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ORIGINAL ARTICLE

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A new halogenated antidiabetic vanadyl complex, bis(5-iodopicolinato)oxovanadium(IV): in vitro and in vivo insulinomimetic evaluations and metallokinetic analysis

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Abstract A new vanadyl complex, bis(5-iodopicolinato)oxovanadium(IV), $VO(IPA)_2$, with a $VO(N_2O_2)$ coordination mode, was prepared by mixing 5-iodopicolinic acid and $VOSO_4$ at pH 5, with the structure characterized by electronic absorption, IR, and EPR spectra. Introduction of the halogen atom on to the ligand enhanced the in vitro insulinomimetic activity (IC_{50}=0.45 mM) compared with that of bis(picolinato)oxovanadium(IV) (IC_{50}=0.59 mM). The hyperglycemia of streptozotocin-induced insulin-dependent diabetic rats was normalized when $VO(IPA)_2$ was given by daily intraperitoneal injection. The normoglycemic effect continued for more than 14 days after the end of treatment. To understand the insulinomimetic action of $VO(IPA)_2$, the organ distribution of vanadium and the blood disposition of vanadyl species were investigated. In diabetic rats treated with $VO(IPA)_2$, vanadium was distributed in almost all tissues examined, especially in bone, indicating that the action of vanadium is not peripheral. Vanadyl concentrations in the blood of normal rats given $VO(IPA)_2$ remain significantly higher and longer than those given other complexes because of its slower clearance rate. $VO(IPA)_2$ binds with the membrane of erythrocytes, probably owing to its high hydrophobicity in addition to its binding with serum albumin. The longer residence of vanadyl species shows the higher normoglyceric effects of $VO(IPA)_2$ among three complexes with the $VO(N_2O_2)$ coordination mode. On the basis of these results, $VO(IPA)_2$ is indicated to be a preferred agent to treat insulin-dependent diabetes mellitus in experimental animals.

Keywords Vanadyl iodopicolinate · Insulinomimetics · Diabetes mellitus · Blood circulation monitoring EPR · Metallokinetic analysis

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Introduction

Vanadium ions and complexes have been subjects of interest as potential therapeutic agents to treat diabetes mellitus for the last decade of the 20th century [1, 2, 3, 4, 5, 6, 7, 8]. Since we found in 1995 that the bis(picolinato)oxovanadium(IV) $[VO(PA)_2]$ complex with the $VO(N_2O_2)$ coordination mode is a potent agent for treating insulin-dependent diabetes mellitus (IDDM) in rats on daily intraperitoneal (i.p.) injection or oral administration [9], we have used the complex as a leading compound for developing more active agents with hypoglycemic effects. By introducing an electron-donating group such as a methyl group into the pyridine ring of the picolinate ligand, the bis (6-methylpicolinato)oxovanadium(IV) $[VO(6MPA)_2]$ complex was prepared (Fig. 1) [10]. The in vitro insulin-mimetic activity of $VO(6MPA)_2$ in terms of the inhibitory effect of free fatty acid (FFA) release from isolated rat adipocytes treated with epinephrine was enhanced [10]. Based on these in vitro results, the complex was administered to experimental animals. The hyperglycemia of not only rats with IDDM [10, 11, 12] but KKA^y mice with hereditary non-insulin-dependent diabetes mellitus (NIDDM) [13] was normalized by daily i.p. injection or on oral administration of this complex.

On the other hand, we examined the effect of the introduction of an electron-withdrawing group such as halogen atoms on the same picolinate ligand in order

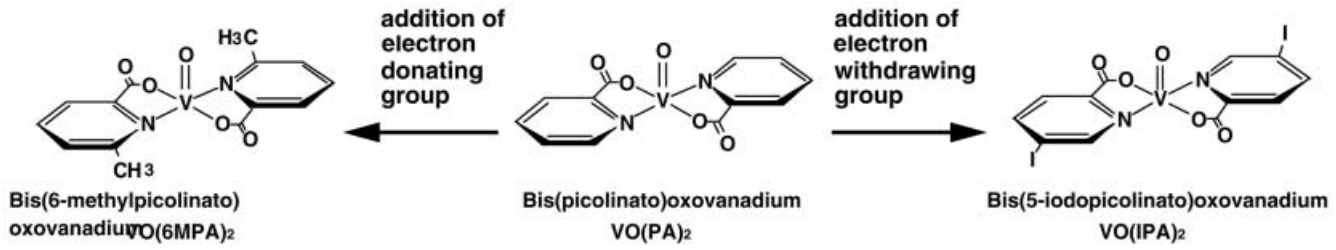


Fig. 1 Structures of bis(5-iodopicolinato)oxovanadium(IV) [$VO(IPA)_2$] and its related complexes

to clarify the structure-activity relationship of the complexes. A new complex, bis(5-iodopicolinato)oxovanadium(IV) [$VO(IPA)_2$] (Fig. 1), was prepared and it was found that the complex exhibits a stronger in vitro insulinomimetic activity than $VO(PA)_2$ or $VO(6MPA)_2$. This paper reports the synthesis, in vitro, and in vivo insulinomimetic activities of the complex as well as the pharmacokinetic features of vanadyl species due to the complex by using a recently developed method of blood circulation monitoring (BCM) EPR [14, 15, 16]. On the basis of the results, we propose here the usefulness of the introduction of a halogen on to the ligand in order to find insulinomimetic vanadyl complexes that express a higher level of activity.

