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Pharmacokinetic Analysis of Free Radicals by *in vivo* BCM (Blood Circulation Monitoring)-ESR Method

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In pharmacokinetic studies, a variety of analytical method including radioisotopic detection and HPLC (high performance liquid chromatography) has been used. In the present investigation, we developed *in vivo* BCM (Blood Circulation Monitoring)-ESR method, which is a new technique with a conventional X-band ESR spectrometer for observing stable free radicals in the circulating blood of living rats under anaesthesia. Both 5-(PROXYL derivatives) and 6-(TEMPO derivatives) membered nitroxide spin probes with various types of substituent functional group were used. After physico-chemical properties of the spin probes such as hyperfine coupling constant (A-value), g-value and partition coefficient as well as chemical stability of the compounds in the fresh blood were obtained, the *in vivo* BCM-ESR method was performed in normal rats. Several pharmacokinetic parameters such as half-life of the probes, distribution volume, total body clearance and mean residence time were obtained and discussed in terms of their chemical structures. In addition, clearance of a spin probe was related to the urine concentration. The BCM-ESR method was found to be very useful to observe free radicals at the real time. By time-dependent ESR signal decay of spin probes, pharmacokinetic parameters were obtained.

Keywords: X-band ESR, blood circulation monitoring, nitroxide radicals, rat, pharmacokinetics, radical clearance

Abbreviations: TEMPO: 2, 2, 6, 6-tetramethyl-piperidine-1-oxyl, 4-hydoxy-TEMPO: 4-hydoxy-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl, 4-amino-TEMPO: 4-amino-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl, 4-oxo-TEMPO: 4-oxo-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl, 4-carboxy-TEMPO: 4-carboxy-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl, 4-(2-iodeacetamido)-TEMPO: 4-(2-iodeacetamido)-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl, 4-maleimido-TEMPO: 4-maleimido-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl, 4-phosphonooxy-TEMPO: 4-phosphonooxy-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl, 3-carboxy-PROXYL: 3-carboxy-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl, 3-carbamoyl-PROXYL: 3-carbamoyl-2, 2, 5, 5-tetramethyl-pyrrolidine-1 -oxyl, 3-cyano-PROXYL: 3-cyano-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl, 3-amino-PROXYL: 3-amino-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl, 3-aminomethyl-PROXYL. 3-aminomethyl-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl, 3-(2-bromoacetamido)-PROXYL 3-(2-bromoacetamido)-2, 2, 5, 5-tetramethylpyrrolidine-1-oxyl, 3-(2-iodoacetamido)-PROXYL: 3-(2-iodoacetamido)-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl, 3-(isothiocyanatomethyl)-PROXYL: 3- (isothiocyanatomethyl)-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl, 3-maleimido-PROXYL: 3-maleimido-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl, 3-[3-(2-bromoacetamido) propyl -carbamoyl]-PROXYL: 3-[3-(2-bromoacetamido)-propylcarbamoyl]-2, 2, 5, 5-tetramethylpyrrolidine-1-oxyl, 3-{2-[2-(2-bromoacetamido) ethoxy] ethyl} carbamoyl-PROXYL: 3-{2-[2-(2-bromoacetamido)-ethoxy] ethyl} carbamoyl-2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl, BSA: bovine serum albumin, KRB buffer: Krebs Ringer bicarbonate buffer, BCM-ESR: blood circulation monitoring-electron spin resonance,

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INTRODUCTION

Recently, it has been revealed that *in vivo* free radicals such as active oxygen species including superoxide anions, hydroxyl radicals and nitric oxides as well as bio-paramagnetic metal ions are deeply related to development and treatment of various diseases or maintenance of physiological homeostasis.[1,2] These chemical species are in general paramagnetic compounds, and thus they are the target molecules of ESR measurement which detects only the paramagnetic state.[3] To understand the physiological roles of these paramagnetic species, it is necessary to measure them *in vivo*.

In vivo ESR measurement was started in 1975 by Feldman et al. who detected free radicals such as 4-hydroxy-TEMPO.[4] In ESR measurement for whole body, a prevention of microwave absorption by water molecule is indispensable. Therefore, L-band ESR operated at low frequency (1 GHz), which enables to detect paramagnetic species in aqueous samples, has been developed.[5–8] However, available L-band ESR spectrometers are too insensitive to detect paramagnetic species generated *in vivo*.

On the other hand, radioisotopes are generally used for *in vivo* pharmacokinetic analysis, but a special institution is required to use radioisotopes. Therefore, it is very important to develop *in vivo* pharmacokinetic analysis method without using radioisotopes. Then, we develop the pharmacokinetic methods with both X- and L-band ESR spectrometries instead of radioisotopes. In the present paper, we propose an *in vivo* BCM (Blood Circulation Monitoring)-ESR method, which measures stable free radicals in continuously circulating blood in rats. In this study, we aimed a fundamental development of *in vivo* pharmacokinetic method by X-band ESR with nitroxide radicals to find relationships between physical properties of spin-probes and pharmacokinetic parameters. Before us, a direct ESR method has been reported by Wang et al.,[9,10] who observed ascorbic acid radical in the circulating blood of rat. But we report here a systematic and quantitative BCM-ESR method monitoring nitroxide radicals administrated to rats, which in turn develops a new pharmacokinetic method by ESR.

