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Spinnokinetic Analyses of Blood Disposition and Biliary Excretion of Nitric Oxide (NO)-Fe(II)-*N*-(Dithiocarboxy)sarcosine Complex in Rats: BCM-ESR and BEM-ESR Studies

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Nitric oxide (NO) is well known to have a wide variety of biological and physiological functions in animals. On the basis of the fact that Fe(II)-dithiocarbamates react with NO, a Fe(II)-*N*-(dithiocarboxy)sarcosine complex (Fe(II)-DTCS) was proposed as a trapping agent for endogenous NO. However, quantitative pharmacokinetic investigation for NO-Fe(II)-dithiocarbamate complexes in experimental animals has been quite limited. This paper describes the results on the quantitative pharmacokinetic features of a NO-Fe(II)-*N*-DTCS in both the blood and bile of rats following intravenous (i.v.) administration of the complex. For this purpose, we applied two *in vivo* methods, i.e. (1) *in vivo* blood circulation monitoring-electron spin resonance (BCM-ESR) which previously developed, and (2) *in vivo* biliary excretion monitoring-electron spin resonance (BEM-ESR). We monitored real-time ESR signals due to nitrosyl-iron species in the circulating blood and bile flow. The ESR signal due to NO-Fe(II)-DTCS was stable in biological systems such as the fresh blood and bile. In *in vivo* BCM- and BEM-ESR, the pharmacokinetic parameters were calculated on the basis of the two-compartment and hepatobiliary transport models. The studies also revealed that the compound is widely distributed in the peripheral organs and partially excreted into the bile. We named a kinetic method to follow spin concentrations as spinnokinetics and this method will be useful for detecting and quantifying the endogenously generated NO in Fe(II)-DTCS administered animals.

Keywords: Nitric oxide-iron(II)-*N*-(dithiocarboxy)sarcosine complex; BCM-ESR; BEM-ESR; Blood disposition; Biliary excretion; Spinnokinetics

Abbreviations: BCM-ESR, blood circulation monitoring-electron spin resonance; BEM-ESR, biliary excretion monitoring-electron spin resonance; NO-Fe(II)-DTCS, nitrosyl-iron(II)-*N*-(dithiocarboxy)-sarcosine complex; RSA, rat serum albumin; Hb, bovine hemoglobin; trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; Asc, sodium ascorbate; GSH, reduced glutathione; AUC_{blood}, area under the concentration curve in the blood; MRT_{blood}, mean residence time in the blood; CL_{tot}, total body clearance; Vd_{ss}, steady-state distribution volume in the body; $Vd_{central}$, distribution volume of central compartment; $Vd_{peripheral}$, distribution volume of peripheral compartment; Q_{bile}, bile flow rate; CL_{bile}, biliary excretion clearance; k_{tr}, the transfer rate constant from the blood space to the bile duct; Ae_{bile}, biliary excretion amount; MET_{bile}, mean biliary excretion time; MTT_{liver}, mean hepatic transit time

INTRODUCTION

Nitric oxide (NO), a simple diatomic free radical, has been shown to play many biological, physiological, and pathophysiological roles in biological systems, which in turn regulate the vascular, neurotransmitter, and host defense systems.[1–4] To investigate the biological effects of NO in animals, we need *in vivo* NO detection to know the quantity and distribution of NO in cells and organs. Electron spin resonance (ESR) spectrometry combined with a spin-trapping technique has been applied to detect *in vitro* and *in vivo* NO in real time, because this method has higher reliability for sensitive and specific detection of NO than other analytical techniques.[5–9]

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On the basis of the fact that Fe complexes with dithiocarbamate derivatives reacted to NO with high affinities,[10–13] the Fe(II)-*N*-(dithiocarboxy)sarcosine complex (Fe(II)-DTCS) as a highly water-soluble NO-trapping agent[14] was proposed for the use in endogenous NO in biological systems.[15] NO-Fe(II)-DTCS formed in live animals was detectable at room temperature by both X- and L-band ESRs,[15–18] because of the distinctive three line ESR spectrum and its stability.

In vivo ESR detection of NO by a spin-trapping technique has been successful, however, quantitative interpretation for the detected NO-Fe(II)-dithiocarbamates was difficult due to the successive formation and decomposition of NO-Fe(II)-dithiocarbamate complexes.[19] Most of the previous studies simply described the detection of NO-adducts in animals, except for some studies on the quantification of NO using Fe(II)-dithiocarbamate complexes in *in vitro* aqueous solution[20] and *ex vivo* organs removed from animals.[21,22] In particular, the fast decay of a NO-adduct at the high concentrations made it difficult to monitor the ESR signals,[23] because the decay rate of a NO-Fe(II)-dithiocarbamate depended on its concentration.

Therefore, when we aim at determining the physiological levels of NO in living systems using ESR spectrometry, the pharmacokinetic analysis for the distribution and decomposition of a NO-Fe(II)-dithiocarbamate complex is essential.[9,19,23] However, previous papers in which ESR signal intensities were monitored in the body of animals by using L-band ESR determined only half-lives of signal intensities due to NO-Fe(II)-dithiocarbamate complexes. For the purpose of developing the quantitative pharmacokinetics, we applied the *in vivo* blood circulation monitoring-ESR (BCM-ESR) with a more sensitive X-band ESR than L-band.[24–27] In addition, we developed an *in vivo* biliary excretion monitoring-ESR (BEM-ESR), following the direct ESR measurement of NO-Fe(II)-D-*N*-methylglucamine dithiocarbamate (NO-Fe(II)-MGD) in the bile flow of rats by Kotake *et al.*[28] Such methods enabled us to quantitatively analyze the ESR signals due to NO-Fe(II)-DTCS in the circulating blood and the bile flow of normal rats. The proposed methods, which we named as spinnokinetics in the present study, may be useful for detecting and quantifying endogenous NO generated in animals administered Fe(II)-DTCS spin-trap.

EXPERIMENTAL

Materials

Sterilized physiological saline was purchased from Otsuka Pharmaceutical Industries (Tokushima, Japan). Nembutal sodium injection (pentobarbital: 50 mg/ml) was obtained from Abbott Laboratories Co. (North Chicago, IL, USA). Heparin sodium injection was obtained from Shimizu Pharmaceutical Co. (Osaka, Japan). Rat serum albumin (RSA) and bovine hemoglobin (Hb) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Sodium ascorbate (Asc) and reduced glutathione (GSH) were from Wako (Osaka, Japan). All other reagents were of the highest grade commercially available and were used without further purification. Disodium *N*-(dithiocarboxy)sarcosine and nitric oxide-iron(II)-*N*-(dithiocarboxy)sarcosine complex (NO-Fe-DTCS) were prepared as reported previously.[14]

Animals

Male Wistar rats (8 weeks old) weighing 250 g were purchased from Shimizu Experimental Material Co. (Kyoto, Japan) and maintained on a light/dark cycle in the central animal facility before use. All animals were given free access to standard rat chow and water prior to the experiments. All experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and performed according to the guidelines for animal experimentation of KPU.