Materials and methods

Chemicals

α-Picoline, collagenase, carbon disulfide, and vanadyl sulfate ($VOSO_4 \cdot nH_2O$, VS) were obtained from Wako Pure Chemicals (Tokyo, Japan). $VOSO_4$ was standardized complexometrically using EDTA and determined as $VOSO_4 \cdot 3.2H_2O$. Bovine serum albumin (BSA; fraction V), (±)-epinephrine hydrochloride (Epi), streptozotocin (STZ), and insulin (bovine pancreatic) were purchased from Sigma (St. Louis, Mo., USA). Rat serum albumin (RSA; fraction V), bovine apo- and holo-transferrin (Tf) were purchased from Wako Pure Chemicals (Tokyo, Japan). Nembutal sodium injection (pentobarbital: 50 mg/mL) was obtained from Abbott Laboratories (North Chicago, Ill., USA.). Heparin sodium injection was obtained from Shimizu Pharmaceutical (Osaka, Japan). Other reagents were of the highest purity commercially available.

Preparation and characterization of the $VO(IPA)_2$ complex

5-Iodopicolinic acid (IPA) was synthesized from α-picoline as reported by Riley et al. [17]. $VO(IPA)_2$ was prepared by mixing IPA and $VOSO_4$ at a 2:1 molar ratio of the ligand:metal ion in an aqueous solution at pH 5.0, and the solution was stirred for 60 min. The precipitate was filtered and washed several times with pure water and diethyl ether, and then dried in a vacuum desiccator. The yield of the complex was 69% on the basis of the ligand. The complex was soluble in 5% DMSO and stable in 5% acacia suspension. The prepared complex was characterized by elemental analysis and physical methods such as visible absorption, IR absorption, and EPR spectroscopies (Table 1). Visible and EPR spectra were measured for 5% DMSO aqueous solutions of the complex using Shimadzu UV-260 (Kyoto, Japan) and JEOL JES-RE1X (Tokyo, Japan) spectrometers, respectively. EPR spectra were recorded at room and liquid nitrogen temperatures. Instrumental conditions were as follows: modulation frequency of 100 kHz, modulation amplitude width of 0.63 mT, microwave power of 5 mW. The standards were tetracyanoquinodimethane lithium salt (TCNQ-Li) (g=2.00252) and Mn(II) in MgO [magnetic field between the third and fourth signals due

Table 1 Physicochemical properties of $VO(IPA)_2$ and its related complexes[a]

Complex	MW	Solvent	EPR parameters						IR spectrum
			g-value			A-value (10^{-4} cm^{-1})			ν(V=O) (cm^{-1})
			g_0	$g_{\|}$	$g_{\perp}$	A_0	$A_{\|}$	$A_{\perp}$	
$VO(PA)_2$	329	H_2O	1.980	1.945	1.998	93.4	168.2	53.5	980
$VO(6MPA)_2$	357	H_2O	1.981	1.941	2.002	92.2	164.0	53.4	948
$VO(IPA)_2$	599	5% DMSO	1.976	1.945	1.991	92.2	166.9	52.7	968

	Visible absorption maxima (nm) (ε, mM^{-1} cm^{-1})		Partition coefficient (P)	Elemental analysis			
	λ_1 (ε_1)	λ_2 (ε_2)			C (%)	H (%)	N (%)
$VO(PA)_2$	731.0 (3.2×10^{-2})	576.0 (1.4×10^{-2})	0.330	Calcd.	43.04	3.03	8.32
				Found	43.78	3.06	8.51
$VO(6MPA)_2$	749.0 (4.9×10^{-2})	620.0 (2.5×10^{-2})	0.595	Calcd.	49.57	3.45	8.18
				Found	49.56	3.54	8.26
$VO(IPA)_2$	728.0 (2.7×10^{-2})	547.6 (2.6×10^{-2})	–	Calcd.	24.06	1.67	4.68
				Found	24.16	1.68	4.44

[a]The values obtained are the mean of duplicate measurements

to Mn(II), 8.69 mT]. The microwaves were calibrated using a Takeda Riken frequency counter, TR 5212 (Tokyo, Japan). IR spectra were measured using a Shimadzu FTIR-408 on a KBr disk.