MATERIALS AND METHODS

Reagents

3-Carboxy-PROXYL, 3-carbamoyl-PROXYL, TEMPO, 4-hydroxy-TEMPO and 4-amino-TEMPO were purchased from Sigma Chemical Co. 3-Cyano-PROXYL, 3-aminomethyl-PROXYL, 3-(2-bromoacetamido)-PROXYL, 3-(2-iodeacetamido) -PROXYL, 3-(isothiocyanatomethyl)-PRO-XYL, 3-maleimido-PROXYL, 3-[3-(2-bromoacetamido) propylcarbamoyl-PROXYL, 3-{2-[2-(2-bromoacetamido) ethoxy] ethyl} carbamoyl-PROXYL, 4-oxo-TEMPO, 4-carboxy-TEMPO, 4-(2-iodeacetamido)-TEMPO and 4-maleimido-TEMPO were obtained from Aldrich Chemical Co. All spin-label agents at a concentration of 10mM were dissolved in physiological saline (0.9% NaCl). Pentobarbital (50mg/ml) and BSA (bovine serum albumin, fraction V) were purchased from Dainabot Co. and Sigma Chemical Co., respectively. Other reagents were of the highest purity commercially available.

Animals

Male Wistar rats (9–10 weeks old) weighing 300–350g were purchased from Simizu Experimental Material Co. (Kyoto, Japan).

BCM-ESR Method

Rats were anaesthetized by intraperitoneal injection of pentobarbital (50mg/kg body weight). Animals were put on a hand-made warmer for rat. Heparinized polyethylene tubes of 0.5mm inner diameter were inserted into the femoral artery and vein to make a circuit, which was connected to an ESR cell (Fig. 1).[9,10] ESR cell used was a glass 10μl Drummond capillary (Drum-

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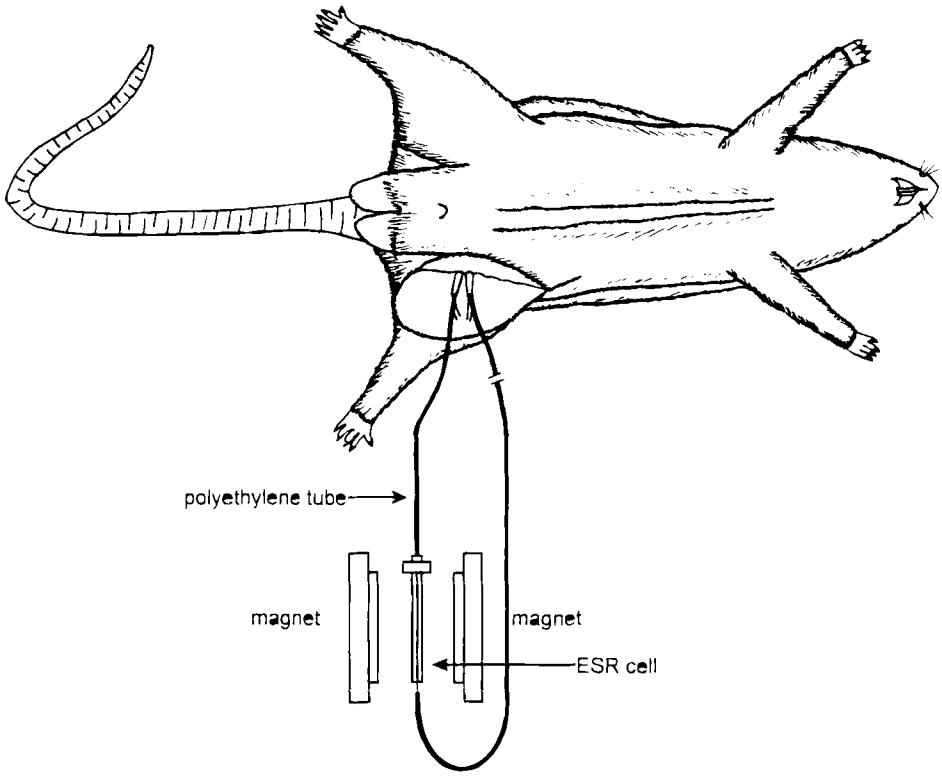

FIGURE 1 *In vivo* blood circulation monitoring -ESR (BCM-ESR) method. The rats were anaesthetized by *i.p.* injection of pentobarbital at a dose of 50mg/kg and operated, in which polyethylene tubes were inserted into the femoral artery and vein through a capiraly for ESR measurement.

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mond Scientific Company, Pennsylvania, USA), which was ESR signal free under the experimental conditions. The length from artery to the center of ESR cavity was 40cm and the total length from artery to vein was 80cm. The blood circulation rate was checked using an artificial air bubble in the circulating blood. Immediately after the injection of a spin-label solution at a dose of 10 μmol/kg body weight into the femoral vein of a rat, ESR spectra were measured at every 20 sec for 3–5min. ESR spectra were recorded with a JEOL REIX ESR spectrometer at a modulation frequency of 100KHz, modulation amplitude of 0.1mT and microwave of 5.0mW. The spectrometer was calibrated with an ADVANTEST microwave counter, R5372. Other instrumental conditions were as follows: Magnetic field 336.5 ± 5.0mT and response 0.03 sec. Concentrations of spin-label agents in the blood were calculated from each calibration curve by monitoring the central peak height of the triplet signal due to nitroxide radicals dissolved in the fresh blood of rats. In pharmacokinetic study monitoring the ESR signal intensity, which depends on the concentration of spin label agents in blood, one or two compartment model analysis method was used.[11,13] Following equations were used (Fig. 2).