Stability and ESR Parameters of NO-Fe(II)-DTCS in the Fresh Blood, Erythrocytes, Serum, Bile, Urine of Rats, and 3% RSA Solution

The stability of NO-Fe-DTCS in the fresh blood, erythrocytes, serum, bile, urine of rats, and 3% RSA solution was evaluated in terms of the paramagnetic disappearance rate constants as well as the changes of ESR spectral patterns. NO-Fe-DTCS dissolved in a phosphate buffer (pH 7.4), which was degassed by a N_2 purge in advance, was added to each vehicle of rats at a final concentration of 100 μM and incubated at 37°C in a water bath for 15 min. To prevent the blood and erythrocytes from hemolysis during the incubation period, we added glucose at a final concentration of 5 mM to the fresh blood and erythrocytes before addition of NO-Fe-DTCS. After an aliquot of each solution transferred into a quartz 20 μl capillary (Eiko-sha Co., Osaka, Japan), which was fixed in the cavity of the ESR system, ESR spectra were immediately recorded every 5 min by means of the X-band ESR spectrometer JES-RE-1X (JEOL, Tokyo, Japan) under the following conditions: frequency, 9.4 GHz; microwave power, 5.0 mW; modulation frequency, 100 kHz; modulation amplitude width, 0.1 mT; receiver gain, 1000; response, 0.03 s; scanning time, 1 min; magnetic field 330 ± 10 mT. Data were collected and analyzed by

an ESPRIT ESR Data System (JEOL, Tokyo, Japan). ESR parameters of NO-Fe(II)-DTCS (100 μM) dissolved in each vehicle were calculated from the obtained ESR spectra measured under the same conditions as those for stability experiments. A hyperfine coupling constant (A_0-value) and g_0-value were obtained from the spectra, where the A_0-values were calculated as the means on the basis of selected magnetic field measurements.

Stability of NO-Fe(II)-DTCS in the Hemolyzed Blood and Erythrocytes, Hemoglobin, GSH, Trolox, and Ascorbate

The stability of NO-Fe-DTCS (100 μM) dissolved in the hemolyzed blood and erythrocytes, hemoglobin (Hb) with both intact and reduced states, GSH, trolox, and ascorbate (Asc) solutions were evaluated by the paramagnetic disappearance rate constant in the same manner as described above. Each vehicle was prepared as follows. The blood was hemolyzed by pre-incubation at 37°C for 30 min without glucose before the addition of NO-Fe-DTCS. The hemolyzed erythrocytes were prepared by washing with 0.2% NaCl solution followed by the addition of same volume of 1.6% NaCl solution. Bovine Hb (50–250 μM) and reducing agents (1 mM) such as GSH, trolox, and Asc were dissolved in a phosphate buffer (pH 7.4). A Hb solution with a reduced state was prepared by mixing 4 times molar concentration of Asc.

BCM-ESR Method of NO-Fe(II)-DTCS in Rats

The spinnokinetic features of nitrosyl-iron species in the blood of normal rats receiving an intravenous (i.v.) bolus injection of NO-Fe(II)-DTCS at a dose of 10 μmol/kg of body weight were analyzed by *in vivo* BCM-ESR.[24–27] Briefly, rats were anesthetized by intraperitoneal (i.p.) injection of pentobarbital at a dose of 50 mg/kg body weight and kept at 35°C on a Deltaphase Isothermal Pad (Model 39 DP, Braintree Scientific, Inc., Braintree, MA, U.S.A.). Heparinized polyethylene tubes (0.5 mm i.d., 10 cm length, Natsume Co., Osaka, Japan) were cannulated into the left femoral artery and vein. The free ends of these cannulae were joined with heparinized silicone tubes (0.5 mm i.d., 30 cm length) to make a blood circuit outside the body, which was directly connected to an ESR cell (a quartz 20 μl capillary tube, Eiko-sha Co., Osaka, Japan). Blood from the femoral artery was returned and recirculated to the femoral vein after flowing through the ESR cell supported by the rat's own heartbeat and blood pressure without depletion. NO-Fe(II)-DTCS was dissolved at 5 μmol/ml in a physiological saline, which was degassed by a N_2 purge in advance. After an i.v. bolus injection through a second cannula in the right femoral vein, ESR spectra were measured at room temperature every 30 s using an X-band ESR spectrometer JES-RE-1X (JEOL, Tokyo, Japan). Instrumental conditions for the BCM-ESR measurements were as follows: frequency, 9.4 GHz; microwave power, 5.0 mW; modulation frequency, 100 kHz; modulation amplitude width, 0.1 mT; receiver gain, 1000; response, 0.03 s; scanning time, 20 s; interval time 10 s between measurements. Ranges of the external magnetic field were 330.0 ± 5 mT, and the central magnetic field was adjusted to coincide with the field of the central signal due to nitrosyl-iron(II) species. Data were collected and analyzed by an ESPRIT ESR Data System (JEOL, Tokyo, Japan).

To determine the absolute concentrations of NO-Fe(II)-DTCS, 20 μl of NO-Fe(II)-DTCS dissolved in the blood of untreated rats was applied to the ESR cell used for BCM-ESR. Instrumental conditions for the measurements were the same as those for the BCM-ESR measurements. Calibration curves were obtained by monitoring the signal intensities of the central peak due to nitrosyl-iron(II) species, which were newly prepared using the fresh blood spiked with NO-Fe(II)-DTCS every experiment. The linear relationships between ESR signal intensities and concentrations of NO-Fe(II)-DTCS were found in the range of 10–200 μM (S/N ratio ≥ 3) in the blood, where the correlation coefficients with linear regression were greater than 0.999 for five concentrations of three repeated measurements. The detection limit at S/N ratio of 2 was 5 μM in the blood.

Spinnokinetic parameters for the disposition of NO-Fe(II)-DTCS were obtained from the concentration curves in the blood on the basis of a two-compartment model. The equation of a two-compartment model $[C_{blood}(t) = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t)]$ was fitted to each individual profile of the determined concentrations of NO-Fe(II)-DTCS using a nonlinear least-squares regression program MULTI,[29] which was newly rewritten with Microsoft Fortran. $C_{blood}(t)$ is the blood concentration, α and β are the apparent rate constants, A and B are the corresponding zero time intercepts of concentrations, and t is time. The spinnokinetic parameters such as area under the concentration curve in the blood (AUC_{blood}), mean residence time in the blood (MRT_{blood}), total body clearance (CL_{tot}), steady-state distribution volume in the body (Vd_{ss}), and distribution volume of central ($Vd_{central}$) and peripheral ($Vd_{peripheral}$) compartments were calculated from the fitted results by using the following equations: $AUC_{blood} = A/\alpha + B/\beta$, $MRT_{blood} = (A/\alpha^2 + B/\beta^2)/(A/\alpha + B/\beta)$, $CL_{tot} = D_{iv}/(A/\alpha + B/\beta)$, $Vd_{ss} = D_{iv} \cdot (A/\alpha^2 + B/\beta^2)/(A/\alpha + B/\beta)^2$, $Vd_{central} = D_{iv}/(A + B)$, and $Vd_{peripheral} = Vd_{ss} - Vd_{central}$, where D_{iv} is the i.v. bolus dose of NO-Fe(II)-DTCS.[30]

Stability and ESR Parameters of NO-Fe(II)-DTCS in the Circulating Blood of Rats Treated with i.v. Injection

The stability of NO-Fe-DTCS in the circulating blood of rats treated with i.v. injection was evaluated in terms of the ESR spectral patterns and ESR parameters due to NO-Fe(II)-DTCS at liquid nitrogen temperature. After the end of BCM-ESR measurement, blood was obtained from the arterial cannule at 15 min after the administration. To prevent the blood from hemolysis, a glucose solution at a final concentration of 5 mM was added to the blood. After 100 μl of the blood was transferred into a quartz ESR tube (JEOL, Tokyo, Japan), ESR spectra were recorded at liquid nitrogen temperature by means of X-band ESR spectrometer JES-RE-1X under the following conditions: frequency, 9.2 GHz; microwave power, 5.0 mW; modulation frequency, 100 kHz; modulation amplitude width, 0.63 mT; receiver gain, 500; response, 0.03 s; scanning time, 4 min; magnetic field 330 ± 25 mT. Data were collected and analyzed by an ESPRIT ESR Data System. ESR parameters of NO-Fe(II)-DTCS such as $g_{\perp}$- and $g_{//}$-values were calculated from the obtained ESR spectra.