Evaluation of in vitro insulin-mimetic activity

The insulin-mimetic activity of the complex was evaluated by in vitro experiments [18], in which the inhibition of FFA release from isolated rat adipocytes treated with epinephrine was estimated. Briefly, isolated rat adipocytes (2.7×10^6 cells/mL), prepared as described [18], were preincubated at 37 °C for 0.5 h with three different concentrations of the vanadyl compound in 1 mL KRB buffer (120 mM NaCl, 1.27 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.75 mM KCl, 1.2 mM KH_2PO_4, and 24 mM $NaHCO_3$; pH 7.4) containing 20 mg bovine serum albumin (BSA, Sigma, USA). A 10 μM epinephrine solution was then added to the reaction mixture and the resulting solutions were incubated at 37 °C for 3 h. The reactions were stopped by soaking in ice water and the mixtures were centrifuged at 12,000 rpm for 1 min. For the outer solution of the cells, FFA levels were determined using an FFA kit (Wako Pure Chemicals, Tokyo, Japan). From the curve for the complex concentration-dependent inhibitory effect on FFA release from isolated rat adipocytes treated with epinephrine, the IC_{50} value, which shows the 50% inhibitory concentration of the complex, was determined.

Evaluation of in vivo antidiabetic activity

Diabetes was induced in male Wistar rats, weighing 190–210 g, by a single intravenous (i.v.) injection of freshly prepared STZ (40 mg/kg body weight) in 0.1 M citrate buffer (pH 5.0). Blood samples for analysis of serum glucose were obtained from the tail vein of the rats and serum glucose levels were measured using the glucose oxidase method (glucose C-II test; Wako Pure Chemicals). STZ rats with a blood glucose level of 14.3–26.9 mM (257–484 mg/dL) at 1 week after STZ administration were used for the experiments. STZ rats were given the $VO(IPA)_2$ complex suspended in 5% acacia daily at doses of 5.0 mg (98 μmol) V/kg body weight for the first 3 days, 2.5 mg (49 μmol) V/kg body weight for the following 2 days, and then 1.0 mg (19.6 μmol) V/kg body weight for the following 9 days by intraperitoneal (i.p.) injection. After the administration of the complex for 14 days, the rats were maintained for 14 days under usual conditions. On days 0, 14, and 28 of the experiments, the serum insulin, FFA, and blood urea nitrogen (BUN) of rats treated with the $VO(IPA)_2$ complex were measured at 10:00–11:00 a.m. using the GLAZYME insulin-EIA test, NEFA C-test Wako, and UN B-test Wako (Wako Pure Chemicals), respectively.

Determination of total vanadium concentrations in STZ rats treated with the $VO(IPA)_2$ complex

STZ-induced diabetic male Wistar rats, weighing 200 g, were given a suspension of $VO(IPA)_2$ complex in 5% acacia by i.p. injection at doses of 98 μmol V/kg body weight for the first 3 days, 49 μmol V/kg body weight for the following 2 days, and then 19.6 μmol V/kg body weight for the following 9 days. After no administration for 14 days, the rats were sacrificed under anesthesia with ether and organs such as the liver, spleen, pancreas, kidney, lung, heart, brain, blood, serum, adipose, and bone were removed and lyophilized. Vanadium was determined by neutron activation analysis (NAA) at the Research Reactor Institute of Kyoto University using the peak area of 1434.1 keV based on the $^{51}V(n,\gamma)^{52}V$ reaction (half-life of ^{52}V, 3.75 min) [18].

Metallokinetic analysis of $VO(IPA)_2$ by BCM-EPR

Recently, we have proposed a new pharmacokinetic analysis method combined with an EPR spectrometer and named it "in vivo blood circulation monitoring EPR (BCM-EPR)" using rats under anesthesia [14, 15, 16, 19, 20]. By use of the in vivo BCM-EPR method, we examined the metallokinetic feature of vanadyl species in the rats receiving $VO(IPA)_2$ at a dose of 0.5 mg (9.8 μmol) V/kg of body weight, and compared the results of $VO(IPA)_2$ with those of its related complexes [15]. Briefly, rats were anesthetized by i.p. injection of pentobarbital and kept at 35 °C on a Deltaphase Isothermal Pad (Model 39 DP, Braintree Scientific, Braintree, USA). Heparinized polyethylene tubes were cannulated into the left femoral artery and vein. The free ends of these cannulas were joined with heparinized silicone tubes to make a blood circuit outside the body, which was directly connected to an EPR cell (a quartz 20 μL capillary tube). Blood from the femoral artery was returned and recirculated to the femoral vein after flowing through the EPR cell by the rat's own heartbeat and blood pressure without depletion. $VO(IPA)_2$ dissolved in physiological saline containing 5% DMSO was given by single i.v. injection to the rats, and EPR spectra were measured at room temperature every 30 s using an X-band EPR spectrometer, and data were collected in a computer system. The disappearance of the EPR signal due to vanadyl species in the blood was plotted against the time following the administration of the compounds. To determine the concentrations of vanadyl species, 10 μL of each vanadyl complex dissolved in the blood of untreated rats was applied to the EPR cell. Each calibration curve obtained by monitoring the signal intensities of the central peak due to the corresponding vanadyl complex was freshly prepared by using the fresh blood spiked with each complex. Metallokinetic parameters for $VO(IPA)_2$ and its related complexes were obtained as follows: a two-compartment model [$C_b = A\exp(-\alpha t) + B\exp(-\beta t)$] was fitted to each individual profile of the determined concentrations of $VO(IPA)_2$ or its related complexes using a nonlinear least squares regression program, MULTI [21], where C_b is the blood concentration, α and β are the apparent rate constants, A and B are the corresponding zero time intercept, and t is time. The pharmacokinetic parameters such as area under the concentration curve (AUC), mean residence time (MRT), total clearance (CL_{tot}), and distribution volume (V_d) were calculated from the fitted results on the basis of the following equations: $AUC = A/\alpha + B/\beta$, $MRT = (A/\alpha^2 + B/\beta^2)/(A/\alpha + B/\beta)$, $CL_{tot} = dose/(A/\alpha + B/\beta)$, and $V_d = dose(A/\alpha^2 + B/\beta^2)/(A/\alpha + B/\beta)^2$, where dose is the amount of vanadyl complexes intravenously administered [22].