One compartment analysis. $t_{1/2} = \ln 2/K$ ($t_{1/2}$: half life, K: clearance rate constant), $V = D/C_0$ (V: distribution volume, D: dose, C_0: concentration in blood at the administration), CLtot = K × V

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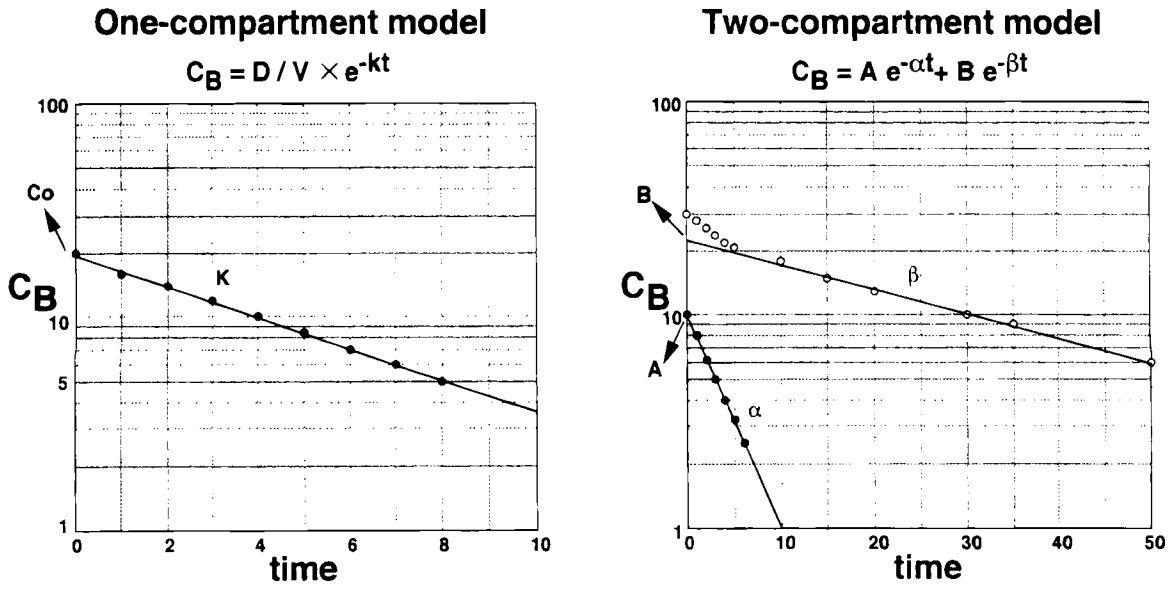

FIGURE 2 Compartment model analyses by pharmacokinetic studies. The pharmacokinetics of spin-label agents were analyzed with one- or two-compartment model analysis. PROXYL-derivatives and 4-maleimido-TEMPO were analyzed by two-compartment model, and other TEMPO-derivatives were by one-compartment model. In each equation, C_B values represent the concentration of a spin-label agent in the circulating blood of a rat.

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(CLtot = total body clearance), AUC = D/CLtot (AUC: area under the blood concentration vs. time curve) and MRT = 1/K (MRT: mean residence time).

Two compartment analysis. AUC = $A/\alpha + B/\beta$ (A: concentration of distribution phase (α phase) in blood, α: clearance rate constant of distribution phase, B: concentration of clearance phase (β phase) in blood, β: clearance rate constant of clearance phase), MRT = $(A/\alpha^2 + B/\beta^2)/AUC$, CLtot = D/AUC, Vss = MRT × CLtot (Vss: volume of distribution at steady state) and $t_{1/2}$ = MRT × ln2. In the analyses of PROXYL-derivatives and 4-maleimido-TEMPO, two-compartment model analysis was used and in others one-compartment model analysis was applied. ESR signal intensity due to PROXYL-derivatives were decreased in a two phase pattern, which was fitted to the clearance curve of two-compartment model. While, ESR signal intensity due to TEMPO-derivatives gave a linear pattern, which was fitted to the clearance curve of one compartment model. However, the signal due to 4-maleimido-TEMPO, which binds with thiol-containing proteins in the circulating blood, decayed showing a two phase clearance curve, thus the clearance curve was analyzed by two-compartment model.

Stability of Spin-Label Agents

Stability of a spin-label agent was evaluated with its half life in the fresh blood of rats. A spin-label agent (final concentration, 200nmol/ml) dissolved in physiological saline (0.9% NaCl) was added to the fresh blood of rats and mixed. An aliquot of the solution was transferred into a capillary, which was fixed in the cavity of ESR. ESR spectra were recorded at room temperature under the following conditions: Magnetic field 336.5 ± 5.0mT, response 0.03 sec, modulation fre-

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quency 100KHz, modulation amplitude 0.1mT and microwave power 5.0mW.

Interaction of Spin-Label Agents with a Protein

A spin-label agent (4-maleimido-TEMPO) (200 nmol/ml) was added to BSA solution (4.3g/dl) dissolved in KRB buffer (pH 7.4), which contains 120mM of NaCl, 1.27mM of $CaCl_2$, 1.2 mM of $MgSO_4$, 4.75mM of KCl, 1.2mM of KH_2PO_4 and 24mM of $NaHCO_3$. ESR spectrum was measured at room temperature under the same conditions as for those of stability experiments.

ESR Parameters of Spin-Label Agents in the Blood of Rats

A spin-label agent was dissolved in the fresh blood of rat (10nmol/ml), and ESR spectra were measured at room temperature under the same conditions as for those of stability experiments. Hyperfine coupling constant (A-value) and g-value were calculated from the spectra.

Partition Coefficient of Spin-Label Agents

The partition coefficient of spin-label agent was also determined by ESR method with a conventional method in KRB buffer (pH7.4)/n-octanol system for 6 hours. Obtained partition coefficient is shown as follows. P = [O: concentration of spin probe in n-octanol]/[W: concentration of spin probe in KRB buffer].

Recovery of Nitroxide Radical by Ferricyanide

3-Carboxy-PROXYL dissolved in physiological saline was injected at a dose of 10μmol/kg body weight in the tail vein of rats without anaesthesia. The blood of rats was collected for 3–360 min and the concentration of 3-carboxy-PROXYL in the blood was determined by ESR method. On the other hand, the serum (50μl) was separated from each blood, and ESR spectrum was recorded at room temperature after addition of 10μl ferricyanide solution (60mM).

Excretion of Spin-Label Agents in the Urine of Rat

3-Carboxy-PROXYL dissolved in physiological saline was injected at a dose of 10μmol/kg body weight in the tail vein of rat without anaesthesia. After the administration, the urine was collected every two hours, and the concentration of 3-carboxy-PROXYL in the urine was determined by ESR method. On the other hand, ESR spectrum of each urine (50μl) was measured after addition of 10μl ferricyanide solution (60mM).

