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In Vivo L-Band ESR and Quantitative Pharmacokinetic Analysis of Stable Spin Probes in Rats and Mice

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Free radical species in animals have been measured by X-band ESR spectrometric method on a block of organs or a portion of homogenized samples. However, a nondestructive *in vivo* ESR measurement has been realized by using a recently developed L-band ESR spectrometry. With this L-band ESR method, we measured ESR spectra in animals, who received stable nitroxide radicals. L-band ESR spectra were observed at the upper abdomen of mice as well as at the heads of mice and rats at various ages immediately after the intravenous injections of nitroxide radicals such as 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (4-hydroxy-TEMPO) and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-carbamoyl-PROXYL), in which ESR measurements of the radicals were performed noninvasively at the real time. On the basis of the observed time-dependent free radical clearance curves, the following important results were obtained: (1) Free radical clearances were able to analyze by the pharmacokinetic method. (2) The radicals at the head of mice, given 4-hydroxy-TEMPO, were determined quantitatively by a new analytical method using L-band ESR for the first time. (3) The elimination of the radical was found to be saturated in mice. (4) The clearance rate constant of 4-hydroxy-TEMPO detected at the head of mice was decreased in dose- and age-dependent manners. While, no age-dependent clearance rate constant of 4-hydroxy-TEMPO was observed at the upper abdomen of mice. (5) Ratios of the amount of the detected radicals to that of the administered radicals were decreased age-dependently, but they were independent of the dose of the radicals, suggesting the age-dependent decrease of distribution capacity ratio of the radical at the head of animals. (6) Clearance rate constants of 4-hydroxy-TEMPO and 3-carbamoyl-PROXYL, that were estimated by X- and L-band ESR for the collected blood of mice and rats, were found to be remarkably smaller than those in whole living animals observed by *in vivo* L-band ESR method. The results suggest that the clearance of the nitroxide radical is relevant to the alteration of the radical in animals following the change of organ distribution and metabolism. (7) Both the radical and its corresponding hydroxylamine, which is the reduced form of the radical, were detectable by X-band ESR method in the collected urine of mice and rats without and with an oxidizing agent, respectively.

On the basis of the results on L-band ESR spectrometry, the first quantitative pharmacokinetic analysis of stable spin probes in animals is proposed.

Keywords: In vivo L-band ESR, stable nitroxide radical, pharmacokinetic analysis, spin clearance

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INTRODUCTION

Recently, free radical species such as organic radicals, active oxygen species and paramagnetic transition metal ions in living systems have been thought to take part in many biochemical and physiological processes involving aging, development of malignant tumors, many types of disease or defense of organisms.[1,2] ESR spectroscopy has been proved to detect extremely unstable free radical species.[3] For example, the spin trapping method by X-band ESR is widely used to detect the active oxygen species in *in vitro* systems,[4–6] in which unstable free radical species are trapped with spin trapping reagents and converted to the corresponding stable nitroxide radicals. Although the spin trapping method is very useful, it is impossible to detect directly free radical species in living systems. Therefore, *in vivo* measurement is needed to clarify the roles of free radical species in living animals. Feldman *et al.* measured invasively X-band ESR spectrum in the liver of rats treated with a nitroxide radical.[7] On the other hand, we have proposed a blood circulation monitoring-ESR (BCM-ESR) method in living rats receiving nitroxide radicals to analyze their pharmacokinetic features.[8] In general, X-band ESR method is ineffective in detecting free radical species in living animals and aqueous solution at room temperature, since X-band microwave (around 9 GHz) is absorbed by water molecule which has a high dielectric constant.[9] Whereas, L-band microwave (around 1 GHz) has an advantage with a little dielectric loss by water molecule. L-band ESR method is thus expected to be very useful for measuring free radical species or paramagnetic metal ions in living animals or in aqueous solution at room temperature. Recently, we have reported that coordination structures of Cu(II) complexes in aqueous solution at room temperature can be estimated by L-band ESR method.[10,11] However, at present the sensitivity of available L-band ESR apparatus is not sufficient to detect endogenous free radical species or paramagnetic substances in a living animal. Therefore, stable nitroxide radicals as spin-label agents (spin probes) administered to rats or mice have been used to measure the radicals in animals by *in vivo* L-band ESR method.[12–15] In such investigations, the free radicals were not analyzed quantitatively, in which spin clearance rate constant, spectral pattern, or imaging of the radicals have been reported. More recently, nitric oxide (•NO) generated in rats treated with lipopolysaccharide (LPS) has been trapped by high amount of Fe(II) complexes, and the imaging of endogenous •NO was reported by L-band ESR method.[16,17]

Since nitroxide radicals involving stable spin probes and •NO are susceptible to molecular dioxygen, superoxide anion radical and biological redox systems,[18–22] the measurement of nitroxide radicals in living animals may provide valuable information on their biological functions. For instance, decay features of nitroxide radicals administered to animals are assumed to express their physiological or pathological conditions.[23] However, the fundamental investigation on the nitroxide radicals by L-band ESR method is essentially very few. Therefore, we have attempted to study fundamentally the *in vivo* L-band ESR spectrometry on stable nitroxide radicals in whole animals. Further, we have tried to determine the radicals quantitatively by a new method as proposed here. Before us, a pharmacokinetic analysis of nitroxides has been reported, however, the quantitative analytical method has not been sufficiently examined.[24] The purpose of the present paper is to report the first trial on the quantitative pharmacokinetic analysis of different types of spin-labeled nitroxide compound, such as 4-hydroxy-TEMPO (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) and 3-carbamoyl-PROXYL (3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl), on the basis of the L-band ESR measurements at the head and the upper abdomen of animals at several ages.

EXPERIMENTAL

Materials

4-Hydroxy-TEMPO and 3-carbamoyl-PROXYL were purchased from Wako-Pure Chemical Industry (Osaka, Japan) and Sigma Chemical Co. (USA), respectively, and were used without further purification. These probes were dissolved at appropriate concentrations in physiological saline (0.9% NaCl solution). Nembutal sodium injection (pentobarbital: 50 mg/ml) was obtained from Abbott Laboratories Co. (USA). Heparin sodium injection was obtained from Shimizu Co. (Osaka, Japan). Potassium hexacyanoferrate(III) and manganese sulfate were purchased from Nakalai Tesque Inc. (Kyoto, Japan). Capillary tube (10 μl) was obtained from Drummond Scientific Co. (USA). Other reagents were of the highest purity commercially available.

Animals

Male Std-ddy mice (4, 6, 10, 17, 37, and 52 weeks old) and male Wistar rats (3, 4, 6, and 10 weeks old) were purchased from Shimizu Experimental Material Co. (Kyoto, Japan) and used in the experiments. Mice and rats were anesthetized by intraperitoneal (i.p.) injection of pentobarbital at the dose of 50 mg/kg body weight. Mice or rats were euthanized by i.p. injection of high dose of pentobarbital, immediately after *in vivo* measurements of the radicals.

Methods

Administration of Nitroxide Radicals in Animals

Anesthetized mice or rats were fixed on a hand-made Teflon holder. A spin-labeled nitroxide compound dissolved in a saline was intravenously (i.v.) bolus administered into the tail vein by using a needle furnished with polyethylene tube and 1 ml syringe, and L-band ESR spectra due to the radicals were measured immediately after the injection. The ages of animals, dose schedule, and measurement site of the radicals in the body were summarized in Table I. In L-band ESR measurements of the radicals at the upper abdomen of mice, 4-hydroxy-TEMPO was administered to mice aged 4, 6, 17, 37, and 52 weeks at the dose of 1.0 mmol/kg body weight, and 3-carbamoyl-PROXYL was administered to mice aged 4 weeks at the same dose. In the measurements of the radicals at the head of mice, 4-hydroxy-TEMPO was administered to mice aged 4, 6, 17, and 52 weeks at the doses of 0.13 (only 17 weeks old), 0.25, 0.5, and 1.0 mmol/kg body weight. In case of mice at the ages of 10 and 37 weeks, 4-hydroxy-TEMPO was given at the doses of 0.25 and 0.5 mmol/kg body weight, respectively. Rats at the ages of 3, 4, 6, and 10 weeks received 4-hydroxy-TEMPO at the dose of 0.25 mmol/kg body weight in the measurements of radicals at the head.