EPR parameters of $VO(IPA)_2$ in the fresh blood, serum, albumin, and transferrin

EPR parameters of $VO(IPA)_2$ in fresh blood, and in the erythrocytes, serum of rats, RSA, and Tf were calculated from EPR spectra measured at room (22 °C) and liquid nitrogen (77 K) temperatures. Concentrations of RSA and Tf were prepared to be 4% and 0.4% w/v, respectively, according to their physiological levels in rat serum. Instrumental conditions of EPR measurement were as follows: microwave power of 5 mW, modulation frequency of 100 kHz, modulation amplitude width of 0.63 mT, receiver gain of 500 for 22 °C and 40 for 77 K, response of 0.03 s, scanning time of 4 min, and magnetic field of 330± 100 mT. $VO(IPA)_2$ (10 μL) dissolved in saline containing 5% DMSO was added to 490 μL of each vehicle and mixed to a final concentration of 500 nmol/mL. After an aliquot of each solution was transferred into a quartz flat cell or tube, EPR spectra were immediately recorded using the X-band EPR. Hyperfine coupling constants (A_0 value) and g_0 values were obtained from the spectra at room temperature, where the A_0 values were estimated as the means on the basis of selected magnetic field measurements. $A_{\parallel}$, $A_{\perp}$, $g_{\parallel}$, and $g_{\perp}$ values were obtained from the spectra at 77 K, where the $A_{\parallel}$ and $A_{\perp}$ values

were estimated as the means on the basis of the spectral regions for the |5/2| and |7/2| hyperfine components, respectively [23, 24].

To study the components in the blood where $VO(IPA)_2$ is mainly distributed, the EPR spectra of $VO(IPA)_2$ dissolved in the rat fresh blood, in its serum separated by centrifugation, and lysates and membranes of its erythrocytes separated by hemolysis after removing its serum were measured. NaCl solution (0.2%) as the same volume as the removed serum was added to the pellet of erythrocytes to hemolyze the erythrocytes, and their lysates and membranes were separated by centrifugation.

Statistical analysis

All experimental results are presented as the mean value±standard deviation. Statistical analysis was performed by analysis of variance (ANOVA) at a 1% or 5% significance level of the difference.

Results and discussion

The structure of the new complex $VO(IPA)_2$ was characterized by elemental analysis, visible absorption, IR, and EPR spectra. The physicochemical parameters of $VO(IPA)_2$ together with the related complexes [10, 25] are summarized in Table 1. In the visible absorption spectra of $VO(IPA)_2$ dissolved in 5% DMSO solution, two absorption bands characteristic of the vanadyl state were observed. In the IR spectra of $VO(IPA)_2$, the band due to the V=O stretch was found at 968 cm^{-1}. The EPR spectrum of $VO(IPA)_2$ in 5% DMSO solution at 77 K indicated the formation of two vanadyl species, a major species and a minor species found in trace amounts, probably due to the presence of *trans* and *cis* isomers in solution (Fig. 2). EPR parameters of the dominant form were g_0=1.976, A_0=92×10^{-4} cm^{-1}; $g_{||}$=1.945, $A_{||}$=167×10^{-4} cm^{-1}; $g_{\perp}$=1.991, $A_{\perp}$=53×10^{-4} cm^{-1}. From these data, $VO(IPA)_2$ was indicated to form a square pyramidal structure [23]. The partition coefficient of $VO(IPA)_2$ was not determined owing to its insolubility in *n*-octanol or $CHCl_3$.