BEM-ESR Method of Analyzing NO-Fe-DTCS in Rats

The spinnokinetic features of nitrosyl-iron species excreted in the bile of normal rats receiving i.v. bolus injection of NO-Fe(II)-DTCS at the same dose as those for the BCM-ESR were analyzed by *in vivo* BEM-ESR. The abdomen of a rat, which was anesthetized by i.p. injection of pentobarbital (50 mg/kg) and kept at 35°C on a warming pad, was opened through a middle incision (2 cm), and the bile duct was cannulated with a PE-10 polyethylene tube (0.3 mm i.d., 0.6 mm o.d., 40 cm length, Beckton & Dickinson, Parsippany, NJ, USA).[31] After the abdomen was sutured, the anesthetized rat was placed on the countertop above the electromagnet of the X-band ESR spectrometer, and the free end of the bile cannula was directly connected to an ESR cell (a quartz 20 μl capillary tube). Bile from the liver naturally flowed through the ESR cell at a flow rate of 100 ± 10 μl/min/kg of body weight. After an i.v. administration of NO-Fe(II)-DTCS, ESR spectra were measured at room temperature every 70 s using an X-band ESR spectrometer. Instrumental conditions for BEM-ESR measurements were the same as those for the BCM-ESR, except for the receiver gain, 100, and the scanning time, 1 min. Data were collected and analyzed by an ESPRIT ESR Data System. The bile flow rates were measured every 1 min by the bile volumes collected from the ESR cell. The void time from the outlet of the bile duct to the outlet of the cannula, which was calculated from dividing the cannula volume by the bile flow rate, was subtracted from the biliary excretion data.

To determine the absolute concentrations of NO-Fe(II)-DTCS, 20 μl of NO-Fe(II)-DTCS dissolved in the bile of untreated rats was applied to the ESR cell. Instrumental conditions for the measurement were the same as those for the *in vivo* measurements. Calibration curves obtained from the central signal intensities due to nitrosyl-iron(II) species were newly prepared using the fresh bile spiked with NO-Fe(II)-DTCS every experiment. The linear relationships between ESR signal intensities and concentrations of NO-Fe(II)-DTCS were found in the range of 0.1–2.0 mM (S/N ratio ≥ 3) in the bile, where the correlation coefficients with linear regression were greater than 0.999 for the four concentrations of three repeated measurements.

Spinnokinetic parameters for the biliary excretion of NO-Fe(II)-DTCS were obtained from the concentration curves in the bile on the basis of the hepatobiliary transport model.[32] Because the equation of the hepatobiliary transport model was defined by a convolution integral in the time domain $\left[C_{\mathrm{bile}}(t) = \int_0^t f_{\mathrm{bile}}(\tau) \cdot C_{\mathrm{blood}}(t-\tau)\mathrm{d}\tau\right]$, the image equation in the Laplace domain $\left[\tilde{C}_{\mathrm{bile}}(s) = \tilde{f}_{\mathrm{bile}}(s) \cdot \tilde{C}_{\mathrm{blood}}(s)\right]$ was fitted to each individual profile of the determined concentrations of NO-Fe(II)-DTCS using a nonlinear least-squares regression program MULTI(FILT).[33] Its program adopted an algorithm of fast inverse Laplace transform (FILT) for the numerical manipulation of the image equation.[34] $C_{\mathrm{bile}}(t)$ is the bile concentration, $f_{\mathrm{bile}}(t)$ is the weight function for the hepatobiliary transport process, and s is the Laplace variable. In the present study, the Laplace transform of $f_{\mathrm{bile}}(t)$ was specified as

$$\tilde{f}_{\mathrm{bile}}(s) = \frac{CL_{\mathrm{bile}}}{Q_{\mathrm{bile}}} \cdot \left(\frac{k_{\mathrm{tr}}}{s + k_{\mathrm{tr}}}\right)^5$$

among several models, where Q_{bile} is bile flow rate, CL_{bile} is biliary excretion clearance, and k_{tr} is the transfer rate constant from the blood space to the bile duct. CL_{bile} and k_{tr} were directly calculated from the fitted results. Other spinnokinetic parameters such as biliary excretion amount (Ae_{bile}), mean biliary excretion time (MET_{bile}), and mean hepatic transit time (MTT_{liver}) were calculated as follows: $Ae_{\mathrm{bile}} = CL_{\mathrm{bile}} \cdot AUC_{\mathrm{blood}}$, $MRT_{\mathrm{bile}} = MRT_{\mathrm{blood}} + MTT_{\mathrm{liver}}$, and $MTT_{\mathrm{liver}} = 5/k_{\mathrm{tr}}$.[32]

Spinnokinetics of NO-Fe-DTCS in Rats Treated with i.v. Continuous Infusion

The spinnokinetic features of nitrosyl-iron species in the blood and bile of normal rats who received i.v. continuous infusion of NO-Fe(II)-DTCS at a dose of

2 μmol/min/kg of body weight were analyzed by BCM- and BEM-ESR. Rats were treated with i.v. infusion under anesthesia. ESR spectra were recorded at room temperature in the same manner as described above. The theoretical curves for the concentrations in the blood and bile after infusion were simulated with the parameters that were calculated from the i.v. bolus data, using FILT algorithm on the basis of the following equations:

$$\left[C_{\text{blood}}(t)=\frac{R_{\text{inf}}}{D_{iv}}\cdot\left[\frac{A}{\alpha}\cdot(1-\exp(-\alpha\cdot t))+\frac{B}{\beta}\cdot(1-\exp(-\beta\cdot t))\right]\right]$$

derived from a two-compartment model,[30] and

$$\left[\tilde{C}_{\text{bile}}(s)=\frac{CL_{\text{bile}}}{Q_{\text{bile}}}\cdot\left(\frac{k_{\text{tr}}}{s+k_{\text{tr}}}\right)^5\cdot\frac{R_{\text{inf}}}{D_{\text{iv}}\cdot s}\cdot\left(\frac{A}{s+\alpha}+\frac{B}{s+\beta}\right)\right]$$