In Vivo L-Band ESR Measurements

ESR spectra due to nitroxide radicals were measured at every 30 s with an L-band ESR spectrometer JES-RE-3L (JEOL, Japan) equipped with a loop-gap resonator of horizontal model and a R3361A Spectrum Analyzer (Advantest, Japan). The cavity of the ESR spectrometer was maintained at a constant temperature (37°C), which was adjusted by water circulating through thermoregulated water bath. In the measurement of ESR spectra at the upper abdomen of mice, a capillary tube containing DPPH powder as the standard material was attached on the surface of the mice body to check the sensitivity of L-band ESR spectrometer. Instrumental conditions for the L-band ESR measurements were as follows: frequency 1.1 GHz, microwave power 1.0 mW for the measurement at the upper abdomen of mice, 15.5 mW for that at the head of mice and rats, modulation frequency 100 kHz, modulation

TABLE I Clearance rate constants and half lives of spin clearance for 4-hydroxy-TEMPO and 3-carbamoyl-PROXYL, monitored at the upper abdomen and head of mice and at the head of rats at various ages

Animals	Age (weeks)	Body wt. (g)	Dose (mmol/kg)	Domain of measurement	k ($\times 10^{-3}$ s^{-1})	$t_{1/2}$ (s)
4-Hydroxy-TEMPO						
mice	4	20	1.0	upper abdomen	11.6 ± 1.1	60.3 ± 5.7
mice	6	30	1.0	upper abdomen	10.8 ± 0.5	64.7 ± 3.3
mice	17	40–45	1.0	upper abdomen	8.7 ± 0.9	80.0 ± 9.6
mice	37	50–55	1.0	upper abdomen	10.2 ± 2.1	70.1 ± 15.0
mice	52	50–60	1.0	upper abdomen	9.9 ± 1.0	70.2 ± 7.3
mice	4	20	0.25	head	19.2 ± 1.6	36.3 ± 3.0
mice	4	20	0.5	head	14.9 ± 0.4	46.5 ± 1.1
mice	4	20	1.0	head	11.5 ± 0.9	60.7 ± 4.8
mice	6	30	0.25	head	12.4 ± 1.0	56.2 ± 4.4
mice	6	30	0.5	head	11.3 ± 0.5	61.9 ± 2.5
mice	6	30	1.0	head	9.1 ± 0.9	76.7 ± 7.0
mice	10	35	0.25	head	11.7 ± 0.1	61.0 ± 6.4
mice	17	40–45	0.13	head	12.9 ± 0.2	53.5 ± 0.9
mice	17	40–45	0.25	head	11.9 ± 0.7	58.1 ± 3.1
mice	17	40–45	0.5	head	10.4 ± 1.4	67.5 ± 8.8
mice	17	40–45	1.0	head	8.8 ± 0.2	80.4 ± 1.6
mice	37	50–55	0.5	head	9.4 ± 0.2	75.6 ± 2.8
mice	52	50–60	0.25	head	9.6 ± 1.3	73.1 ± 9.3
mice	52	50–60	0.5	head	8.5 ± 0.9	82.6 ± 9.2
mice	52	50–60	1.0	head	7.0 ± 0.2	98.5 ± 3.4
rats	3	60–65	0.25	head	10.9 ± 1.7	65.0 ± 9.0
rats	4	90–100	0.25	head	11.0 ± 0.8	63.6 ± 4.3
rats	6	190–200	0.25	head	9.6 ± 0.8	72.6 ± 5.4
rats	10	240–250	0.25	head	8.6 ± 0.6	81.6 ± 4.8
3-Carbamoyl-PROXYL						
mice	4	20	1.0	upper abdomen	(α) 2.1 ± 0.7	356.7 ± 103.4
					(β) 1.1 ± 0.2	629.0 ± 73.9

Data are the mean values ± standard deviations for 3–4 mice or rats.

amplitude width 1 mT, scanning time 20 s, time constant 0.1 s, and interval time 10 s between each measurement. Ranges of external magnetic field at the upper abdomen of mice and at the head of mice and rats were between 36.5 and 46.5 mT and between 34.0 and 44.0 mT, respectively. The central magnetic field was adjusted to coincide with the field of 2nd spectral signal due to the nitroxide radicals. ESR spectral data were collected and analyzed with an ESPRIT ESR Data System (JEOL, Japan) throughout the investigation. The ESR Data System converted the analogue signal outputted by ESR spectrometer into the digital signal which was inputted into a memory device.

Calibration Lines to Determine the Nitroxide Radicals by L-Band ESR

The calibration lines to determine the radicals at the head of mice were obtained by using both 1.0 ml of 100 mM manganese sulfate in a polyethylene tube (1.5 ml) as the standard material and a rubber balloon with fine quality containing each volume of a saline solution (19, 29, 35, 41.5, 51.5, and 54 ml) as a substitute of mice at each age (4, 6, 10, 17, 37, and 52 weeks). Firstly, 1.0 ml of 100 mM Mn^{2+} (polyethylene tube) and a mouse at each age were fixed on a hand-made Teflon holder, and L-band ESR spectra were measured at various receiver gains. Secondly, 1.0 ml of

100 mM Mn^{2+} (polyethylene tube) and the rubber balloon containing the saline were fixed and L-band ESR spectra were measured similarly as above. Thirdly, several concentrations of 4-hydroxy-TEMPO dissolved in a fresh mouse blood (1.0 ml) in a polyethylene tube (1.5 ml), 1.0 ml of 100 mM Mn^{2+} (polyethylene tube), and the rubber balloon containing the saline were fixed in a similar manner, and L-band ESR spectra were measured at various receiver gains. To estimate the amount of the radical, the intensities of the obtained signals for the radical were normalized by using the signal intensity due to Mn^{2+}. In the same way as analysis of ESR spectra for *in vivo* measurements, the intensity of the lowest magnetic field signal among the monitored triplet signal was used to obtain the calibration lines of 4-hydroxy-TEMPO. Instrumental conditions were the same as those for *in vivo* measurements of the radical at the head of mice, as described above. In measurements of ESR spectra of manganese sulfate (Mn^{2+}), scanning time was fixed to be 200 s between the external magnetic field 50.0 ± 50.0 mT, which corresponded to 20 s between 34.0 and 44.0 mT for the measurement of 4-hydroxy-TEMPO.

Pharmacokinetic Analysis of the Spin Probes

The intensity of the lowest magnetic field signal in the triplet L-band ESR spectrum was used for the pharmacokinetic analysis of the disposition of nitroxide radicals. Spin clearance curves of the signal intensity were semilogarithmically plotted against time after the injection of spin probes. One- or two-compartment model was fitted to these curves by using nonlinear least-squares regression,[25–27] and the spin clearance rate constant (k) was estimated from the slope value of the observed clearance curve, which was obtained from curve-fitting. Half life ($t_{1/2}$) was calculated by an equation of $t_{1/2} = \ln 2/k$.[25–27] The initial amount of 4-hydroxy-TEMPO detected at the head initial radical amount (IA) and the spin clearance rate constant (k) were estimated by curve-fitting with nonlinear least-squares regression from the quantitative spin clearance curve for 4-hydroxy-TEMPO monitored at the head of mice. The ratio of the initial amount of the radical detected at the head to that of the administered spin probes as well as the half life ($t_{1/2}$) were also evaluated. An area under the clearance curve of the radical amount detected at the head of mice (AUC_{head}) was calculated by an equation of $AUC_{head} = IA/k$.

Stability of Nitroxide Radicals in the Fresh Blood of Animals

4-Hydroxy-TEMPO or 3-carbamoyl-PROXYL was added to the fresh blood (3.5 μmol/ml) of mice or rats at the age of 4 weeks in a 1.5 ml polyethylene tube treated with 40 unit/ml heparin solution. Blood concentrations of the radicals (3.5 μmol/ml) corresponded to the initial concentration in the circulated blood after i.v. administration of the radicals at the dose of 0.25 mmol/kg body weight. L- and X-band ESR spectra were recorded at every 10 min at 37°C. ESR spectra of both 4-hydroxy-TEMPO and 3-carbamoyl-PROXYL in the fresh blood were measured with both X-band ESR spectrometer JES-RE-3X (JEOL, Japan), and L-band ESR spectrometer equipped with a loop-gap resonator of vertical model and R3361A Spectrum Analyzer. Capillary tubes with a volume of 10 μl were used for measuring X-band ESR spectra and polyethylene tubes with a volume of 1.5 ml were used for measuring the L-band ESR spectra. Instrumental conditions for the X-band ESR measurements were as follows: frequency 9.4 GHz, microwave power 5.0 mW, modulation frequency 100 kHz, modulation amplitude width 0.1 mT, scanning time 2 min, and external magnetic field 335.4 ± 5.0 mT. Instrumental conditions for the L-band ESR measurements were as follows: frequency 1.1 GHz, microwave power 15.5 mW, modulation frequency 100 kHz, modulation amplitude width

1 mT, scanning time 2 min, and external magnetic field 37.5 ± 5.0 mT.