RESULTS

ESR Parameters and Stability of Spin-Label Agents

Stability was estimated by half life ($t_{1/2}$) of a spin-label agent in the fresh blood of rats. The peak height of ESR signal due to a nitroxide radical incubated with the blood was decreased time-dependently, but both hyperfine coupling constant (A-value) and g-value were not changed (Table I). Half lives of PROXYL-derivatives in the fresh blood of rats were found to be in the range of 1.3–44.5 hr, being long enough compared with those (12.1–76.4 min) of TEMPO-derivatives, as shown in Table II. Half life of the spin-label agent in the blood of rats appeared to depend on the structure of spin-label agent.

Partition Coefficients of Spin-Label Agents

Under physiological conditions, partition coefficient of spin-label agents was determined, as shown in Table III. As judged by the results, both 3-(isothiocyanatomethyl)-PROXYL and TEMPO were found to have a high lipophilic character, while both 3-carboxy-PROXYL and 3-maleimido-PROXYL to have a high hydrophilic character.

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TABLE I Hyperfine coupling constants and g-values of spin-label agents in the fresh blood of rats

Spin-label agents	A (mT)	A (10^{-3} cm^{-1})	g
3-carboxy-PROXYL	1.620	1.517	2.006
3-carbamoyl-PROXYL	1.600	1.499	2.006
3-cyano-PROXYL	1.572	1.472	2.006
3-amino-PROXYL	1.588	1.487	2.006
3-aminomethyl-PROXYL	1.598	1.496	2.006
3-(2-bromoacetamido)-PROXYL	1.594	1.493	2.006
3-(2-iodoacetamido)-PROXYL	1.517	1.421	2.006
3-(isothiocyanatomethyl)-PROXYL	1.614	1.512	2.006
3-maleimido-PROXYL	1.588	1.487	2.006
3-(3-[2-bromoacetamidol] propylcarbamoyl-PROXYL	1.589	1.489	2.006
3-{2-[2-(2-bromoacetamido) ethoxy]ethyl} carbamoyl-PROXYL	1.592	1.491	2.006
TEMPO	1.721	1.611	2.006
4-hydroxy-TEMPO	1.696	1.589	2.006
4-amino-TEMPO	1.682	1.576	2.006
4-oxo-TEMPO	1.690	1.584	2.006
4-carboxy-TEMPO	1.707	1.600	2.006
4-(2-iodoacetamido)-TEMPO	1.721	1.613	2.006
4-maleimido-TEMPO	1.707	1.600	2.006
4-phosphonooxy-TEMPO	1.707	1.600	2.006

TABLE II Stabilities of spin-label agents in the fresh blood of rats

Spin-label agents	$t_{1/2}$ (min)	K (min^{-1})
TEMPO	37.4 ± 3.3	0.019 ± 0.002
4-hydroxy-TEMPO	34.9 ± 2.9	0.020 ± 0.002
4-amino-TEMPO	12.1 ± 0.8	0.058 ± 0.004
4-oxo-TEMPO	29.5 ± 2.2	0.023 ± 0.002
4-carboxy-TEMPO	76.4 ± 4.4	0.009 ± 0.001
4-(2-iodoacetamido)-TEMPO	24.3 ± 1.4	0.029 ± 0.002
4-maleimido-TEMPO	57.8 ± 2.6	0.012 ± 0.001
4-phosphonooxy-TEMPO	36.4 ± 0.2	0.019 ± 0.000
Spin-label agents	**$t_{1/2}$ (hr)**	**K (hr^{-1})**
3-carboxy-PROXYL	17.6 ± 4.8	0.043 ± 0.014
3-carbamoyl-PROXYL	10.5 ± 1.1	0.067 ± 0.008
3-cyano-PROXYL	2.6 ± 0.1	0.262 ± 0.010
3-amino-PROXYL	6.5 ± 0.2	0.107 ± 0.004
3-aminomethyl-PROXYL	6.9 ± 0.2	0.100 ± 0.002
3-(2-bromoacetamido)-PROXYL	1.3 ± 0.0	0.533 ± 0.000
3-(2-iodoacetamido)-PROXYL	7.3 ± 0.3	0.095 ± 0.004
3-(isothiocyanatomethyl)-PROXYL	16.4 ± 10.7	0.042 ± 0.028
3-maleimido-PROXYL	44.5 ± 22.2	0.016 ± 0.007
3-(3-[2-bromoacetamido] propylcarbamoyl-PROXYL	9.1 ± 1.2	0.076 ± 0.010
3-{2-[2-(2-bromoacetamido) ethoxy]ethyl} carbamoyl-PROXYL	7.1 ± 1.4	0.098 ± 0.019

Concentration of each spin-label agent was 200 nmol/ml (final concentration) in the fresh blood of rats. Stability were evaluated with their half lives or clearance rate constants of spin-label agents in the fresh blood of rats.