in the Laplace domain which was derived from a convolution integral of $f_{\text{bile}}(t)$ and $C_{\text{blood}}(t)$.[32,33] $C_{\text{blood}}(t)$ is the blood concentration, R_{inf} and D_{iv} are the i.v. infusion rate and bolus dose of NO-Fe(II)-DTCS, respectively, A and B are the corresponding zero time intercepts of concentrations, α and β are the apparent rate constants, and t is time. $C_{\text{bile}}(t)$ and $\tilde{C}_{\text{bile}}(s)$ are the bile concentration and its Laplace transform, $f_{\text{bile}}(t)$ and $\tilde{f}_{\text{bile}}(s)$ are the weight function for the hepatobiliary transport process and its Laplace transform which was specified as

$$\tilde{f}_{\text{bile}}(s)=\frac{CL_{\text{bile}}}{Q_{\text{bile}}}\cdot\left(\frac{k_{\text{tr}}}{s+k_{\text{tr}}}\right)^5,$$

respectively, and s is the Laplace variable. CL_{bile} is the biliary excretion clearance, Q_{bile} is the bile flow rate, and k_{tr} is the transfer rate constant from the blood space to the bile duct. The simulation lines from the bolus data were examined to reproduce comparably the infusion data for the determined concentrations of nitrosyl-iron species in the blood and bile.

Statistical Analysis

All experimental results are presented as the mean values ± standard deviations (SD) for four experiments. Statistical analysis was performed by analysis of variance (ANOVA) at a 1 or 5% significance level of the difference.

RESULTS

In Vitro ESR of NO-Fe(II)-DTCS in the Fresh Blood, Erythrocytes, Serum, Bile, Urine of Rats, and 3% RSA Solution

We examined the stability of NO-Fe(II)-DTCS in the fresh blood, erythrocytes, serum, bile, urine of rats, and 3% RSA solution at 37°C by both paramagnetic disappearance rate constants and changes of ESR spectral patterns due to the presence of nitrosyl-iron species. No changes were observed with respect to the signal intensity and the spectral pattern in each vehicle with increasing time. The hyperfine coupling constant (A_0-value) and g_0-value with 1.26 mT and 2.04, respectively, in all vehicles, coincided with the reported values (Fig. 1).[10,14] Therefore, nitrosyl-iron states in NO-Fe(II)-DTCS were stable in the biological fluids such as the blood, serum, bile, and urine under the experimental conditions.

An isotropic ESR spectral pattern exhibited the existence of NO-Fe(II)-DTCS unbound to the erythrocytes, while the anisotropic spectral patterns in serum and RSA solution exhibited NO-Fe(II)-DTCS bound to serum proteins including RSA. These results showed that the added NO-Fe(II)-DTCS existed in at least two species such as free and protein-bound forms in the blood. On the other hand, the added NO-Fe(II)-DTCS existed in free form in the bile and urine.

In Vitro Stability of NO-Fe(II)-DTCS in the Hemolyzed Blood and Erythrocytes, Hb, GSH, Trolox, and Asc

When NO-Fe(II)-DTCS was added to the hemolyzed blood or hemolyzed erythrocytes, the ESR spectra were changed with time. Then, we measured the *in vitro* stability of NO-Fe(II)-DTCS in terms of paramagnetic disappearance rate constants in the hemolyzed blood and erythrocytes, as well as the intracellular components such as Hb, GSH, and Asc at 37°C (Table I). NO-Fe(II)-DTCS exhibited long lifetime in the normal blood and erythrocytes, while those in the hemolyzed blood and erythrocytes lost their paramagnetism quickly, the half-lives of NO-Fe(II)-DTCS in the hemolyzed blood and erythrocytes being 1.7–3.5 min. Then, *in vitro* stability of NO-Fe(II)-DTCS was examined in the presence of Hb (50–250 μM) and reducing agents (1 mM) such as GSH, Asc, and trolox as a α-tocopherol analog. The paramagnetism of NO-Fe(II)-DTCS was lost in the Hb concentration-dependent manner ($t_{1/2}$ = 2.9–18.6 min). In addition, ESR spectra of NO-Fe(II)-DTCS in the presence of intact and reduced Hb exhibited neither ESR signals due to nitrosyl-iron species nor those due to the NO-Hb at liquid nitrogen temperature. These results suggested that NO-Fe(II)-DTCS with Hb lost its paramagnetism and changed to diamagnetic species by oxidation under an aerobic condition.

Spinnokinetic Parameters of NO-Fe(II)-DTCS in the BCM-ESR Method

Although no ESR signal was observed in the circulating blood before the administration of

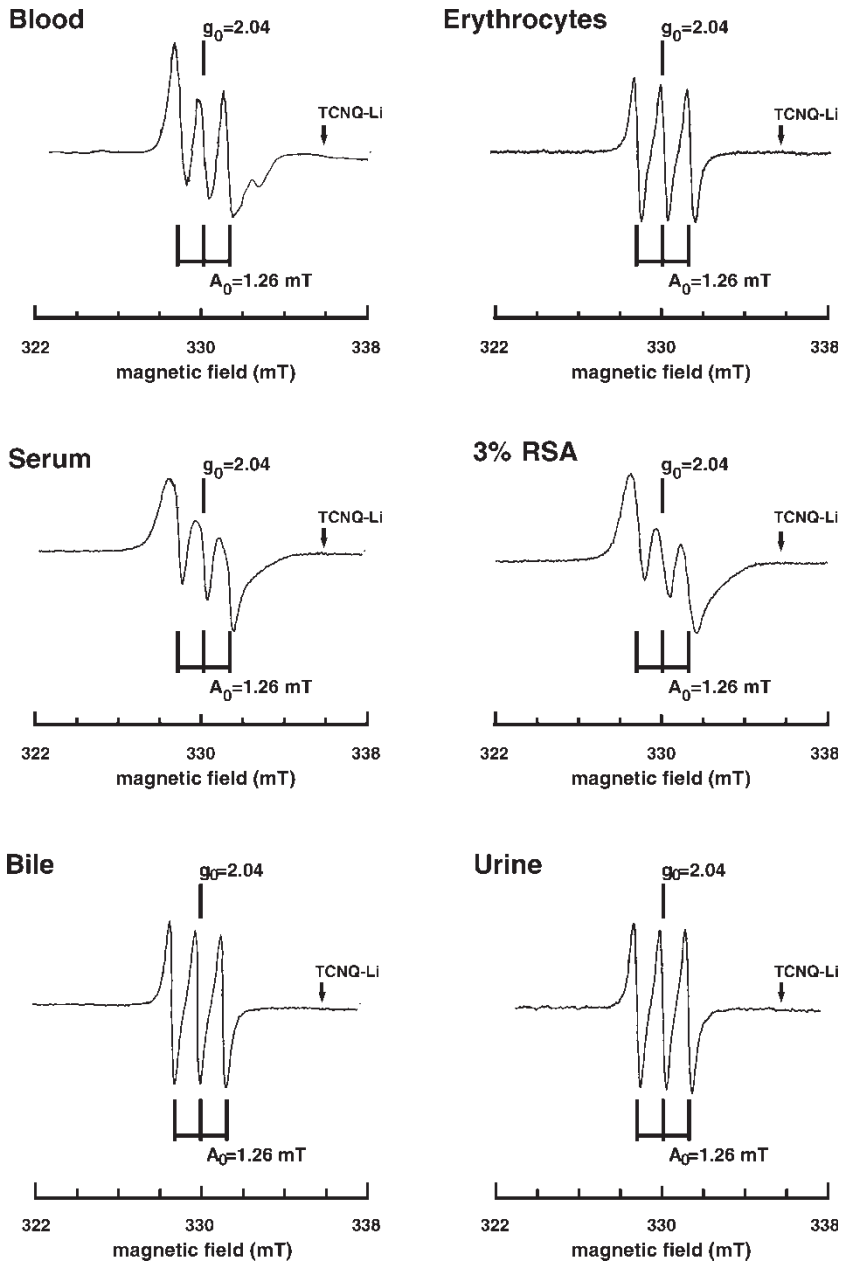

FIGURE 1 ESR spectra of NO-Fe(II)-DTCS (100 μM) dissolved in the fresh blood, erythrocytes, serum, bile, urine of rats, and 3% RSA solution. ESR spectra were recorded at room temperature after incubation of each sample at 37 °C for 15 min.