Excretion of Nitroxide Radicals into the Urine of Animals

Mice and rats at the age of 4 weeks received i.v. injection of 4-hydroxy-TEMPO at the dose of 1.0 mmol/kg body weight. The urine was collected at every 2 h after the administration. A portion of each collected urine was treated with a 1.0 M potassium hexacyanoferrate(III) to reoxidize the one-electron reduced form of 4-hydroxy-TEMPO and the ESR spectra were measured immediately. The concentrations of 4-hydroxy-TEMPO in the urine with or without potassium hexacyanoferrate(III) were determined by X-band ESR spectra at room temperature (22°C). Capillary tubes with a volume of 10 μl were used to measure X-band ESR spectra of 4-hydroxy-TEMPO in the urine. Instrumental conditions for the measurements were as follows: frequency 9.4 GHz, microwave power 5.0 mW, modulation frequency 100 kHz, modulation amplitude width 0.1 mT, scanning time 20 s, and external magnetic field 335.4 ± 5.0 mT. ESR spectral data were collected and analyzed with an ESPRIT ESR Data System (JEOL, Japan) throughout the investigation.

Statistical Analysis

All experimental results were presented as the mean values ± standard deviations from 3 to 4 mice or rats. The statistical evaluation was performed by analysis of variance (ANOVA) at a 1% or 5% significant level of the difference.

RESULTS

Calibration Lines by L-band ESR

L-band ESR spectra for a standard manganese (Mn^{2+}) solution put on the head of mice at 4 weeks age and for that put on the rubber balloon containing 19 ml of saline solution are shown in Figure 1(a) and (b), respectively. When Mn^{2+} plus a nitroxide radical (4-hydroxy-TEMPO) were put on the rubber balloon, a well separated ESR spectrum due to Mn^{2+} and the nitroxide radical was observed (Figure 1(c)). The signal intensity of Mn^{2+} was not influenced by the nitroxide radical. Also, the signal intensity of radical was hardly affected by Mn^{2+} (Figure 1(d)). Furthermore, almost no difference was observed in the

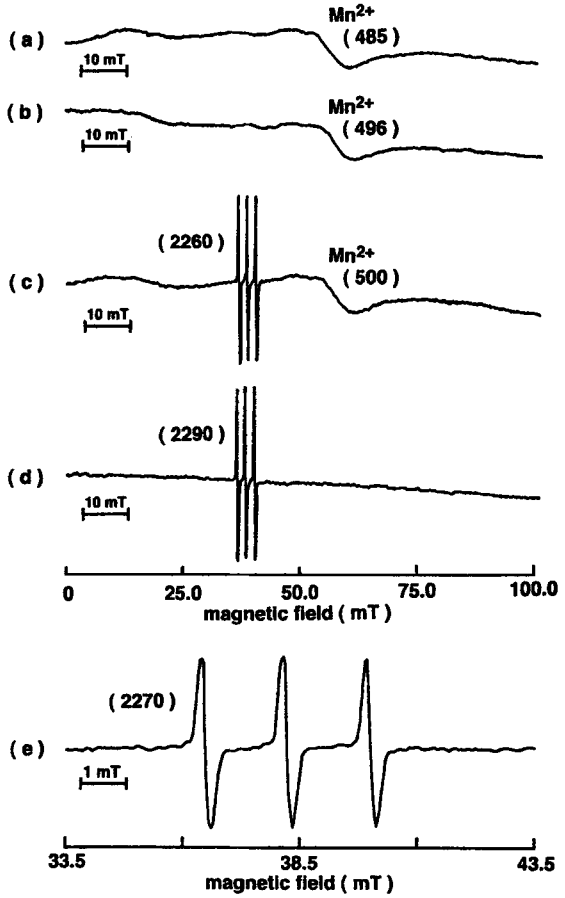

FIGURE 1 ESR spectra of 100 mM Mn^{2+} (1.0 ml) or/and 1.0 mM 4-hydroxy-TEMPO (1.0 ml) (a) Mn^{2+} put on the head of mouse at the age of 4 weeks, (b) Mn^{2+} put on a rubber balloon containing 19 ml of saline solution, (c) Mn^{2+} and 4-hydroxy-TEMPO put on a rubber balloon containing 19 ml of saline solution, (d) 4-hydroxy-TEMPO put on a rubber balloon containing 19 ml of saline solution and (e) 4-hydroxy-TEMPO under same conditions as for (c). ESR spectra of (a), (b), (c), and (d) were measured at magnetic field of 50.0 ± 50.0 mT, and that of (e) was 38.0 ± 5.0 mT. The values in parentheses represent ESR signal intensity at arbitrary unit measured on the ESR spectrometer.

signal intensity of the radical between the magnetic field range of 50.0 ± 50.0 and 39.0 ± 5.0 mT as well as the scanning time for 3 min 20 s and 20 s as shown in Figure 1(e). Thus, the calibration lines of 4-hydroxy-TEMPO normalized with standard Mn^{2+}, as shown in Figure 2, were successfully obtained to determine the radicals. Slopes of the calibration lines decreased with increase of saline volume in the rubber balloons, which may reflect the age of mice in terms of body weight.

In Vivo L-Band ESR Measurement

Figure 3 shows L-band ESR spectra measured at every 30 s at the head of a mouse (age: 6 weeks), who received i.v. injection of 4-hydroxy-TEMPO at the dose of 0.5 mmol/kg body weight. A triplet signal with equal intensities characteristic of the nitroxide radical was observed. Among the signal, small but regular signals, probably due to the respiration of the animal, were observed. They disappeared with death of the animal during ESR measurement as observed before.[13] The characteristic ESR signals decreased gradually with time after the radical administration, keeping the spectral pattern without appearance of new signals during measurement. The clearance rate constants (k) and half lives ($t_{1/2}$) for all examined systems are summarized in Table I. The area under the clearance curve due to the radicals

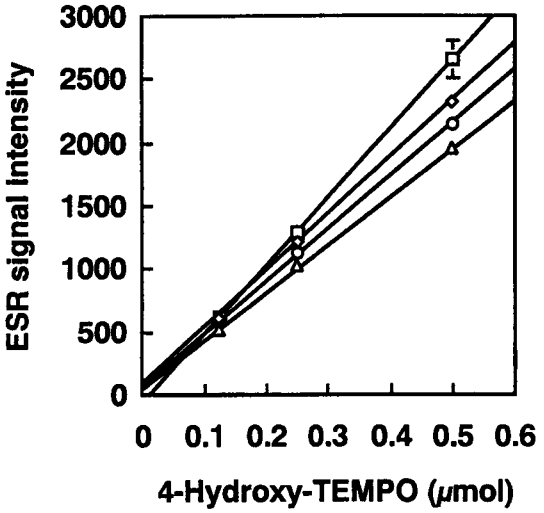

FIGURE 2 Calibration lines of 4-hydroxy-TEMPO. ESR spectral intensity due to the radical put on a rubber balloon containing different volume of saline solution as substitutes of mice at various ages was normalized with that of the standard Mn^{2+}. Volume of saline solution: (□) 19 ml, (◇) 29 ml, (○) 44 ml, and (△) 54 ml.

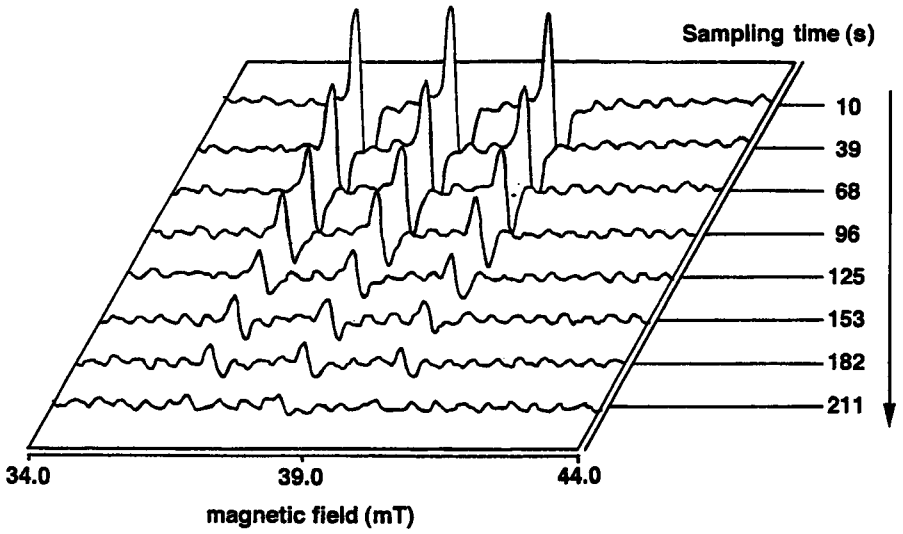

FIGURE 3 L-band ESR spectra at every 30 s at the head of a mouse (6 weeks old) given 4-hydroxy-TEMPO at the dose of 0.5 mmol/kg body weight by intravenous injection.