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TABLE III Partition coefficients of spin-label agents

Spin-label agents	P = O/W
3-carboxy-PROXYL	0 ± 0
3-carbamoyl-PROXYL	0.63 ± 0.02
3-cyano-PROXYL	4.79 ± 0.04
3-amino-PROXYL	0.54 ± 0.02
3-aminomethyl-PROXYL	0.37 ± 0.01
3-(2-bromoacetamido)-PROXYL	5.35 ± 0.09
3-(2-iodoacetamido)-PROXYL	9.36 ± 0.16
3-(isothiocyanatomethyl)-PROXYL	45.83 ± 3.99
3-maleimido-PROXYL	0.04 ± 0.02
3-{2-[2-(2-bromoacetamido) ethoxy]ethyl}carbamoyl-PROXYL	1.60 ± 0.13
TEMPO	46.75 ± 6.70
4-hydroxy-TEMPO	4.32 ± 0.09
4-amino-TEMPO	0.94 ± 0.11
4-oxo-TEMPO	1.99 ± 0.03
4-carboxy-TEMPO	0.02 ± 0.01
4-(2-iodoacetamido)-TEMPO	14.72 ± 0.25
4-maleimido-TEMPO	0.22 ± 0.02
4-phosphonooxy-TEMPO	0.15 ± 0.04

Pharmacokinetic Parameters of Spin-Label Agents by BCM-ESR Method

Before administration of a spin-label agent, any ESR signal was not observed in the blood, but after 3 min of the administration, a triplet signal due to a nitroxide radical was observed. Thus the ESR signals observed in the blood were collected every 30 sec, in which ESR signals were decreased gradually and not observed after 360 min of the administration. Fig. 3 shows ESR spectra of 4-hydroxy-TEMPO and 4-maleimido-TEMPO obtained during BCM-ESR methods, together with that of 4-maleimido-TEMPO dissolved in KRB buffer. Line shape of ESR spectrum due to 4-hydroxy-TEMPO during BCM-ESR measurement is isotropic, but that of 4-maleimido-TEMPO during BCM-ESR method is broad compared with the line shape in KRB buffer. Similar broad ESR spectra were also observed for 3-(isothiocyanatomethyl)-PROXYL, 3-maleimido-PROXYL and spin-label agents containing halogen atoms. Time-dependent decay of the ESR signals due to the spin-label agents was observed, which enabled the pharmacokinetic analysis for each agent. Pharmacokinetic parameters of spin-label agents by BCM-ESR method were summarized in Table IV.

Interaction of Spin-Label Agents with a Protein

ESR spectrum of 4-maleimido-TEMPO was measured in the present of BSA at physiological pH 7.4, as shown in Fig. 4, whose spectrum was similar to the that in the blood of rats (Fig. 3(b)), suggesting occurrence of a covalent binding of the spin label agent with serum proteins.

Recovery of Nitroxide Radical by Ferricyanide

Since a part of a spin-label agent administered has been presumed to be reduced to its hydroxyl amine, which is an ESR-silent form, ferricyanide was added to re-oxidize it to the parent radical form. After separation of serum from the blood, ferricyanide was added to the serum and ESR spectrum was measured. ESR signal intensity was increased by addition of ferricyanide in each serum, as shown in Fig. 5. Increment of signal intensity was 167 ± 11% on average.

Excretion of Spin-Label Agents in Urine

Urine from rats after *i.v.* administration of 3-carboxy-PROXYL was collected every 2 hrs and ESR spectra were measured at room temperature. Before administration of an agent, no ESR signal was observed in the urine, but a triplet signal due to the nitroxide radical was observed after 2hrs of the administration (Fig. 6). The strongest signal intensity was found at 2hrs after the administration, but it decreased. After 10hrs of the administration, the signal was not observed in the urine. When ferricyanide was added to the urine, ESR signal intensity was increased in each collected urine (Fig. 7). In the urine collected for 10 and 18hrs after the administration of the agent, ESR signal was not observed, but after addition of ferricyanide, the signal due to the nitroxide radical was observed in trace amount. Increment of sig-

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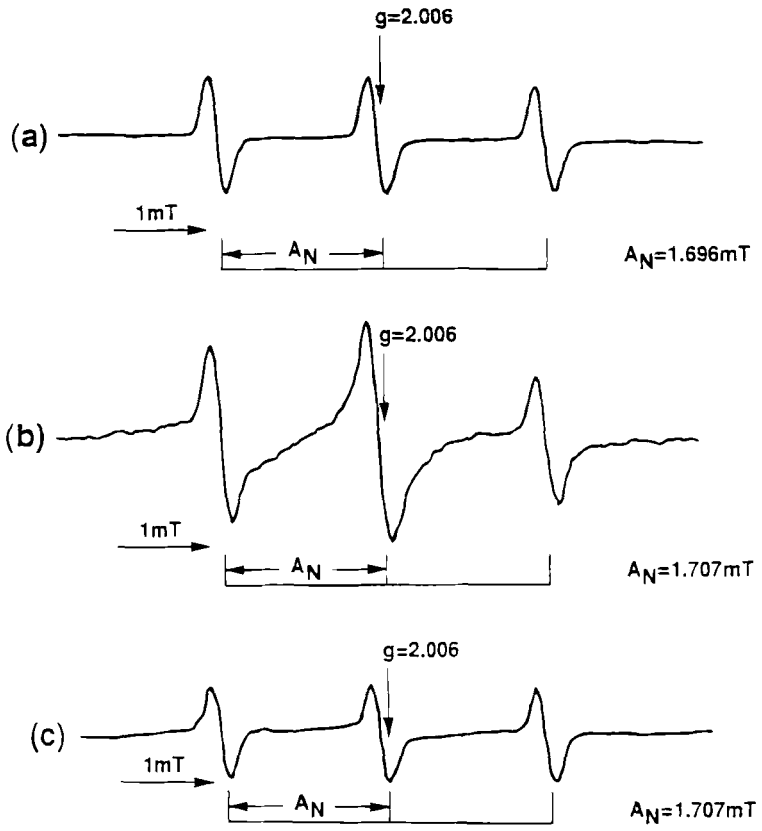

FIGURE 3 ESR spectra of hydroxy-TEMPO and maleimido-TEMPO at room temperature
(a) hydroxy-TEMPO detected by the BCM-ESR method
(b) maleimido-TEMPO detected by the BCM-ESR method
(c) maleimido-TEMPO detected in KRB buffer

nal intensity was 156 ± 10% on average, similarly to the increment observed for the serum. From the results, approximately 25% of the spin-label agent administered is found to be excreted as the parent nitroxide in total urine for 18 hours after the administration of the agent. Therefore, all excretion amount (parent + reduced forms) of the agent in the urine was estimated to be 34 ± 5% of the administered agent.