NO-Fe(II)-DTCS, an ESR signal composed of 3 lines due to the presence of nitrosyl-iron ($NO\text{-}Fe^{2+}$) species developed at 30 s after i.v. injection of the compound. ESR spectra were then collected every 30 s after the administration. The spectral patterns exhibited the same as those for the *in vitro* study in the blood, and the signal intensities due to nitrosyl-iron species decreased with time (Fig. 2a). Concentrations of NO-Fe(II)-DTCS in the circulating blood were determined using a calibration line prepared with the fresh blood of untreated rats. The determination limit for NO-Fe(II)-DTCS was 10 μM in the circulating blood during BCM-ESR measurements. Figure 2b shows the concentration time course for the determined NO-Fe(II)-DTCS in the circulating blood of rats and the simulated curve exhibiting a two-phase pattern. The theoretical curve fitted to the mean data was obtained on the basis of the two-compartment model with nonlinear least squares regression.[24,29] The spinnokinetic parameters for the disposition of NO-Fe(II)-DTCS in the blood are summarized in Table II. AUC_{blood} and MRT_{blood} of NO-Fe(II)-DTCS were calculated to be 0.53 ± 0.02 μmol. min/ml and 4.9 ± 0.5 min, respectively, at a bolus dose of 10 μmol/kg body weight. Vd_{ss}, $Vd_{central}$, and $Vd_{peripheral}$ of NO-Fe(II)-DTCS

TABLE I *In vitro* stability of NO-Fe(II)-DTCS in the fresh blood and erythrocytes of rats, bovine hemoglobin (Hb) with or without ascorbate (Asc), and reducing agents at 37°C

System	Paramagnetic disappearance rate constant (min^{-1})	Half life (min)
Phosphate buffer (pH 7.4)	Very stable	n.d.
Normal blood	Stable at least 15 min	n.d.
Hemolyzed blood	0.200 ± 0.008	3.46 ± 0.13
Normal erythrocytes	Stable at least 15 min	n.d.
Hemolyzed erythrocytes	0.399 ± 0.033	1.74 ± 0.14
Hb		
50 μM	0.037 ± 0.001**	18.6 ± 0.5**
125 μM	0.136 ± 0.007**	5.10 ± 0.25*,**
250 μM	0.238 ± 0.021*	2.91 ± 0.25*
Hb plus Asc		
50 μM	0.068 ± 0.002**	10.2 ± 0.3**
125 μM	0.312 ± 0.022**	2.22 ± 0.16*,**
250 μM	0.455 ± 0.012*	1.52 ± 0.04*
1 mM GSH	Stable at least 60 min	n.d.
1 mM trolox	Stable at least 60 min	n.d.
1 mM Asc	Stable at least 30 min	n.d.

NO-Fe(II)-DTCS was dissolved in each system (pH 7.4) at the initial concentration of 100 μM. *, **: Significantly different at the 5% or 1% level of ANOVA.

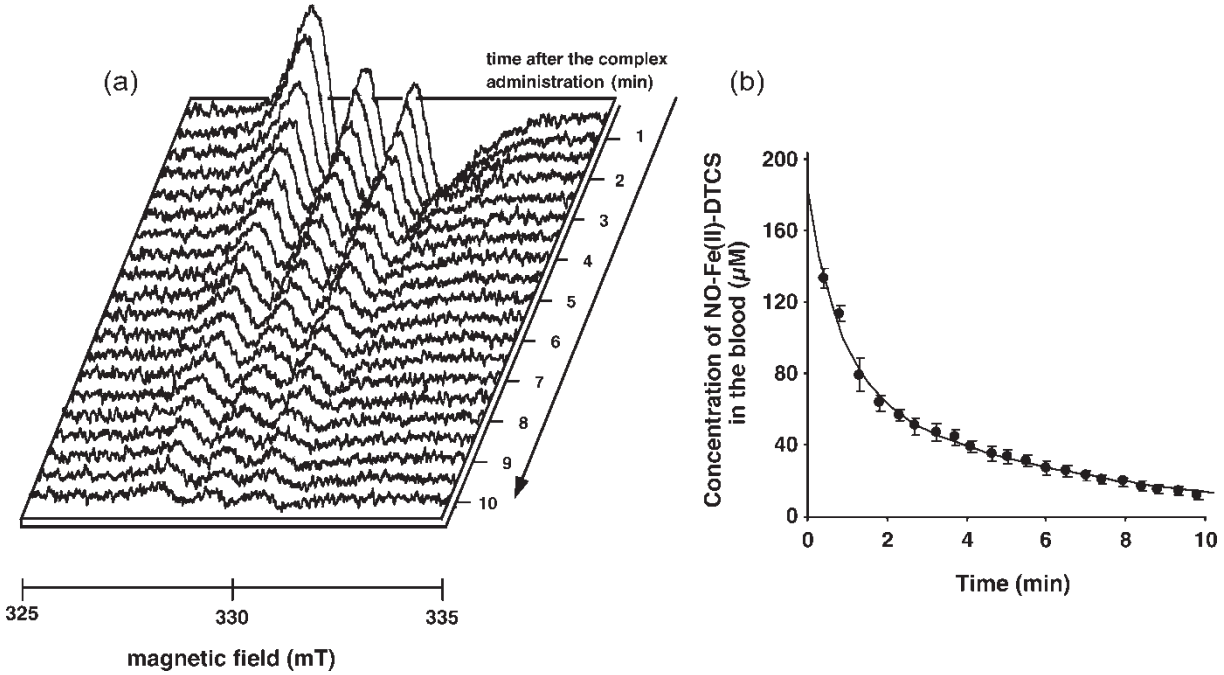

FIGURE 2 The representative result of (a) *in vivo* BCM-ESR due to nitrosyl-iron ($NO\text{-}Fe^{2+}$) species in a rat receiving an i.v. bolus injection of NO-Fe(II)-DTCS and (b) the time course for the concentration of nitrosyl-iron ($NO\text{-}Fe^{2+}$) species in the blood monitored by the BCM-ESR method. Rats were treated with an i.v. bolus injection of NO-Fe(II)-DTCS at a dose of 10 μmol/kg body weight under anesthesia. ESR spectra were recorded at room temperature every 30 s for 10 min after administration. Concentrations of NO-Fe(II)-DTCS in the blood were determined by each calibration line prepared with the fresh rat blood. Data and the simulated curve represent the mean ± SD ($n = 4$) and the theoretical curve fitted to the mean value, respectively.