TABLE II Pharmacokinetic parameters for 4-hydroxy-TEMPO monitored at the head of mice at various ages

Age (weeks)	Body wight (g)	Dose (mmol/kg)	AUC_{head} (μmol · s)	IA (μmol)†	Ratio% (IA/AA)‡	
4	20	0.25	14.61 ± 1.04	0.27 ± 0.03	5.43 ± 0.61	
4	20	0.5	35.69 ± 3.43	0.53 ± 0.04	5.28 ± 0.39	
4	20	1.0	91.88 ± 19.14	0.94 ± 0.03	4.71 ± 0.17	
					av. (5.14 ± 0.38)	
6	30	0.25	27.33 ± 3.28	0.35 ± 0.05	4.60 ± 0.81	
6	30	0.5	59.32 ± 5.07	0.68 ± 0.05	4.52 ± 0.35	
6	30	1.0	152.86 ± 20.04	1.47 ± 0.05	4.91 ± 0.46	
					av. (4.68 ± 0.21)	
10	35	0.25	31.05 ± 3.72	0.38 ± 0.04	4.25 ± 0.40	**
17	40–45	0.13	15.20 ± 1.62	0.19 ± 0.02	3.45 ± 0.34	
17	40–45	0.25	32.21 ± 3.16	0.39 ± 0.02	3.46 ± 0.20	*
17	40–45	0.5	80.93 ± 29.63	0.89 ± 0.27	3.96 ± 1.19	
17	40–45	1.0	200.39 ± 45.03	1.72 ± 0.17	3.81 ± 0.37	
					av. (3.67 ± 0.26)	
37	50–55	0.5	84.80 ± 13.44	0.88 ± 0.07	3.19 ± 0.26	
52	50–60	0.25	50.03 ± 11.13	0.46 ± 0.06	3.06 ± 0.41	
52	50–60	0.5	120.04 ± 27.47	0.95 ± 0.14	3.17 ± 0.48	**
52	50–60	1.0	271.42 ± 31.13	1.90 ± 0.21	3.16 ± 0.68	
					av. (3.13 ± 0.06)	

Data are the mean values ± standard deviations for 3 mice or rats.
*$p < 0.05$, **$p < 0.01$.
†Initial amount of the radical detected at the head of mouse.
‡Ratio (%) of the initial amount of the radical detected to the amount of the administered spin probe.

(AUC_{head}) at the head of mice, initial radical amount (IA), and ratio (%) of the initial radical amount to that of the administered radicals are summarized in Table II. Both data at the head of rats over the ages of 13 weeks and at the upper abdomen of rats were not obtained, due to the limitation of cavity size (i.d.: 35 mm) of L-band ESR spectrometer. Although we monitored the radicals at the head of mice (age: 4 weeks) given the radical at the dose of 0.13 mmol/kg, the parameters were not available exactly because of its rapid clearance.

Comparison of Spin Clearance for the Nitroxide Radicals

Figure 4(a) shows the time-dependent semilogarithmic clearance curve plotted for the signal intensity of the lowest magnetic field in the ESR spectra due to 4-hydroxy-TEMPO, which was detected at the upper abdomen of mouse (age: 4 weeks) receiving the agents at the dose of 1.0 mmol/kg body weight. While, under the same conditions, the clearance curve for 3-carbamoyl-PROXYL gave a two phase form (α and β) with two semilogarithmic straight lines (Figure 4(b)). These results indicate that the clearances of both spin probes are analyzed by the first order kinetics, the clearance rate constant (k) for 4-hydroxy-TEMPO being $(11.6 \pm 1.1) \times 10^{-3}\,s^{-1}$, while those for 3-carbamoyl-PROXYL being (α) $(2.1 \pm 0.7) \times 10^{-3}\,s^{-1}$ and (β) $(1.1 \pm 0.2) \times 10^{-3}\,s^{-1}$ (Table I).

Dose-Dependent Spin Clearance Change of the Nitroxide Radical

The clearance rate constants observed at the head of mice (4, 6, 7, and 52 weeks old), who received 4-hydroxy-TEMPO at various doses, decreased dose-dependently (Figure 5). Also, both IA and AUC_{head} due to the radicals, that were monitored at the head of mice, increased in dose-dependent manners at each age of mice (Table II).

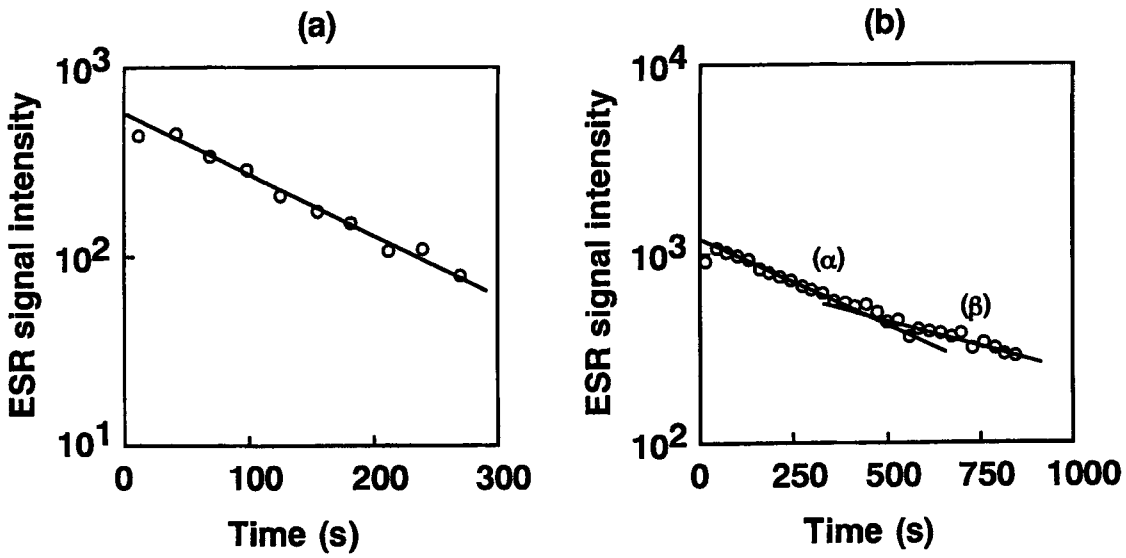

FIGURE 4 Semilogarithmic plots of L-band ESR signal intensity against time, monitored at the upper abdomen of mice (4 weeks old). Mice received 4-hydroxy-TEMPO (a) and 3-carbamoyl-PROXYL (b) at the dose of 1.0 mmol/kg body weight by intravenous injection.

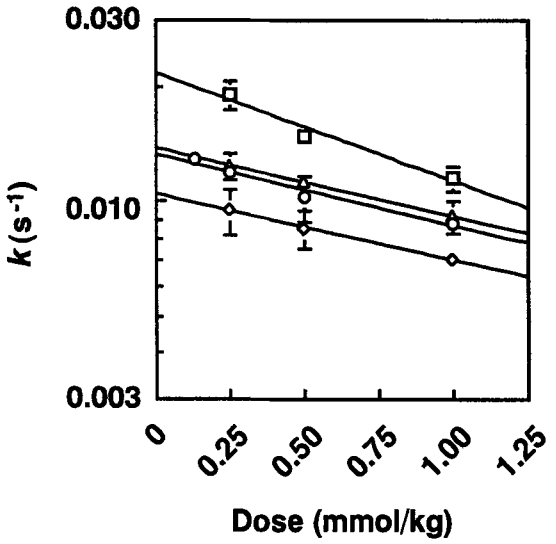

FIGURE 5 Semilogarithmic plots of clearance rate constants (k) against the dose of 4-hydroxy-TEMPO, monitored at the head of mice at following ages: (□) 4 weeks, (△) 6 weeks, (○) 17 weeks, and (◇) 52 weeks old.

However, no dose-dependent difference in the ratio (%) of initial amount of the radicals to that of the administered radicals at each age of mice was observed as shown in Table II.