DISCUSSION

Several spin-label agents in the circulating blood were measured by BCM-ESR method, demonstrating that nitroxide radicals disappeared from the blood, without both changing the ESR line shape and developing new signals. Administered nitroxides are presumed to be reduced to ESR inactive hydroxylamines by active oxygen species like superoxide anion radicals, or reducing agents such as ascorbic acid and glutathione, and lose their paramagnetic properties.[14–22] When ferricyanide, which is known to oxidize hydroxylamine, was added to the blood, ESR signal intensity increased for each collected blood (167 ± 11%) (Fig. 5). Therefore, clearance of nitroxides in the circulating blood was thought to be partially contributed by the reduction of them with reducing components in the blood. However, since the

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TABLE IV Pharmacokinetic parameters for the blood of rats treated with spin-label agents (D = 10μ mol/kg) by *i.v.* injection as determined by BCM-ESR method

Spin-label agents	$t_{1/2}$ (sec)	V (ml/kg)	CLtot (ml/min/kg)	AUC (nmol · min/ml)	MRT (sec)
3-carboxy-PROXYL	165.7 ± 15.2	168.2 ± 15.2	43.0 ± 2.2	236.3 ± 12.5	239.1 ± 22.0
3-carbamoyl-PROXYL	99.2 ± 4.5	145.1 ± 3.0	60.0 ± 2.9	167.7 ± 7.8	143.2 ± 6.7
3-cyano-PROXYL	47.6 ± 0.3	161.2 ± 7.8	140.9 ± 6.4	71.1 ± 3.3	68.6 ± 0.4
3-amino-PROXYL	55.6 ± 4.7	173.7 ± 10.7	130.4 ± 5.9	76.9 ± 3.4	80.2 ± 6.7
3-aminomethyl-PROXYL	71.5 ± 9.5	174.1 ± 9.1	103.0 ± 8.5	98.3 ± 7.9	103.2 ± 13.8
3-(2-bromoacetamido)-PROXYL	12.5 ± 1.2 (min)	79.4 ± 15.3	4.4 ± 0.5	2288.0 ± 280.0	18.0 ± 1.7 (min)
3-(2-iodoacetamido)-PROXYL	5.2 ± 0.5 (min)	147.5 ± 21.4	4.5 ± 1.5	520.8 ± 40.1	35.7 ± 9.1 (min)
3-(isothiocyanatomethyl)-PROXYL	21.7 ± 5.9 (min)	56.7 ± 6.56	1.9 ± 0.5	5612.1 ± 1839.0	31.3 ± 8.5 (min)
3-maleimido-PROXYL	28.2 ± 5.0 (min)	32.8 ± 2.6	0.8 ± 0.2	12390.0 ± 2990.0	40.7 ± 7.1 (min)
3-(3-[2-bromoacetamido] propylcarbamoyl-PROXYL	24.7 ± 7.3 (min)	245.6 ± 64.5	7.1 ± 1.1	1447.2 ± 241.4	35.6 ± 10.6 (min)
3-{2-[2-(2-bromoacetamido) ethoxy] ethyl}carbamoyl-PROXYL	16.3 ± 2.7 (min)	164.1 ± 33.8	7.0 ± 1.9	1493.0 ± 349.0	23.6 ± 3.8 (min)
TEMPO	11.9 ± 0.4	67.5 ± 0.9	236.3 ± 7.8	42.7 ± 1.6	17.3 ± 0.6
4-hydroxy-TEMPO	15.4 ± 0.5	62.7 ± 3.4	169.5 ± 14.7	60.6 ± 4.6	22.3 ± 0.8
4-amino-TEMPO	14.8 ± 1.1	57.6 ± 2.2	163.2 ± 9.2	61.7 ± 3.6	21.3 ± 1.5
4-oxo-TEMPO	8.2 ± 0.2	65.0 ± 1.9	332.0 ± 6.4	30.2 ± 0.6	11.8 ± 0.4
4-carboxy-TEMPO	20.6 ± 0.5	65.2 ± 1.4	132.2 ± 5.7	76.1 ± 3.4	29.8 ± 0.8
4-(2-iodoacetamido)-TEMPO	23.5 ± 5.9 (min)	143.7 ± 2.9	19.3 ± 1.4	520.8 ± 40.1	7.5 ± 0.7 (min)
4-maleimido-TEMPO	31.7 ± 0.8 (min)	82.7 ± 29.7	1.8 ± 0.6	5998.0 ± 2354.0	45.8 ± 1.1 (min)
4-phosphonooxy-TEMPO	21.7 ± 3.6	37.6 ± 6.5	72.3 ± 4.1	138.9 ± 7.6	31.3 ± 5.2

Data are shown as the mean values ± standard deviations for 3 ~ 4 rats.

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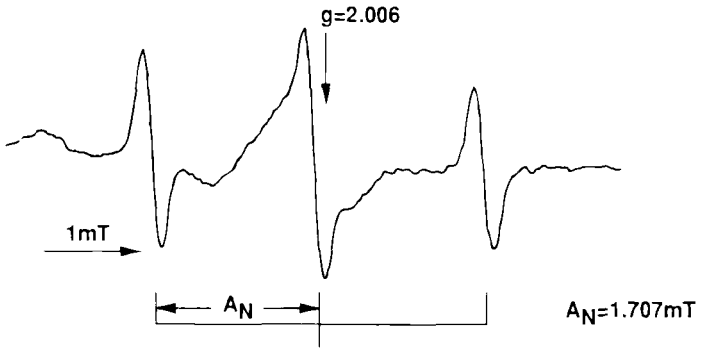

FIGURE 4 ESR spectrum of maleimido-TEMPO detected in BSA solution at room temperature. BSA solution was prepared at pH 7.4 and at a concentration of 4.3g/dl.