TABLE II Spinnokinetic parameters of NO-Fe(II)-DTCS in the blood and bile of rats estimated by the two-compartment model and the hepatobiliary transport model

AUC (μmol.min/ml)	*MRT* (min)	Vd_{ss} (ml/kg)	$Vd_{central}$ (ml/kg)	$Vd_{peripheral}$ (ml/kg)
0.525 ± 0.020	4.91 ± 0.45	93.3 ± 6.0	54.6 ± 1.2	38.7 ± 6.1
CL_{tot} (ml/min/kg)	$t_{1/2,\beta}$ (min)	Ae_{bile} (μmol/kg)	CL_{bile} (ml/min/kg)	MRT_{bile} (min)
19.1 ± 0.7	3.93 ± 0.36	0.842 ± 0.042	1.61 ± 0.08	7.65 ± 0.31

Rats were treated with *i.v.* injection of NO-Fe(II)-DTCS at a dose of 10 μmol/kg body weight under anaesthesia.

were calculated to be 93 ± 6, 55 ± 1, and 39 ± 6 ml/kg, respectively. CL_{tot} of NO-Fe(II)-DTCS was calculated to be 19 ± 1 ml/min/kg.

In Vivo Stability and ESR Parameters of NO-Fe(II)-DTCS in the Circulating Blood of Rats

The stability of NO-Fe(II)-DTCS in the circulating blood of rats at 15 min after i.v. injection was examined by both ESR spectrum and ESR parameters at liquid nitrogen temperature, because they were under detectable levels at room temperature. No ESR spectral changes were observed with respect to the pattern and parameters due to NO-Fe(II)-DTCS in the circulating blood compared with the previous paper.[10] The $g_{\perp}$- and $g_{//}$-values were found to be 2.04 and 2.02, respectively, with the ESR signals due to ceruloplasmin ($g = 2.06$) and organic free radicals ($g = 2.01$) (Fig. 3), as reported previously,[10] suggesting that NO-Fe(II)-DTCS existed stable in the circulating blood of normal rats.

Spinnokinetic Parameters of NO-Fe(II)-DTCS in the BEM-ESR Method

Bile flowed constantly (100 ± 10 μl/min/kg) from the liver, and ESR spectra were successively measured every 70 s after i.v. injection of the compound at room temperature. No ESR signal was observed in the bile flow before the administration of NO-Fe(II)-DTCS, however, due to nitrosyl-iron species ESR signal was observed from 1 to 2 min after the administration and continued for 11–12 min (Fig. 4a). The spectral patterns were isotropic as for *in vitro* study for the bile (Fig. 1). Concentrations of NO-Fe(II)-DTCS in the bile were determined by using a calibration line prepared with the fresh bile of untreated rats. Figure 4b shows that NO-Fe(II)-DTCS were excreted in the bile of rats with the maximum concentration at around 4 min after the administration, together with the simulated curve. The theoretical curve fitted to the mean data was obtained on the basis of the hepatobiliary transport model with nonlinear least squares regression combined with FILT.[32,33] The time corresponding to the highest signal intensities in the bile was delayed in comparison with the time courses of the concentration in the blood (Figs. 2b and 4b), indicating a time lag caused by the hepatobiliary transport from the blood space to the bile duct through the liver. The spinnokinetic parameters for the excretion of NO-Fe(II)-DTCS in the bile are also summarized in Table II. From the curve-fitting method, CL_{bile} and k_{tr} of NO-Fe(II)-DTCS were calculated to be 1.6 ± 0.1 ml/min/kg and 1.8 ± 0.1 min^{-1}, respectively, showing that MTT_{liver} was 2.7 ± 0.1 min. Ae_{bile} and MET_{bile} of NO-Fe(II)-DTCS were calculated to be 0.84 ± 0.04 μmol/kg (8.4 ± 0.4% of a dose) and 7.7 ± 0.3 min, respectively.

Relationship between the Spinnokinetic Feature of NO-Fe(II)-DTCS after a Bolus Injection and that after Continuous Infusion

In many cases of *in vivo* spin-trapping experiments in animals, ESR signals due to spin-adducts gradually increase with time after the administration of spin-trapping agents and attain the maximum levels at a steady-state condition,[7,8,28] in which the formation

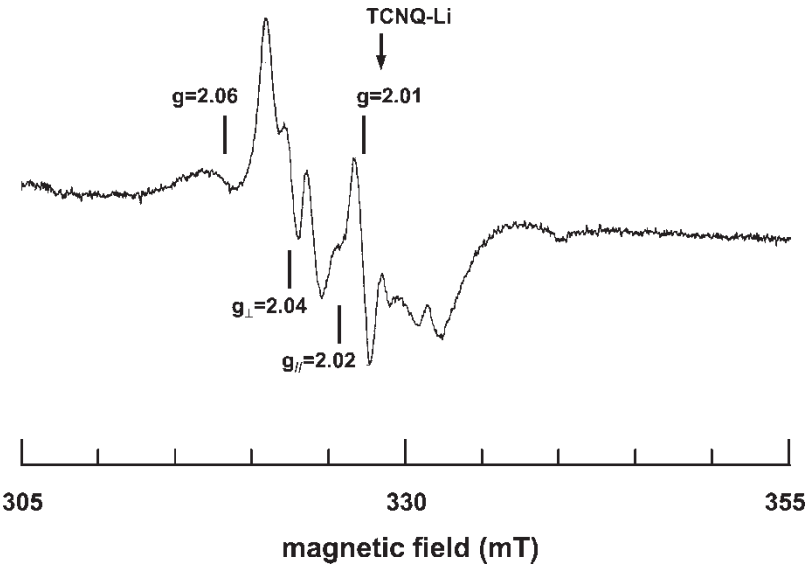

FIGURE 3 ESR spectrum of NO-Fe(II)-DTCS in the circulating blood of rats obtained at 15 min after the i.v. injection. ESR spectrum was recorded at liquid nitrogen temperature. In addition to the ESR spectrum due to nitrosyl-iron species, ESR signals due to ceruloprasmin ($g = 2.06$) and organic free radicals ($g = 2.01$) were observed.