Age-Dependent Spin Clearance Change of the Nitroxide Radical

The clearance rate constants observed at the head of mice (4, 6, 17, 37, and 52 weeks old), who were given 4-hydroxy-TEMPO at the doses of 0.13, 0.25, 0.5, or 1.0 mmol/kg body weight, decreased gradually with increase of the age (Table I and Figure 6(a)). The decrease of spin clearance rate constants (k) depends on the aging of the animals. Figure 6(b) shows a semilogarithmic plot of the spin clearance rate constants against the body weight of mice given 4-hydroxy-TEMPO at the dose of 0.5 mmol/kg body weight, indicating the age-dependent decrease of the spin clearance rate constant. In addition, the ratio (%) of initial amount of the radicals to that of the administered radicals at the head of mice decreased with age of mice, as follows: 5.14 ± 0.38 (%) at 4 weeks old, 4.68 ± 0.21 (%) at 6 weeks old 4.25 ± 0.40 (%) at 10 weeks old, 3.67 ± 0.26 (%) at 17 weeks old, 3.19 ± 0.26 (%) at 37 weeks old, and 3.13 ± 0.06 (%) at 52 weeks old. However, the clearance rate constants obtained by monitoring at the head of rats (3, 4, 6, and 10 weeks old), who received

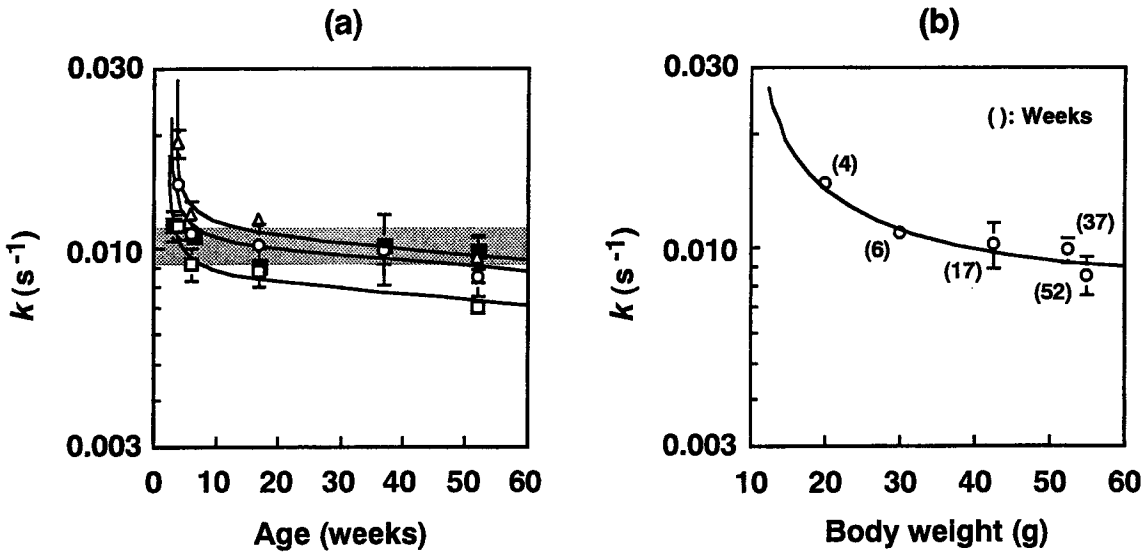

FIGURE 6 (a) Semilogarithmic plots of spin clearance rate constants (k) at the head and the upper abdomen of mice given 4-hydroxy-TEMPO against the age (△) 0.25 mmol/kg body weight (the head), (○) 0.5 mmol/kg body weight (the head), (□) 1.0 mmol/kg body weight (the head), (■) 1.0 mmol/kg body weight (the upper abdomen) (b) Semilogarithmic plots of spin clearance rate constants (k) against body weight (g) of mice given 4-hydroxy-TEMPO at the dose of 0.5 mmol/kg body weight. ESR spectra were measured at the head of mice.

4-hydroxy-TEMPO at the dose of 0.25 mmol/kg body weight, were not changed during the aging process (Table I).

Organ-Dependent Spin Clearance of the Nitroxide Radical

The clearance rate constants of the radicals observed at the head of mice, who received 4-hydroxy-TEMPO at the dose of 1.0 mmol/kg body weight, were decreased age-dependently. While, under the same conditions, no remarkable change in the clearance rate constants of the radicals at the upper abdomen of mice was observed with the animal ages examined (Figure 6(a)). The clearance rate constants of the radicals at the head of mice were available at the dose of 0.25 mmol/kg body weight (Table I), while the radicals at the upper abdomen of mice given 4-hydroxy-TEMPO were not detected even at the dose of 0.5 mmol/kg body weight, but detectable at the dose of 1.0 mmol/kg body weight.

Comparison of Spin Clearance of the Nitroxide Radical between Animal Species

The spin clearance rate constants at the heads of mice and rats (4, 6 and 10 weeks old), who received 4-hydroxy-TEMPO at the dose of 0.25 mmol/kg body weight, were found to be different each other (Figure 7). The results indicate that the clearance rates of the radical in mice are faster than those in rats.

Stability of the Nitroxide Radicals in the Fresh Blood of Animals

Both X- and L-band ESR spectra of 4-hydroxy-TEMPO (3.5 μmol/ml each) or 3-carbamoyl-PROXYL (3.5 μmol/ml each) in the fresh blood collected from mice or rats aged of 4 weeks were measured at different incubation time. The signal intensities decreased very gradually as observed before,[13] in which both clearance curves for 4-hydroxy-TEMPO and 3-carbamoyl-PROXYL

gave a single phase and each spectral pattern was unchanged during the experiments. The differences in clearance rate constants of 4-hydroxy-TEMPO and 3-carbamoyl-PROXYL in the fresh blood of mice or rats were insignificantly observed between X- and L-band ESR methods (Table III). While, significant differences in the half lives of 4-hydroxy-TEMPO in the fresh blood were observed between the fresh blood of mice and rats. The half life of 3-carbamoyl-PROXYL in fresh blood of mice was approximately 21 h, being remarkably longer than that of 4-hydroxy-TEMPO.

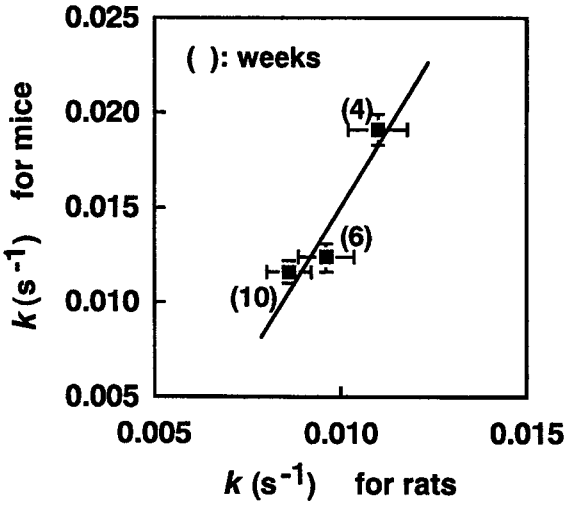

FIGURE 7 Relationship of spin clearance rate constants (k) for mice and rats given 4-hydroxy-TEMPO at the dose of 0.25 mmol/kg body weight. L-band ESR spectra were measured at the head of both animals.

Excretion of the Nitroxide Radical in the Urine of Animals

The urine of mice and rats after i.v. administration of 4-hydroxy-TEMPO were collected every 2 h for 24 h and X-band ESR spectra were measured at room temperature (22°C). ESR signal was not observed in the urine before administration of 4-hydroxy-TEMPO. However, a triplet signal due to the nitroxide radical was observed in the urine of mice and rats, who received the agent at the dose of 1.0 mmol/kg body weight. The strongest signal intensity of the radicals in the urine of mice and rats was observed at 2 h after the administration, but it decreased within 4 h (Figure 8). Total amount of the radical due to 4-hydroxy-TEMPO in the urine excreted from mice and rats was about 1.0% and 0.3% of the administered dose respectively. Since the spin clearance of the nitroxide radical has been found to be due to the one-electron reduction of the corresponding radical,[8,15,21] we added an oxidizing agent, potassium hexacyanoferrate(III), to each collected urine and found remarkable increases of ESR signal intensity due to the existence of the corresponding hydroxylamine as the reduced form of the radical (Figure 8). Mice have been found to excrete most of the hydroxylamine for 4 h after the radical administration. In contrast, the signal due to the nitroxide radical in the urine of rats was observed even after 24 h. Thus, the total amounts of the radical and its reduced form excreted from mice and rats were estimated to

TABLE III Stability of 4-hydroxy-TEMPO and 3-carbamoyl-PROXYL in the fresh blood of mice and rats as determined by X- and L-band ESR methods

Spin-label agent	Animal	ESR method	k ($\times 10^{-3}$ min^{-1})	$t_{1/2}$ (min)
4-Hydroxy-TEMPO	mice	X-band	20 ± 4	35 ± 6 *
	mice	L-band	21 ± 5	35 ± 8 * *
	rats	X-band	14 ± 2	51 ± 6 *
	rats	L-band	14 ± 2	49 ± 6 *
3-Carbamoyl-PROXYL	mice	X-band	0.55 ± 0.01	1265 ± 13
	mice	L-band	0.55 ± 0.08	1286 ± 176

Final concentration of spin probe was 3.5 μmol/ml in the blood. Data are the mean values ± standard deviations for 3 mice or rats.
*$p < 0.05$.