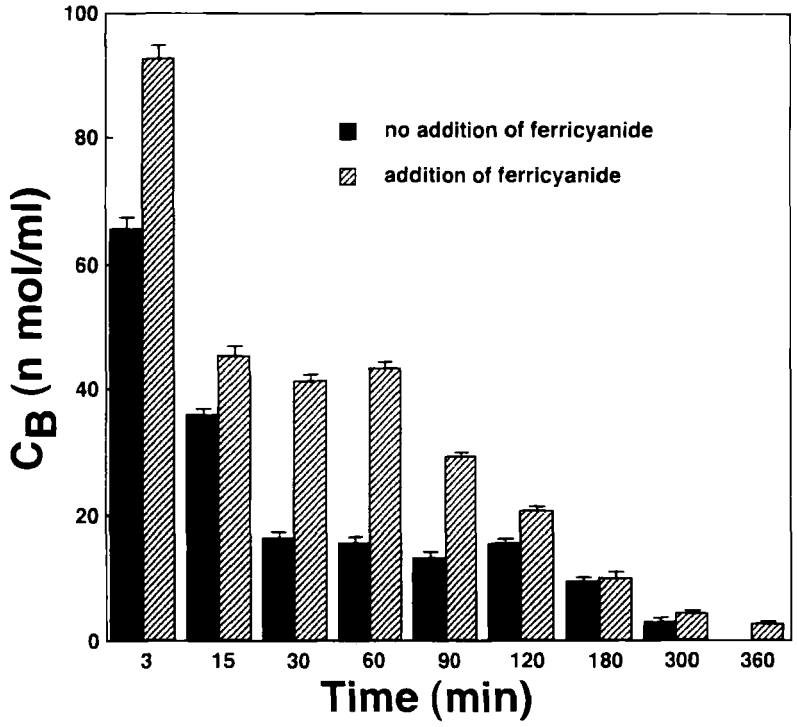

FIGURE 5 Time-dependent clearance of 3-carboxy-PROXYL from the blood of rats and recovering of radicals after addition of ferricyanide to the blood. The ferricyanide was added to the serum separated from the blood collected. The rats were received *i.v.* injection of 3-carboxy-PROXYL (10μmol/kg) and they were not anaesthetized during the experiment. C_B (nmol/ml) indicates the concentration of spin-label agent (3-carboxy-PROXYL) in the blood.

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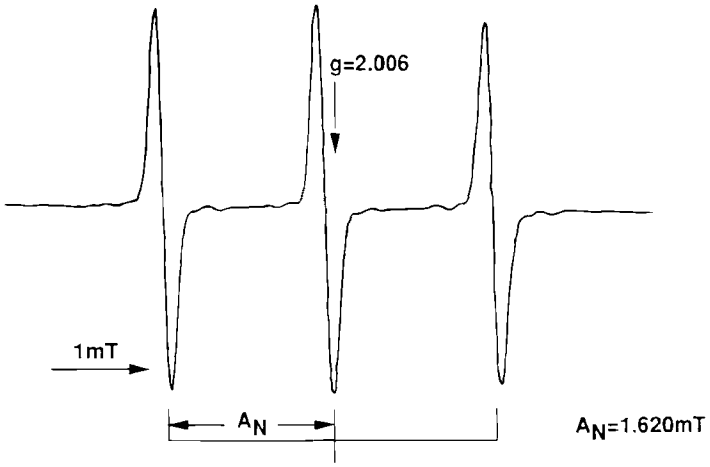

FIGURE 6 ESR spectrum at room temperature of 3-carboxy-PROXYL detected in the urine for 2 hours after the administration of the spin-label agents by *i.v.* injection.

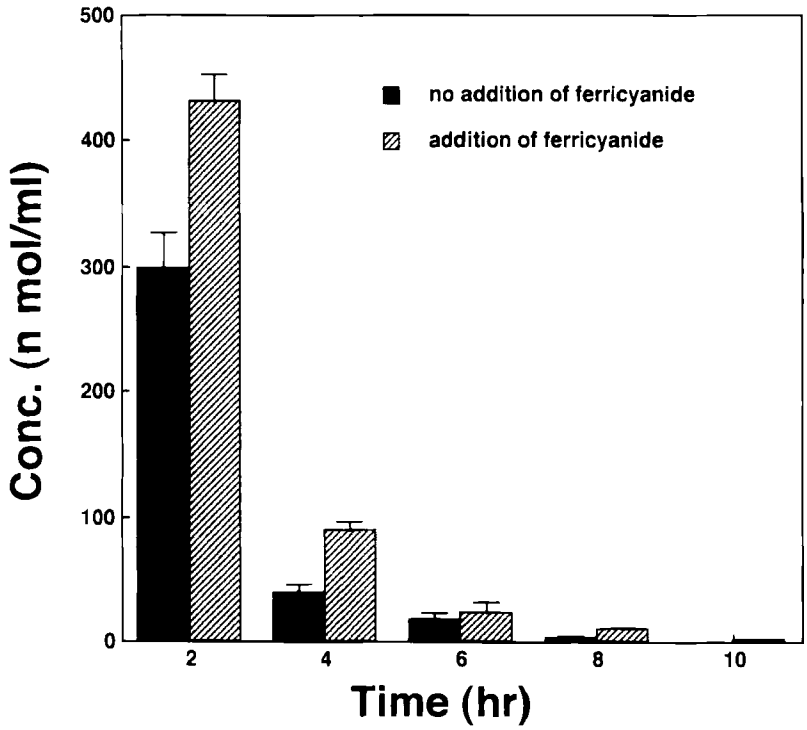

FIGURE 7 Clearance of 3-carboxy-PROXYL from the urine of rats and recovering of radicals after addition of ferricyanide to the urine. The rats were given *i.v.* injection of 3-carboxy PROXYL (10μmol/kg) and were not anaesthetized during the experiment.