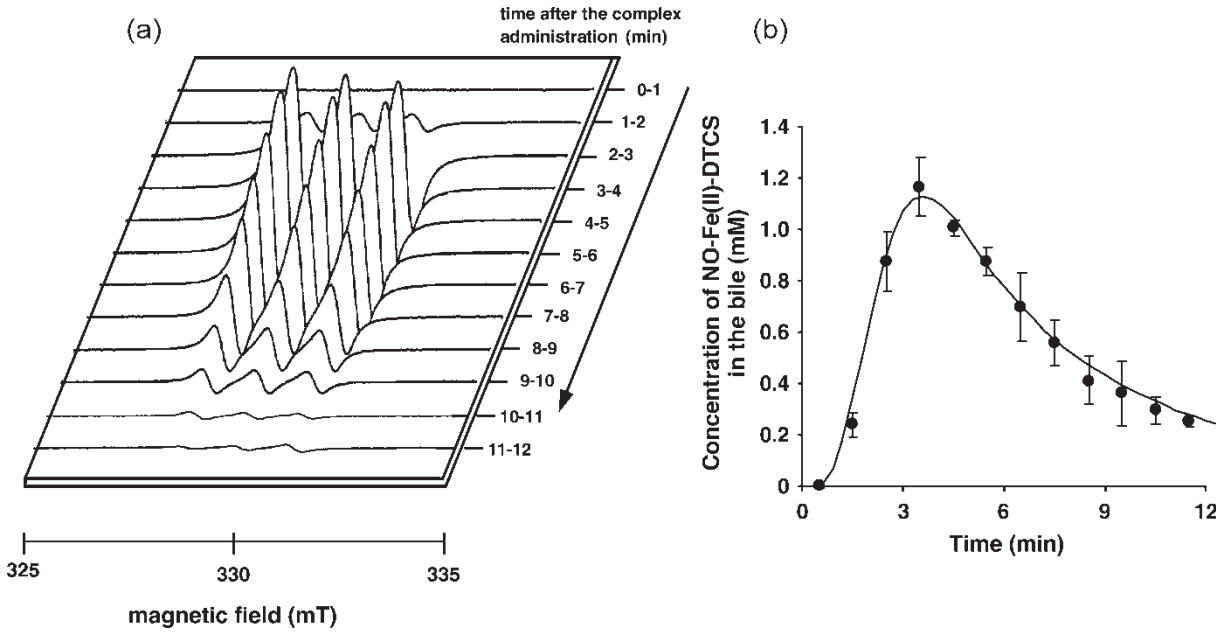

FIGURE 4 The representative results of (a) *in vivo* BEM-ESR due to nitrosyl-iron ($NO\text{-}Fe^{2+}$) species in a rat receiving an i.v. bolus injection of NO-Fe(II)-DTCS and (b) the time course for the concentration of nitrosyl-iron ($NO\text{-}Fe^{2+}$) species in the bile monitored by the BEM-ESR method. Rats were treated with an i.v. bolus injection of NO-Fe(II)-DTCS at a dose of 10 μmol/kg body weight under anesthesia. ESR spectra were recorded at room temperature every 70 s for 15 min after the administration. Concentrations of NO-Fe(II)-DTCS in the bile were determined by each calibration line prepared with the fresh rat bile. Data and the simulated curve represent the mean ± SD ($n = 4$) and the theoretical curve fitted to the mean value, respectively.

and decomposition of spin-adducts in the body achieve equilibrium. When we attempt to quantify NO-Fe(II)-DTCS due to endogenous NO trapped with Fe(II)-DTCS in animals, the kinetic relationship of the spin adduct NO-Fe(II)-DTCS between the steady-state and nonsteady-state after the bolus administration should be evaluated. However, it remains unclear whether increasing ESR signal intensities due to nitrosyl-iron species in the blood and bile of rats receiving a continuous infusion of NO-Fe(II)-DTCS depend on the pharmacokinetic parameters calculated from its disposition after a bolus injection. As a result, we next examined the theoretical curves for increasing concentrations of NO-Fe(II)-DTCS during the infusion.

Rats were given a continuous i.v. infusion of NO-Fe(II)-DTCS at a dose of 2 μmol/min/kg body weight under anesthesia, and ESR spectra were recorded at room temperature every 30 s in the blood and every 70 s in the bile for 15 min after administration. The observed time course for the concentrations of nitrosyl-iron species in the blood by BCM-ESR and that in the bile by BEM-ESR coincided well with the theoretical curves simulated with the parameters in the bolus administration (Fig. 5). The results indicated that the spinnokinetic features of NO-Fe(II)-DTCS in both the blood and bile were quantitatively analyzable on the basis of the two-compartment and hepatobiliary transport models, respectively, as those in a rapid administration.

DISCUSSION

ESR signals due to NO-Fe(II)-DTCS spiked to the fresh blood, erythrocytes, serum, bile, and urine of untreated rats were unchanged over 15 min (Fig. 1 and Table I), and the nitrosyl-iron species existed as the free and protein-bound forms in the blood (Fig. 1). However, the signal intensities decreased rapidly with the half-lives of 3.5 and 1.7 min in the hemolyzed blood and erythrocytes, respectively, (Table I) indicating that NO-Fe(II)-DTCS lost the paramagnetism by the reaction with Hb in both time- and Hb concentration-dependent manners (Table I). On the other hand, ESR signals due to nitrosyl-iron species in endogenous reducing agents such as GSH and Asc were very stable over 30 min.

The concentration of Hb in the blood of animals is reported to be 2.0–2.3 mM, which is much higher than the concentrations used in the present *in vitro* experiments.[35] Thus, it may be difficult to detect ESR signals due to nitrosyl-iron species in the circulating blood of animals with hemolysis. However, NO-Fe(II)-DTCS is considered to be detectable in the blood of live animals, which includes some endogenous reducing agents, because it was reported not to permeate into erythrocytes through the membrane.[10,14,15]

ESR signals due to nitrosyl-iron species in the circulating blood decreased with time in a two-phase pattern after the bolus administration of NO-Fe(II)-DTCS, as monitored by BCM-ESR (Fig. 2).

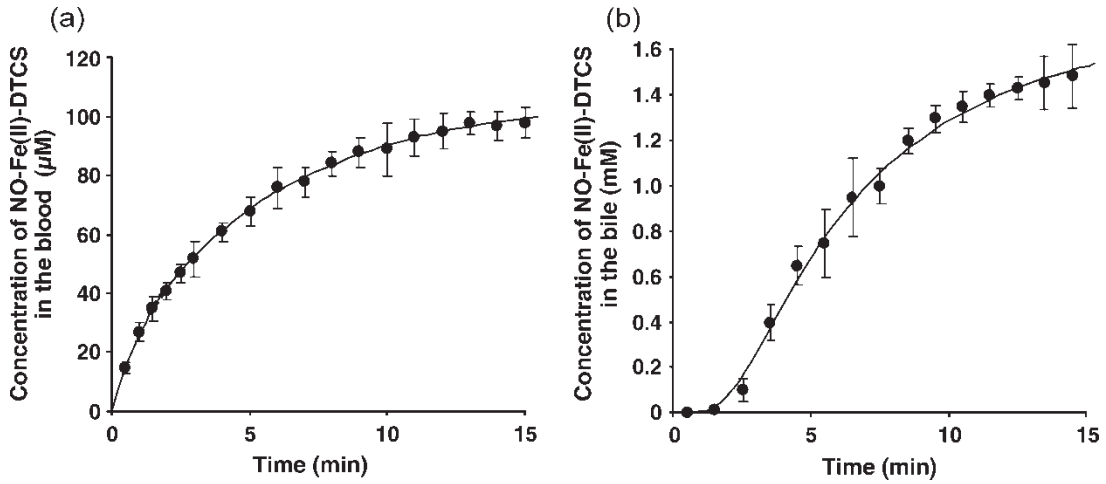

FIGURE 5 Time courses for the concentrations of nitrosyl-iron ($NO\text{-}Fe^{2+}$) species in the (a) blood and (b) bile of rats receiving an i.v. continuous infusion of NO-Fe(II)-DTCS, monitored by the (a) BCM- and (b) BEM-ESR methods. Rats were treated with an i.v. continuous infusion of NO-Fe(II)-DTCS at a dose of 2 μmol/min/kg body weight under anesthesia. ESR spectra were recorded at room temperature every 30 s in the blood and every 70 s in the bile for 15 min after the administration. Data and the simulated curves represent the mean ± SD ($n = 4$) and the theoretical curves calculated from the parameters based on the i.v. bolus data, respectively.