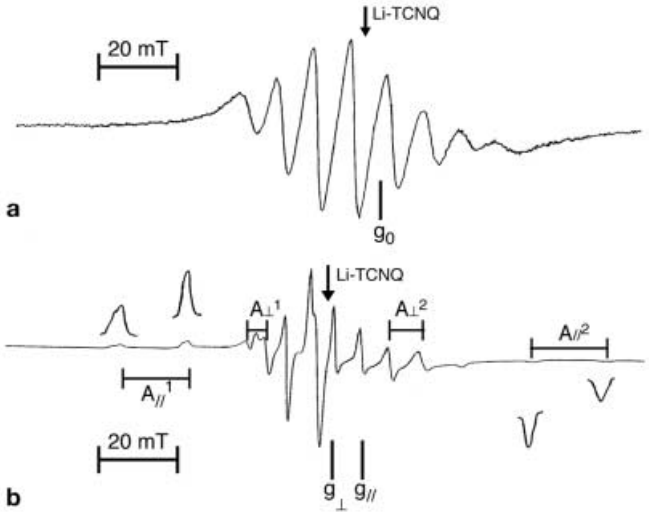


Fig. 2 EPR spectra of $VO(IPA)_2$ in DMSO at room (**A**) and liquid nitrogen (**B**) temperatures

The in vitro insulin-mimetic activity of $VO(IPA)_2$ was examined according to the method previously reported [18]. The dose-dependent inhibitory effects of $VO(IPA)_2$ on FFA release from isolated rat adipocytes treated with epinephrine were observed to be similar to those of $VOSO_4$, $VO(PA)_2$, and $VO(6MPA)_2$ (Fig. 3). The apparent IC_{50} value under the same experimental conditions (Table 2) suggested that introduction of an electron-withdrawing group on the picolinate ligand gives stronger insulinomimetic activity than those of the leading compound $VO(PA)_2$ and of $VO(6MPA)_2$ with an electron-donating methyl group at the sixth position of picolinic acid.

From these in vitro results, we examined the effect of $VO(IPA)_2$ in male Wistar rats with streptozotocin-

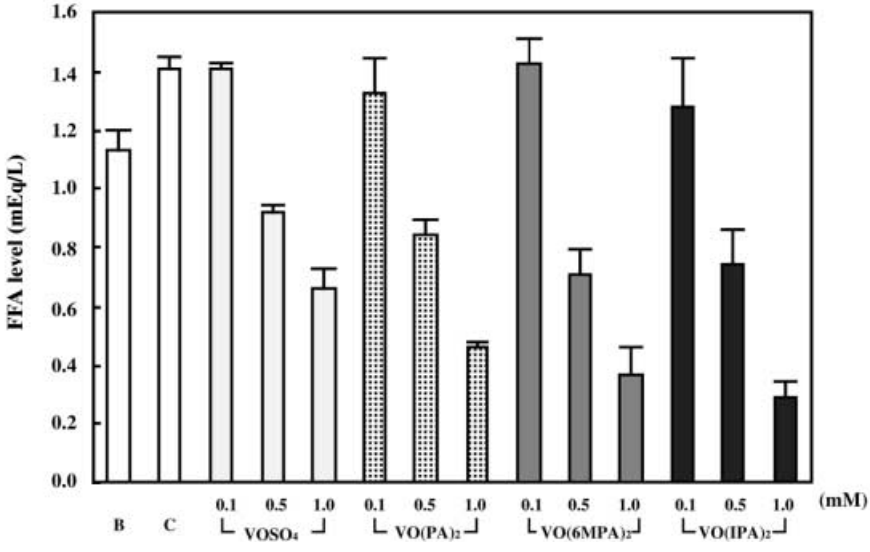


Fig. 3 Inhibitory effects of $VOSO_4$, $VO(PA)_2$, $VO(6MPA)_2$, and $VO(IPA)_2$ on FFA release from rat adipocytes treated with epinephrine in the absence of glucose. Data are expressed as the mean±SD for three experiments. *B* blank, cells only; *C* control, cells plus epinephrine; $VOSO_4$, $VO(PA)_2$, $VO(6MPA)_2$, and $VO(IPA)_2$: cells plus complexes at final concentrations of 0.1, 0.5, and 1.0 mM, respectively

Table 2 Apparent IC_{50} value for the free fatty acid release from isolated rat adipocytes

Complex	IC_{50} (mM)[a]
$VOSO_4$	0.91±0.09[c]
$VO(PA)_2$	0.59±0.05[b,c]
$VO(6MPA)_2$	0.49±0.08
$VO(IPA)_2$	0.45±0.09[b]

[a]Data are expressed as the mean±SD for three experiments
[b]Significantly different: $P<0.05$
[c]Significantly different: $P<0.01$

induced IDDM (STZ rats). When STZ rats received daily i.p. injections of $VO(IPA)_2$ at doses of 5.0 mg (98 μmol) V/kg body weight for the first 3 days, 2.5 mg (49 μmol) V/kg for the following 2 days, and then 1.0 mg (19.6 μmol) V/kg for the following 9 days, the serum glucose levels were normalized within 5 days and were maintained by administration of the complex at lower doses (Fig. 4). It is interesting that the normoglycemic effect of the complex continued at least 14 days after the end of the complex administration.

