical species were also detected by HPLC–ED (Fig. 3). Upon addition of ascorbic acid to the incubation mixture, peaks I-1 and I-2 were absent from the HPLC–ED elution profiles and the ESR signal due to the spin-adducts of nitrosobenzene with retinoic acid radicals also disappeared. This does not seem to be due to reduction of the spin adducts, $-N(R)-O^{\cdot} \rightarrow -N(R)-OH$, because a new peak due to –N(R)–OH, an oxidizable species, was not detected by ED. Instead, it seems to be due to an inhibitory effect of ascorbic acid on the formation of retinoid radicals. Some oxidizable species other than radical species were also detected by HPLC–ED. Although this technique has a disadvantage, *i.e.*, a lack of specificity for radical species, its sensitivity is superior to that of HPLC–ESR by a factor of 10^2–10^3. Thus, we believe that HPLC–ESR and HPLC–ED are complementary methods suitable for the study of radical species.

Studies on other reactions involving radical species are now in progress.

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