In addition, nitrosyl-iron species due to NO-Fe(II)-DTCS existed stable in the circulating blood at the detectable levels (Fig. 3). These results indicated that NO-Fe(II)-DTCS distributed to the organs and then was excreted from the body of rats as the intact chemical form, in the condition that the hemolysis was not observed in the blood of rats receiving NO-Fe(II)-DTCS (data not shown). Approximately, 8% of the dose of NO-Fe(II)-DTCS was excreted in the bile as the intact chemical form over 15 min after the administration (Fig. 4), and excretion of NO-Fe(II)-DTCS in the urine was under detectable levels for 15 min after the administration (data not shown). The excretion of NO-Fe(II)-MGD in the urine was reported to be detectable over 30 min after administration.[21] The urinary excretion process of NO-Fe(II)-DTCS through the kidney might be longer than the biliary excretion process. The explanation remains to be solved in the future.

The biliary excretion of NO-Fe(II)-DTCS demonstrated to be a part of the disappearance of nitrosyl-iron species from the circulating blood and incompletely satisfied the total amount of dose given to rats. Thus, NO-Fe(II)-DTCS incorporated into the blood space was suggested to accumulate in several organ compartments. Yoshimura *et al.* demonstrated that NO-Fe(II)-DTCS i.p. administered to a rat was detectable in the head region of a rat by L-band ESR.[36] Pou *et al.* described that the half-life of NO-Fe(II)-DTCS administered by i.p. injection to a live mouse was 41 min from the decay of ESR signal height in the whole body by L-band ESR, and suggested that the gradual decrease of ESR signals due to NO-Fe(II)-DTCS in the detected region except for the nose and tail of a mouse was primarily due to the oxidation or reduction in organs.[23] These previous findings on the distribution and long residence of NO-Fe(II)-DTCS in organ compartments supported the present results obtained by both BCM- and BEM-ESR methods. In addition, Yokoyama *et al.* showed that NO-Fe(II)-DTCS subcutaneously (s.c.) administered to a live mouse was highly distributed and accumulated in the liver at 40 min after the administration, using the L-band ESR-CT images in the upper abdomen of a mouse as well as the ESR spectra in the removed organs such as liver, gastrointestine, and whole blood of a mouse.[16] Therefore, it was supposed that the eliminated fraction from the circulating blood and the unexcreted fraction of injected NO-Fe(II)-DTCS remained in the distribution and accumulation organ such as the liver.

As shown in Fig. 4, the clearance pattern of NO-Fe(II)-DTCS in the bile of rats started at 1–2 min after injection and reached the maximum at 3 min, which delayed in comparison with the clearance curve in the blood, reflecting a short time-delay for the rapid hepatobiliary transport process of NO-Fe(II)-DTCS through the cytoplasm of liver.[32,37] However, previous studies indicated the long and stable residence of NO-Fe(II)-DTCS in the liver after i.p. or s.c. injection.[16,23] This discrepancy might be resolved after the investigation on the subcellular distribution and chemical speciation of nitrosyl-iron species in the liver. Concentrations of NO-Fe(II)-DTCS in the bile were approximately 10 times higher than those in the blood (Figs. 2 and 4), and thus NO-Fe(II)-DTCS was indicated to concentrate in the bile during the transport through the liver.

Many researchers in the field of *in vivo* ESR have scarcely quantified the paramagnetic species.[24–27] The application of X-band ESR to the real-time *in vivo* ESR measurements is useful not only for highly sensitive detection of ESR signals but also for

the determination of paramagnetic species such as free radicals and metal complexes.[24–27] The importance of our study was both the quantitative determination of *in vivo* observable ESR signals due to a spin-adduct and the evaluation of its pharmacokinetics using both BCM- and BEM-ESR.[24,38] The pharmacokinetic analysis on the basis of BCM- and BEM-ESR provided the spinnokinetic parameters as summarized in Table II.

Our concept for applying the two-compartment model to the spinnokinetic analysis of BCM-ESR was different from that of the conventional compartment model; the central and peripheral compartments were specified as the circulating blood space and other organs, where the paramagnetic species distributed immediately after the i.v. administration.[27] In fact, $Vd_{central}$ of nitrosyl-iron species was calculated to be 54 ± 1 ml/kg (Table II), which coincided well with the anatomical blood volume of rats (54 ml/kg as the reference,[39]). Evidence that NO-Fe(II)-DTCS has longer MRT_{blood} (4.9 ± 0.5 min) and smaller CL_{tot} (19 ± 1 ml/min/kg) than those of the spin probes such as TEMPO and PROXYL derivatives given in rats[24] motivates us to try the *in vivo* quantification of endogenously formed NO using BCM-ESR. In *MRT*, the biliary excretion process of nitrosyl-iron species ($MRT_{bile} = 7.67 \pm 0.3$ min) contributed to the elimination from the circulating blood and the transport through the liver ($MTT_{liver} = 2.7 \pm 0.1$ min). Although only 8% of NO-Fe(II)-DTCS given to rats was excreted in the bile, from the value of CL_{bile} (1.6 ± 0.1 ml/min/kg), the concentrations of nitrosyl-iron species in the bile were approximately 10 times higher than those in the blood. These results indicated that BEM-ESR was a promising method for measuring the *in vivo* generated NO-adduct, as originally described by Kotake *et al.*[28]

When NO-Fe(II)-DTCS was administered to rats by i.v. continuous infusion, the concentrations of nitrosyl-iron species in the blood and bile increased with time by 15 min after the administration (Fig. 5). Spinnokinetic features of nitrosyl-iron species in the blood and bile during infusion of NO-Fe(II)-DTCS were quantitatively explained by the theoretical curves for the concentrations simulated from parameters by BCM- and BEM-ESR. Based on the results on NO-adducts in the blood and bile of rats, the detection and determination of endogenously generated NO in rats receiving Fe-DTCS complex will be possible by the proposed methods. Our study for this purpose is currently underway.

In conclusion, we examined the paramagnetic disappearance of NO-Fe(II)-DTCS in several biological systems such as the fresh blood of untreated rats, and NO-Fe(II)-DTCS was found to be very stable in the biological fluids except for the hemolyzed blood. We also examined the disposition of NO-Fe(II)-DTCS in the circulating blood and its excretion in the bile of rats using real-time *in vivo* BCM- and BEM-ESR, respectively. The results indicated that the elimination of nitrosyl-iron species in the blood was attributable to the distribution in the organs and the excretion to the bile. *In vivo* BCM- and BEM-ESR are, thus, proposed to be useful for investigating the quantitative spinnokinetics of a stable spin-adduct in the blood and bile of rats, respectively. The proposed methods will provide effective tools not only for detecting a NO-adduct formed endogenously[15,23] but also for evaluating quantitatively its organ distribution and elimination.

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