Normalization of the serum glucose levels of STZ rats was supported also by the improvements of FFA and BUN levels at 28 days (Table 3). However, the insulin levels were still low, indicating that the effect of $VO(IPA)_2$ is not peripheral as observed previously [9, 10].

Total vanadium distribution in STZ rats treated with $VO(IPA)_2$ for 14 days and then without the complex administration was examined using neutron activation analysis (NAA), which is one of the most reliable of the many methods for vanadium determination [26]. Vanadium was found to be accumulated in almost all tissues examined, especially in bone, kidney, spleen, adipose, liver, and pancreas in this order in rats given $VO(IPA)_2$ (Fig. 5). It was reported that the highest tissue vanadium levels in rats treated with an organic vanadyl complex, bis(maltolato)oxovanadium(IV), were in bone with intermediate levels in kidney and liver, and low levels in plasma, muscle, and fat [27], where rats were chronically fed bis(maltolato)oxovanadium(IV) in the drinking water for 25 weeks and organs were removed at termination of this chronic feeding study. With biodistribution of total vanadium levels in the tissues, our results evaluated from NAA are in close agreement with the previous report for bis(maltolato)oxovanadium(IV) [27]. In contrast, in rats treated with VS, vanadium was found at the highest levels in the kidney, followed by the liver or bone and pancreas in this order [28], suggesting that the $VO(IPA)_2$ complex has lower renal toxicity than VS [29, 30] in administered periods after i.p. injection.

$VO(IPA)_2$ contains two atoms of iodine per molecule, and the amount due to iodine occupies 43.0% of $VO(IPA)_2$ by molecular weight. As iodine is an essential trace element in mammals, the effect of $VO(IPA)_2$ on iodine levels in animals needs to be considered by chronic administration. In this study, the maximum and minimum daily doses (98 and 19.6 μmol/kg) of $VO(IPA)_2$ correspond to 25 and 5 mg/kg of iodine per day, respectively. This iodine load certainly exceeds an optimal iodine intake of 2–5 μg/kg per day recommended by the WHO [31]. However, these doses of $VO(IPA)_2$ showed no toxicity to rats during and after the administered periods. Actually, all organs including the thyroid glands of rats after the admin-

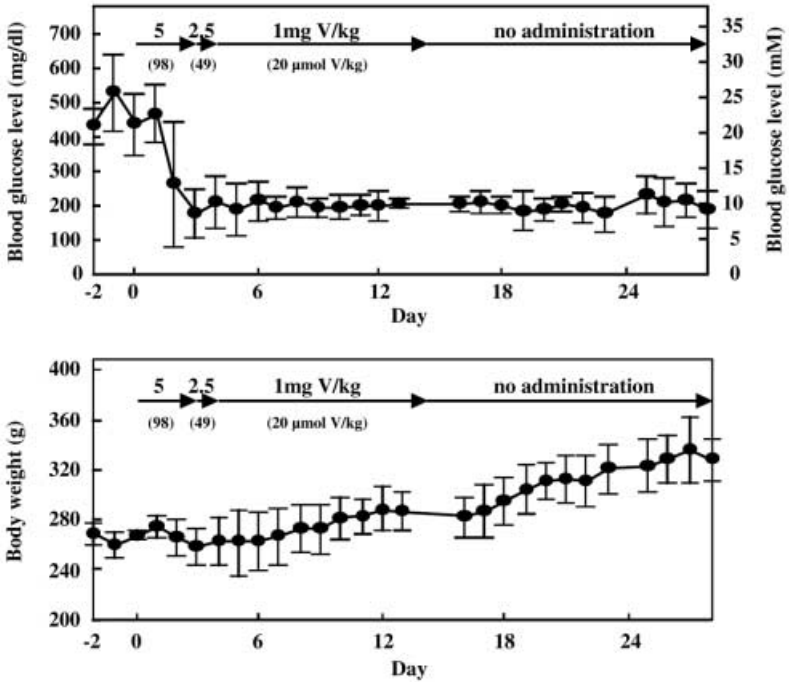


Fig. 4 Changes of blood glucose levels and body weights in STZ rats given the $VO(IPA)_2$ complex by daily i.p. injection. Data are expressed as the mean±SD for five rats

Table 3 Serum parameters in STZ rats given $VO(IPA)_2$ by i.p. injection[a]