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spin disappearance in the fresh blood was very slow (Table II), we speculated that the reduction process is not a major factor for spin clearance in the circulating blood. On the other hand, when the ESR spectrum of the urine was measured after the administration of a spin-label agent, ESR signals due to the nitroxide radical were observed (Fig. 6). In case of 3-carbamoyl-PROXYL, approximately 34% of the administrated agent was excreted as both parent nitroxide and hydroxyl amine for 18 hours after the administration. These results suggest the occurrence of excretion path way from the kidney for the radical.

ESR line shape of 4-hydroxy-TEMPO in circulating blood was found to be in an isotropic form (Fig. 3(a)), indicating that the nitroxide radical exists as the free form. While, ESR spectrum of 4-maleimido-TEMPO in circulating blood was broad (Fig. 3(b)), thus the thermodynamical movement of the radical was suggested to be inhibited. Similar broad spectra during BCM-ESR method were observed for 3-(isothiocyanatomethyl)-PROXYL, 3-maleimido-PROXYL, and spin-label agents with halogen atoms. Broadening of spin-label agents might be related to an occurrence of interactions between the agent and proteins in the blood. Then, the interaction was examined under physiological conditions. ESR spectrum similar to the signal during BCM-ESR method was obtained in the system of 4-maleimido-TEMPO and BSA (Fig. 4), suggesting that the spin-label agent binds covalently with proteins in the blood. Thus we concluded that the clearance process of spin-label agents in circulating blood is contributed by several factors such as covalent binding with blood proteins, incorporation to organs, reductions in blood and organs and excretion to the urine.

We analyzed the pharmacokinetics of the spin-label agents, considering the interaction of them with proteins in the blood (Table IV). Obtained pharmacokinetic parameters of spin-label agents appeared to depend on their structures,[23] the half lives of PROXYL-derivatives being long compared with those of TEMPO-derivatives except for the agents which interact with proteins in the blood. Spin-label agents with maleimido group or halogen atoms interact with proteins in the blood, as judged by the line shape of ESR spectra. Since it is well known that compounds with maleimido group or halogen atoms bind easily with SH group of proteins, the spin-label agents with those functional group bind with proteins or enzymes through the SH groups in the blood and circulate without receiving the reduction in the blood of rats. In fact, the reduced form of a spin-label agent in the blood and urine accounts approximately 40% of the agent administered (Figs. 5 and 7). The clearance process of spin-label agents from the blood was thus suggested to be contributed by the incorporation of them to other organs rather than the reduction of the agents. Fig. 8 depicts the ratio (%) of total nitroxide radicals found in the blood and urine after the administration of 3-carbamoyl-PROXYL. When the total blood volume of rats (300–350 g) is supposed to be 22 ml on average,[24] the ratio of the agents found in the blood and urine of rats forms about 13% and 20%, respectively. Thus, the remaining 67% of the agent might be incorporated in other organs or metabolized to unknown diamagnetic compounds. Thus, it is necessary to examine more detail the fate of spin-label agents in organs of rats. Further investigations are under way.

The distribution volumes (V ml/kg body weight) of PROXYL-derivatives were to be relatively large compared with those of TEMPO-derivatives (Table IV), suggesting that the incorporated ratio of the agents (PROXYL-derivatives) to the organs were to be large compared with those of TEMPO-derivatives. 3-Maleimido-PROXYL gave the smallest distribution volume among other PROXYL-derivatives, which indicates that *in vivo* distribution of 3-maleimido-PROXYL is limited without distributing to the organs. In conclusion, we analyzed the pharmacokinetics of spin-label agents by BCM-ESR method. The concentration of a spin-label agent in the blood was found to relate closely to the chemical structure as well as the functional group on them. On the basis of the present results, we propose here that BCM-ESR

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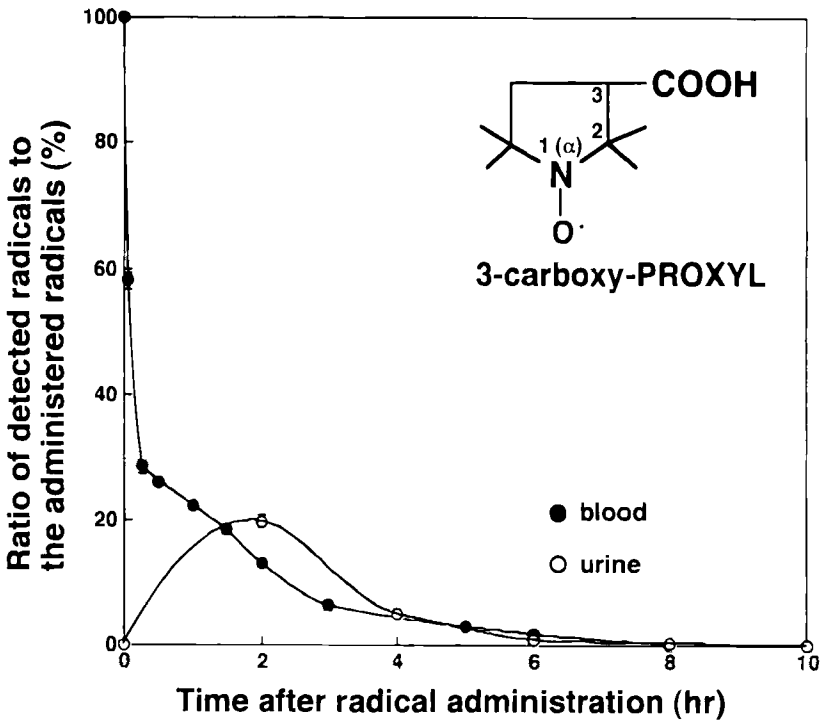

FIGURE 8 Time-dependent ratio of detected radicals to the administered spin-label agent in the blood and urine.

method is an useful and new method to analyze the pharmacokinetics of paramagnetic compounds in living animals.

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