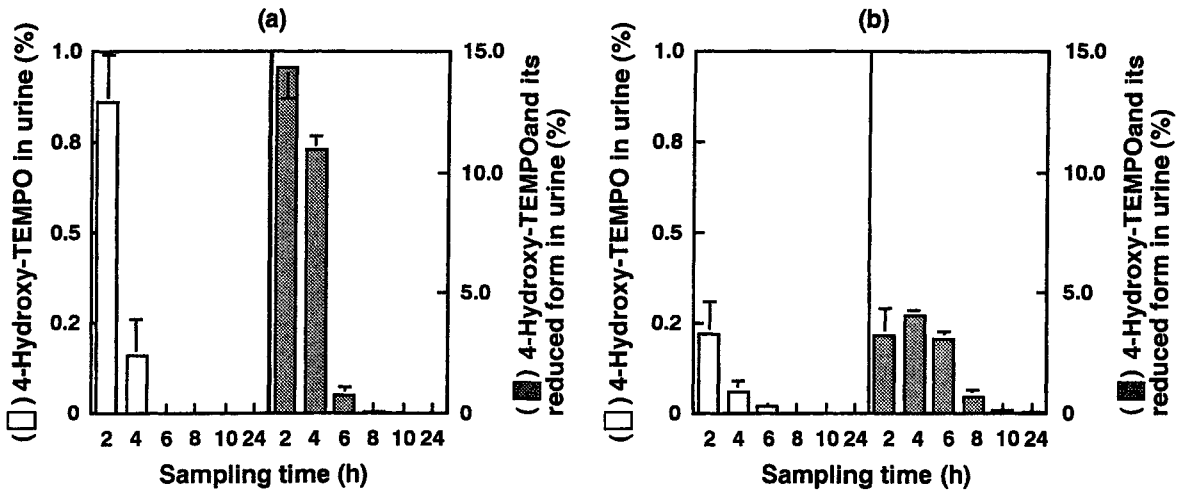

FIGURE 8 Spin clearances due to 4-hydroxy-TEMPO and its reduced form from the urine of (a) mice and (b) rats (4 weeks old). Mice and rats received 4-hydroxy-TEMPO at the dose of 1.0 mmol/kg body weight. (□) ratio (%) of the observed amount of the radical to that of the administered spin probe, (■) ratio (%) of recovering amount of the radical by addition of potassium hexacyanoferrate(III) to that of the administered spin probe.

be approximately 26% and 11% of the administered dose, respectively.

DISCUSSION

By *in vivo* L-band ESR method, we were able to measure the radicals due to the stable spin probes at the upper abdomen of mice as well as at the head of mice and rats. Since it has been thought to be difficult to analyze the radicals quantitatively in a living animal, we have tried and established the quantitative pharmacokinetic analysis of the radicals in animals.

Calibration Lines

We used a phantom as a model of a whole mouse or the head of a rat to obtain the calibration lines. Since 1.0 ml of 100 mM manganese sulfate in a polyethylene tube (1.5 ml) gave constant ESR spectral pattern and signal height under the conditions used, the solution was successfully used in the present quantitative experiments. Calibration lines were drawn by the simultaneous ESR measurements of manganese (Mn^{2+}) as standard substance, several concentrations of 4-hydroxy-TEMPO and a rubber balloon with different volume of saline, which corresponds to the body weight of animals with different ages (Figure 1). The slope of the lines decreased with increase of the volume of saline solution in a rubber balloon (Figure 2). We also observed age-dependent decrease of the signal intensity due to Mn^{2+}, measured at the head of mouse (data not shown). From these results, it was concluded that dielectric loss of the applied microwave increases with increase of the amount of water existing in the measurement site, and the obtained calibration lines are useful to analyze the clearance of ESR signals due to the radicals administered to animals.

Stability of the Spin Probe and Spin Clearance due to Organ Distribution and Metabolism

We analyzed the real time pharmacokinetics of the nitroxide radicals in mice and rats at various ages by *in vivo* L-band ESR method. When the animals received i.v. injection of 4-hydroxy-TEMPO, the ESR signal disappeared with time (Figure 3), but

the spectral pattern was unchanged during the *in vivo* measurements. In general, administered nitroxide radicals are presumed to be reduced to ESR inactive hydroxylamines by active oxygen species like superoxide anion radicals or endogenous reducing agents such as NAD(P)H, ascorbic acid, and glutathione, and lose their paramagnetic properties.[15,28,29] Thus, the stability of nitroxide radicals in the fresh blood was examined in advance. The radicals in the fresh blood were found to be stable in terms of the spin clearance rate constant (k), compared with the behavior in *in vivo* measurements (Tables I and III), indicating that the paramagnetic loss of the radicals in circulating blood is not caused by the reductions with the components of the blood, but affected by the transport to the organs. Thus, we suggest that clearance of the radicals is relevant to the alteration of the radicals in animals involving the changes of organ distribution and metabolism.

Chemical Form of the Spin Probe in Animals

ESR pattern with equal 3 intensity signals due to 4-hydroxy-TEMPO was recorded throughout the present study as shown in Figure 3. When the radical binds with proteins or other biomolecules, the 1st and 3rd signals become small and show broadening because of the restriction of the thermal molecular movement.[8,30] Consequently, the observed nitroxide radicals are thought to exist as the free form in animals.

ESR Monitoring Sites

The spin clearance rate constants for 4-hydroxy-TEMPO detected at the upper abdomen of mice at each age were unchanged and independent of the age of mice (Figure 6). *In vivo* L-band ESR detects the radicals at the measurement site of an alive animal together with those in the blood vessel. Since it has been reported that 4-hydroxy-TEMPO and 3-carbamoyl-PYROXYL cannot permeate through the blood brain barrier because of their high hydrophilicity,[31,32] the radical circulating in the blood vessel is measurable at the head of mice. However, the radical transferred to several organs such as liver is monitored at the upper abdomen of mice simultaneously with that in the blood vessel. Spin clearance time courses of the radical should be sufficiently different from between the blood vessel and some organs, and the overlapping clearance curves attributed to several organs may explain the distinction of the spin clearance rate constants, which depend on the measurement domain in the animal. Recently, it was reported that free radicals generated endogenously such as nitric oxide could be detected at the abdomen of mouse or rat by using both spin trapping agents and *in vivo* L-band ESR methods.[16,17,33,34] These results suggest that it requires to select the best measurement domains according to the purpose of each L-band ESR study. In consideration of the influence of dielectric loss due to water molecule on the irradiation of microwave, accurate measurement is indispensable to analyze quantitatively the kinetic behavior of radicals in a mouse at the upper abdomen.

Spin Clearances of the Spin Probes

(1) *Chemical Structure of the Spin Probes*

The spin clearance of 4-hydroxy-TEMPO was analyzed by the first order kinetics with monophase, while that of 3-carbamoyl-PROXYL was by the first order kinetics with α and β phases (Figure 4), indicating that the spin clearance of the nitroxide radicals depends on the difference in chemical structure such as a six-member ring or a five-member ring structure.[35] This result corresponds to that of BCM-ESR method, which has been proposed from our laboratory.[8]

(2) *Dose-Dependency*

Since the clearance rate constants decreased dose-dependently (Figure 5), we estimated

AUC_{head} due to the radicals detected at the head of mice. AUC_{head} increased nonlinearly with the dose of administered radicals. The results indicate that (i) the ability to eliminate the nitroxide radicals in animals, such as enzymes and endogenous reducing agents, is saturated with increase of the dose, and (ii) the nature of nitroxide radical disposition is capacity-limited and best described in terms of Michaelis–Menten kinetics. It has been assumed that nitroxide radicals are eliminated from animal body according to the first order linear kinetics,[13–15] however, we first confirmed the fact by using present L-band ESR method and pharmacokinetic analysis, in which the nitroxide radicals disappear from animal body according to the nonlinear kinetics.

(3) *Nonlinear Pharmacokinetic Model Analysis*

We analyzed the spin clearance curves in more detail by using one-compartment model with Michaelis–Menten elimination kinetics[36] and evaluated the pharmacokinetic parameters contributing to the nonlinear elimination kinetics of the nitroxide radical (Figure 9). The simultaneous multilines fitting of three spin clearance curves at three different doses was attempted to estimate the pharmacokinetic parameters. The maximum elimination rate (V_{max}) and Michaelis constant (K_m) were calculated by nonlinear least-squares regression with MULTI(RUNGE),[37] and spin clearance rate constants (k) under the linear conditions were calculated by dividing the