Treatment	Days after the first administration	Glucose (mM)	Insulin (pM)	FFA (mEq/L)	BUN (mM)
Normal rats	–	6.4±0.3	155.4±9.6	0.38±0.06	5.0±0.2
STZ rats	–	23.9±3.5	30.6±22.8	0.66±0.25	7.1±1.1
STZ rats+ $VO(IPA)_2$	0	23.8±5.0[c,d]	32.5±24.2	0.66±0.25	7.1±1.1[c]
	14	10.7±0.6[c]	23.1±16.8	0.75±0.18[b]	8.3±2.2[b]
	28	7.9±2.9[d]	19.6±8.9	0.36±0.06[b]	3.6±1.1[b,c]

[a]Data are expressed as the mean±SD for five rats
[b]Significantly different: $P<0.05$
[c]Significantly different: $P<0.01$
[d]Significantly different: $P<0.01$

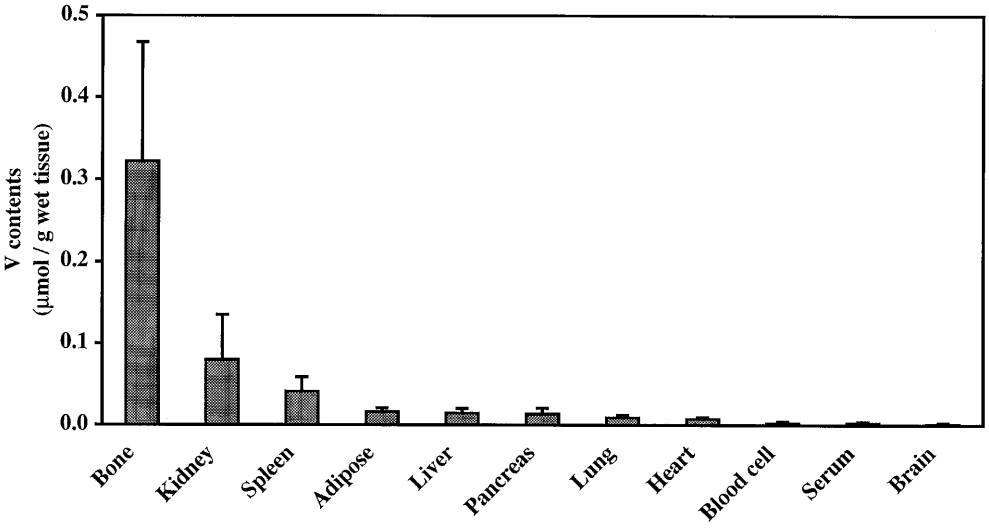


Fig. 5 Organ distribution of total vanadium in STZ rats treated with $VO(IPA)_2$ by daily i.p. injection for 14 days and then without administration of the complex for 14 days. After 14 days of administration and an additional 14 days of no administration, rats were sacrificed under anesthesia with ether, and organs were removed and lyophilized. Data are expressed as the mean±SD for four rats

istration periods were unchanged anatomically, and no significant changes in the wet tissue weights of thyroid glands were observed between pre-administration (15.7±1.4 mg) and post-administration (14.7±0.9 mg) of $VO(IPA)_2$ to STZ rats. Therefore, $VO(IPA)_2$ is considered not to be de-iodinated under the present experimental conditions, and not to cause thyroid dysfunction.

Metallokinetic features of paramagnetic vanadyl species in the blood of normal rats receiving $VO(IPA)_2$ were analyzed using the BCM-EPR method [15, 19, 20]. Concentrations of vanadyl species in the blood of rats given $VO(IPA)_2$ decreased time-dependently after i.v. administration (Fig. 6). Thus the metallokinetic parameters for $VO(IPA)_2$, its related complexes, and $VOSO_4$ as the positive control were obtained, and are summarized in Table 4. We found previously that the EPR signals of the vanadyl compounds added to the fresh blood of untreated rats were very stable over time [15]. Therefore, the vanadyl complexes are in a stable oxidation state in the in vitro fresh blood. However, EPR signals due to vanadyl species in the circulating blood decreased time-dependently after the administration of $VO(IPA)_2$. This indicates that a major factor for the disappearance of EPR signals in the circulating blood due to vanadyl species is not the participation of redox processes of vanadyl species in the circulating blood, but their distribution to the tissues and elimination from the body. Thus, vanadyl species taken up into the blood are distributed to the short- and long-stay tissues, and then accumulated there or re-distributed in the bone, liver, and kidney.

The metallokinetic parameters such as AUC and MRT values for $VO(IPA)_2$ indicated that vanadyl concentrations in the blood of rats given $VO(IPA)_2$ remain significantly higher and longer than those given other complexes [15]. Such real time EPR analyses of vanadyl species revealed that the clearance rate of vanadyl species from the blood of rats given $VO(IPA)_2$ is significantly lower than those given other complexes in terms of total clearance (CL_{tot}), being 2.7 mL/min/kg in $VO(IPA)_2$-treated rats and 10–15 mL/min/kg in rats treated with other complexes (Table 4). It has been observed that the shortest residence times of vanadyl compounds were calculated to be 5–7 min in the blood [32]. With respect to the residence times (MRT) of VS in the blood, our results evaluated from BCM-EPR are in good agreement with those of other earlier studies [32, 33].