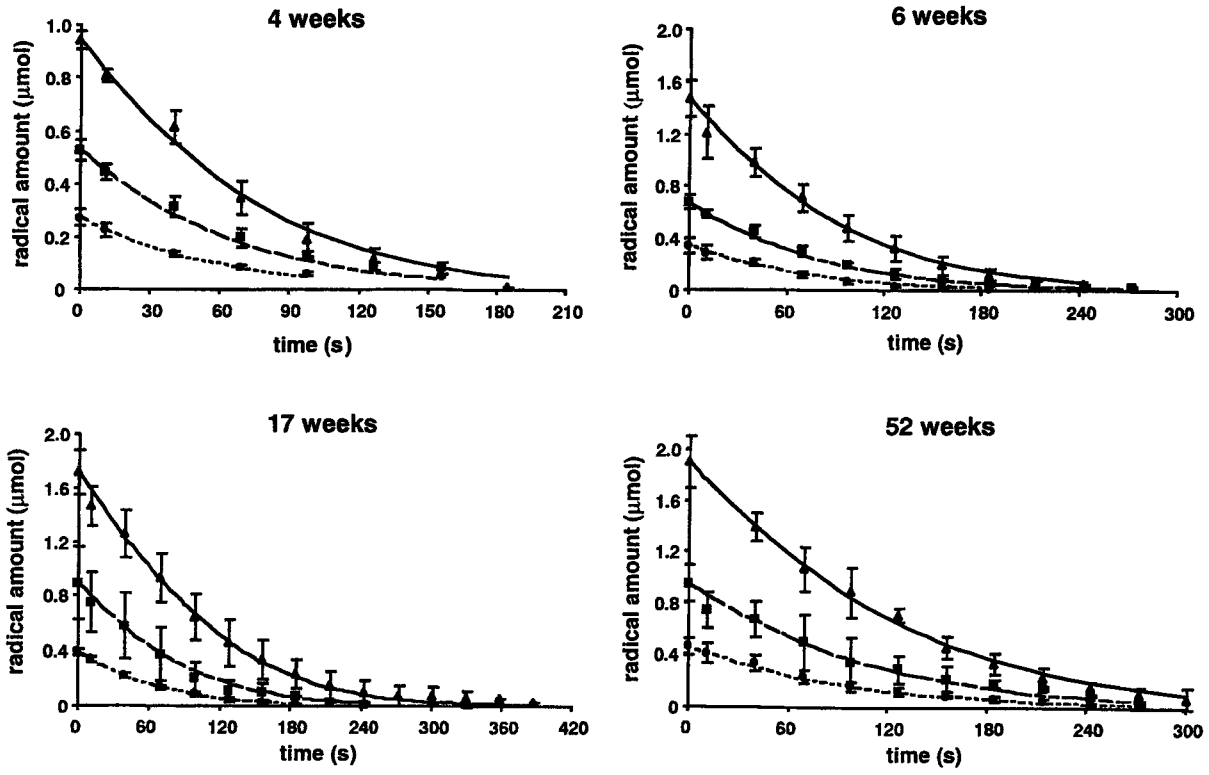

FIGURE 9 4-Hydroxy-TEMPO detected at the head of mice at various ages. (▲) 1.0 mmol/kg, (■) 0.5 mmol/kg, and (●) 0.25 mmol/kg and the fitted curves ((—) 1.0 mmol/kg, (– – –) 0.5 mmol/kg, (- - -) 0.25 mmol/kg) following the analyses by one-compartment model with Michaelis–Menten elimination kinetics.

maximum elimination rate (V_{max}) by Michaelis constant (K_m) at the head of mice at each age.

(4) *Age-Dependency*

Spin clearance rate constant was estimated to be $18.7 \times 10^{-3}\,s^{-1}$ (4 weeks old), $15.2 \times 10^{-3}\,s^{-1}$ (6 weeks old), $17.0 \times 10^{-3}\,s^{-1}$ (17 weeks old), and $11.4 \times 10^{-3}\,s^{-1}$ (52 weeks old) (Table IV). The spin clearance rate constants at each age of mice obtained from the sophisticated nonlinear model analysis were comparable with those from the linear one-compartment model analysis at the lowest dose of 0.25 mmol/kg body weight. However, the spin clearance rate constants at each age of mice obtained from the linear model decreased dose-dependently and deviated from those values determined by the nonlinear model. Therefore, it is suggested that (i) the biological ability to eliminate the radicals in animals will be essentially capacity-limited, (ii) elimination capacity of the radicals is saturated dose-dependently, and (iii) the nonlinear effects appear in the disposition of the nitroxide radicals with increase of doses of the administered radicals. On the other hand, the spin clearance rate constants for the radicals at the head of mice given 4-hydroxy-TEMPO decreased age-dependently (Figure 6). Since three values such as the maximum elimination rate (V_{max}), Michaelis constant (K_m), and the spin clearance rate constant (k) obtained by the Michaelis–Menten elimination model decreased with the age of mice (Table IV), the reducing systems of the radicals in animals including many relating enzyme activities have been suggested to be reduced with aging of animals.

The spin clearance rate constants for 4-hydroxy-TEMPO at the head of rats at the ages of 3, 4, 6, and 10 weeks were not significantly different under the present experimental conditions (Table I). It was reported that the spin clearance rate constants of the radical in rats were almost constant among the ages of 4, 6 and 9 weeks, but the constants for the radicals in rats aged of 11, 14, and 16 weeks were significantly smaller than those in rats aged of 4, 6, and 9 weeks.[38]

(5) Distribution Volume

The initial amount ratios of the detected radical to the dose of the administered 4-hydroxy-TEMPO (IA/AA) were almost constant and independent of the dose at each age of mice (Table II). It thus indicates that the ratio represents the ratio of distribution volume at the head of mice to that in animals if instantaneous equilibrium is achieved in the transfer of the nitroxide radical in the blood circulation of whole body. Since the ratio (IA/AA) decreases significantly with the age of mice, the increment of distribution volume in mice would be larger than that of distribution volume at the head of mice with the age.

Animal Species-Dependency in Metabolism and Urinary Excretion of the Spin Probe

The spin clearance rates constants for 4-hydroxy-TEMPO monitored at the head of mice are larger than those at the head of rats (Figure 7), suggesting the presence of animal species-dependent difference in the radical clearance between mice

TABLE IV Pharmacokinetic parameters for 4-hydroxy-TEMPO detected at the head of mice

Age (weeks)	V_{max} (nmol·s^{-1}/kg)	K_m (nmol/kg)	k^* ($\times 10^{-3}\,s^{-1}$)
4	1.64 ± 0.66	87.5 ± 44.0	18.7
6	1.41 ± 0.35	92.3 ± 29.0	15.2
17	1.14 ± 0.13	66.8 ± 12.2	17.0
52	0.74 ± 0.14	64.7 ± 15.0	11.4

Data were analyzed by Michaelis–Menten elimination model.
*$k = V_{max}/K_m$.

and rats. To confirm these species-dependent difference in detail, urine excretion of the nitroxide radical in mice and rats were examined with X-band ESR method. The excretion rates of both the nitroxide radical and its hydroxylamine as one-electron reduced form in mice were faster than those in rats (Figure 8). In addition, excretion ratios of both the compounds to the administered dose in mice were larger than those in rats. These results also suggest the presence of animal species-dependent difference in metabolism and urinary excretion of the nitroxide radical between mice and rats. Detectable radicals excreted to the urine were found to be the intact and its one-electron reduced form, which was reoxidized by addition of potassium hexacyanoferrate(III). Ratios of the radical undetected in the urine to the dose in mice and rats were 74% and 89%, respectively (Figure 8). Large portions of the administered radical remain in animals as the diamagnetic metabolites or are excreted to the urine as the forms, which cannot revert to the nitroxide radical by the reoxidation. Thus, animal species-dependent difference in urinary excretion of the radical between mice and rats was also observed in the present study. It is necessary to examine the absorption, metabolism, and excretion of the nitroxide radical in mice and rats in the future.

CONCLUSION

Pharmacokinetics of the spin probes in mice and rats at various ages were analyzed noninvasively at the real time by *in vivo* L-band ESR method. The disposition of the radicals observed by *in vivo* L-band ESR is significantly different between the nitroxide radicals and depends on the structures and doses of the spin probes, the species and ages of animals, and the measurement sites of the animal body. Dose-dependent nonlinear kinetics of 4-hydroxy-TEMPO in mice were evaluated quantitatively by pharmacokinetic model analysis and nonlinear least-squares regression. *In vivo* L-band ESR method was thus found to be useful to investigate the disposition of radicals in the whole body of animals. Our present study proposes first the quantitative pharmacokinetic analysis of nitroxide radicals in mice by *in vivo* L-band ESR. L-band ESR is the unique technique that can directly detect free radicals in a living animal, and will be applied to investigate the development of medicines and pharmaceutics, such as pharmacokinetic analysis of new pharmaceutics combined with nitroxide radicals as spin labels instead of radioisotopes.[39]

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References

[1] (a) B. Halliwell and J.M.C. Gutteridge (1989) *Free Radicals in Biology and Medicine*, pp. 416–493, Clarendon Press, Oxford; (b) C.A. Rice-Evans and R.H. Burdern, Ed. (1994) *Free Radical Damage and its Control*, Elsevier, Amsterdam.

[2] (a) J.J.R. Frausto da Silva and R.J.P. Williams (1991) *The Biological Chemistry of the Elements, The Inorganic Chemistry of Life*, Clarendon Press, Oxford; (b) D.M. Taylor and D.R. Williams (1995) *Trace Element, Medicine and Chelation Therapy*, The Royal Society of Chemistry, Cambridge.

[3] (a) H.M. Swartz, J.R. Bolton and D.C. Borg, Ed. (1972) *Biological Applications of Electron Spin Resonance*, Willy-Interscience, New York; (b) A.J. Hoff, Ed. (1989) Advanced EPR, *Applications in Biology and Biochemistry*, Elsevier, Amsterdam.

[4] G.R. Buettner (1987) Spin trapping: ESR parameters of spin adducts. *Free Radical Biology and Medicine*, **3**, 259–303.

[5] G.R. Chalfont, M.J. Perkins and A. Horsefield (1968) A probe for homolytic reactions in solutions. II. The polymerization of styrene. *Journal of the American Chemical Society*, **90**, 7141–7142.

[6] S. Forshult, C. Lagercrantz and K. Torssell (1969) Use of nitroso compounds as scavengers for the study of short-lived free radicals in organic reactions. *Acta Chemica Scandinavica*, **23**, 522–530.

[7] A. Feldman, E. Wildman, G. Bartolinini and L.H. Piette (1975) *In vivo* electron spin resonance in rats. *Physical Medicine Biology*, **20**, 602–612.

[8] K. Takechi, H. Tamura, K. Yamaoka and H. Sakurai Pharmacokinetic analysis of free radicals by *in vivo* BCM (Blood Circulation Monitoring)-ESR method. *Free Radical Research*, **26**, 483–496.

[9] J. Barthel, K. Bachhuber, R. Buchner and H. Hetzenauer (1990) Dielectric spectra of some common solvents in the microwave region. *Chemical Physics Letters*, **165**(4), 369–373.

[10] T. Sawada, K. Fukumaru and H. Sakurai (1995) L-band ESR spectra of copper(II) complexes with CuN_4 configurations. *Biochemical and Biophysical Research Communications*, **216**(1), 154–161.

[11] K. Fukumaru, T. Sawada, N. Nishino and H. Sakurai (1996) Relationship between X- or L-band ESR spectra and coordination structures of copper(II) complexes with a CuO_4 coordination mode. *Chemical and Pharmaceutical Bulletin*, **44**(11), 1989–1997.

[12] J.L. Zweier and P. Kuppusamy (1988) Electron paramagnetic resonance measurements of free radicals in the intact beating heart: A technique for detection and characterization of free radicals in whole biological tissues. *Proceedings of the National Academy of Sciences of the United State of America*, **85**, 5703–5708.

[13] H. Utsumi, E. Muto, S. Masuda and A. Hamada (1990) *In vivo* ESR measurement of free radicals in whole mice. *Biochemical and Biophysical Research Communications*, **172**(3), 1342–1348.

[14] K. Takeshita, H. Utsumi and A. Hamada (1991) ESR measurement of radical clearance in lung of whole mouse. *Biochemical and Biophysical Research Communications*, **177**, 874–880.

[15] Y. Miura, H. Utsumi and A. Hamada (1992) Effects of inspired oxygen concentration on *in vivo* redox reaction of nitroxide radicals in whole mice. *Biochemical and Biophysical Research Communications*, **182**, 1108–1114.

[16] V. Quaresima, H. Takehara, K. Tsushima, M. Ferrari and H. Utsumi (1996) *In vivo* detection of mouse liver nitric oxide generation by spin trapping electron paramagnetic resonance spectroscopy. *Biochemical and Biophysical Research Communications*, **221**, 729–734.

[17] T. Yoshimura, H. Fujii, F. Takayama, K. Oikawa and H. Kamada (1996) *In vivo* EPR detection and imaging of endogenous nitric oxide in lipopolysaccharide-treated mice. *Nature Biotechnology*, **14**, 992–994.

[18] J.F. Glockner, H.C. Chang and H.M. Swartz (1991) *In vivo* oximetry using nitroxide-liposome system. *Magnetic Resonance in Medicine*, **20**, 123–133.

[19] W.K. Subczynski, S. Lukiewicz and J.S. Hyde (1986) Murine *in vivo* L-band ESR spin-label oximetry with a loop-gap resonator. *Magnetic Resonance in Medicine*, **3**, 747–754.

[20] H. Fujii and K. Kakinuma (1990) Studies on the superoxide releasing site in plasma membranes of neutorophils with ESR spin-labels. *Journal of Biochemistyr*, **108**, 292–296.

[21] K. Chen, J.F. Glockner, P.D. Morse, II and H.M. Swartz (1989) Effects of oxygen on the metabolism of nitroxide spin labels in cells. *Biochemistry*, **28**, 2496–2501.

[22] H. Utsumi, A. Shimakura, M. Kashiwagi and A. Hamada (1989) Localization of the active center of nitroxide radical reduction in rat liver microsomes: Its relation to cytochrome P-450 and membrane fluidity. *Journal of Biochemistry*, **105**, 239–244.

[23] F. Gomi, H. Utsumi, A. Hamada and M. Matsuo (1993) Aging retards spin clearance from mouse brain and food restriction prevents its age-dependent retardation. *Life Sciences*, **52**, 2027–2033.

[24] G. Bacic, M.J. Nilges, R.L. Magin, T. Walczak and H.M. Swartz (1989) *In vivo* localized ESR spectroscopy reflecting metabolism. *Magnetic Resonance in Medicine*, **10**, 266–272.

[25] K. Yamaoka, Y. Tanigawara and T. Nakagawa (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. *Journal of Pharmacobio-Dynamics*, **4**, 879–885.

[26] D.J. Cutler (1978) On the definition of the compartment model concept in pharmacokinetics. *Journal of Theory and Biology*, **73**, 329–345.

[27] M. Gibaldi and D. Perrier (1982) Pharmacokinetics 2nd Edn: *Drug and the Pharmaceutical Sciences*, Vol. 15, Marcel Dekker, INC., New York.

[28] K. Maruyama and S. Ohnishi (1974) Spin-label study of the photosynthetic bacterium, *Rhodospirillum Rubrum*. *Journal of Biochemistry*, **75**, 1153–1164.

[29] K. Maruyama and S. Ohnishi (1974) Effects of stearic spin-labels on the photochemical activities of chromatophores from *Rhodospirillum rubrum*. *Journal of Biochemistry*, **75**, 1165–1168.

[30] K. Takeshita, H. Utsumi and A. Hamada (1987) Dynamic properties of the haptenic site of lipid haptens in phosphatidylcholine membranes. Their relation to the phase transition of the host lattice. *Biophysical Journal*, **52**, 187–197.

[31] S. Ishida, S. Matsumoto, H. Yokoyama, N. Mori, H. Kumashiro, N. Tsuchihashi, T. Ogata, M. Yamada, M. Ono, T. Kitajima, H. Kamada and E. Yoshida (1992) An ESR-CT imaging of the head of a living rat receiving an administration of a nitroxide radical. *Magnetic Resonance Imaging*, **10**(1), 109–114.

[32] M. Hiramatsu, K. Oikawa, H. Noda, A. Mori, T. Ogata and H. Kamada (1995) Free radical imaging by electron spin resonance computed tomography in rat brain. *Brain Research*, **697**, 44–47.

[33] J. Jiang, K.J. Liu, X. Shi and H.M. Swartz (1995) Detection of short-lived free radicals by low-frequency electron paramagnetic resonance spin trapping in whole living animals. *Archives of Biochemistry and Biophysics*, **319**(2), 570–573.

[34] J. Jiang, K.J. Liu, S.J. Jordan, H.M. Swartz and R.P. Mason (1996) Detection of free radical metabolite formation using *in vivo* EPR spectroscopy: Evidence of rat hemoglobin thiyl radical formation following administration of phenylhydrazine. *Archives of Biochemistry and Biophysics*, **330**(2), 266–270.

[35] A. Iannone, A. Tomasi and H. Swartz (1990) Metabolism of nitroxide spin labels in subcellular fraction of rat liver I. Reduction by microsomes, *Biochimica et Biophysica Acta*, **1034**, 285–289.

[36] T. Fujimiya, K. Yamaoka and Y. Fukui (1989) Parallel first-order and michaelis–menten elimination kinetics of ethanol. Respective role of alcohol dehydrogenase (ADH), nonADH and first order pathway. *The Journal of Pharmacology and Experimental Therapeutics*, **249**(1), 311–317.

[37] K. Yamaoka and T. Nakagawa (1983) A nonlinear least squares porgram based on differential equations, MULTI(RUNGE), for microcomputers. *Journal of Pharmacobio-Dynamics*, **6**, 595–606.

[38] H. Yokoyama, N. Tsuchihashi, T. Ogata, M. Hiramatsu and N. Mori (1996) An analysis of the intracerebral ability to eliminate a nitroxide radical in the rat after administration of idebenone by an *in vivo* rapid scan electron spin resonance spectrometer. *MAGMA*, **4**(3,4), 247–250.

[39] R. Niwa, R. Konaka, M. Hiramatsu and H. Kamada (1997) Synthesis of spin labels for ESR imaging of living rat head. *Chemical and Pharmaceutical Bulletin*, **45**(5), 923–927.