The slow elimination of vanadium from the circulating blood of rats indicated the different association of the vanadyl complex with the blood components such as serum proteins or erythrocytes between rats

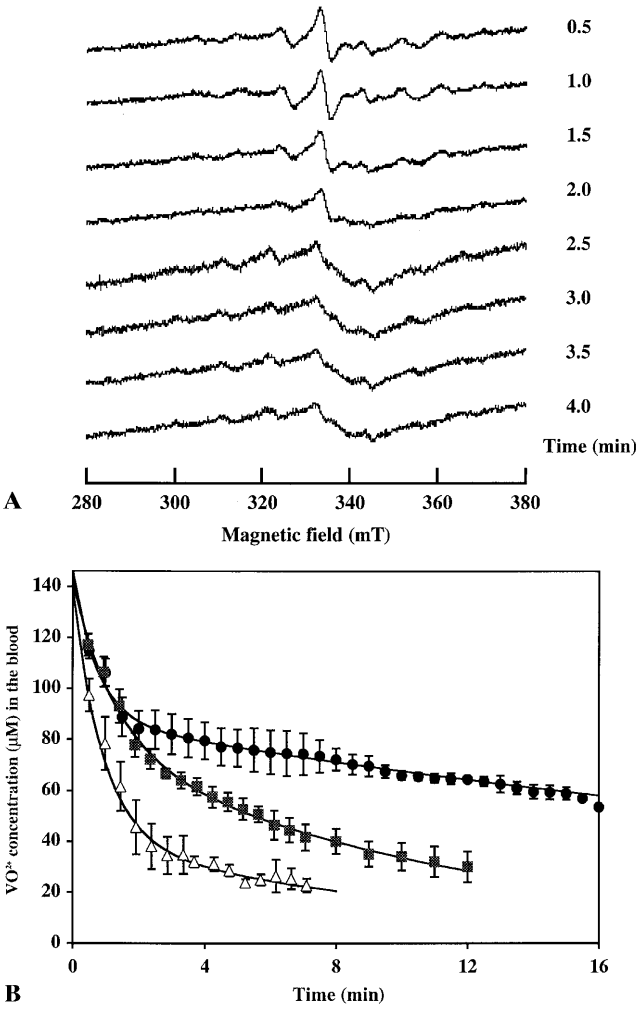


Fig. 6 **A** Results of in vivo BCM-EPR of vanadyl (VO^{2+}) species. Rats were administered $VO(IPA)_2$ intravenously at a dose of 0.5 mg (9.8 μmol) of vanadium/kg body weight under anesthesia. EPR spectra were recorded at room temperature every 30 s following administration of the complex. **B** Time courses of vanadyl (VO^{2+}) concentration in the blood of rats treated with (●) $VO(PA)_2$, (■) $VO(6MPA)_2$, or (△) $VO(IPA)_2$ as monitored using the BCM-EPR method. Data are expressed as the mean±SD for three or four rats and theoretical curves are fitted to those mean values

given $VO(IPA)_2$ and those given other complexes [34]. Thus, we further examined the EPR spectra for the interaction between the complex and the blood components. As shown in Figs. 7 and 8, $VO(IPA)_2$ appeared to interact with both the erythrocyte membrane of rats and RSA. The EPR parameters such as g_0=1.994 and A_0=90.8×10^{-4} cm^{-1} supported the interactions of $VO(IPA)_2$ with the erythrocyte membrane and the serum components (Table 5). These results suggest the long-resident character of $VO(IPA)_2$ in the blood. In contrast, $VO(PA)_2$ and $VO(6MPA)_2$ have been indicated to bind with serum albumin [15]. Therefore, $VO(IPA)_2$ is proposed to have a different chemical character in terms of its interaction with the erythrocyte membrane, probably due to its higher hydrophobicity than other complexes [35]. The higher normoglycemic effect of $VO(IPA)_2$ among the three complexes with the $VO(N_2O_2)$ coordination mode is due to the longer residence of vanadyl species than other complexes in rats given $VO(IPA)_2$.

Concentrations of RSA and Tf were prepared to be 4% (600 μM) and 0.4% (50 μM) w/v in the in vitro study, respectively, according to their physiological levels in rat serum. As shown in Fig. 7, EPR spectra of $VO(IPA)_2$ measured with both holo- and apo-Tf were isotropic, while that with RSA was anisotropic at room temperature. These results suggest that $VO(IPA)_2$ interacts hardly with both holo- and apo-Tf under the physiological conditions. However, Chasteen and co-workers [24, 36] observed anisotropic EPR signals of the vanadyl ion in the presence of high concentrations (0.3–1 mM) of transferrin. Thus, we conclude at present that the vanadyl complex $VO(IPA)_2$ binds with RSA under physiological conditions.

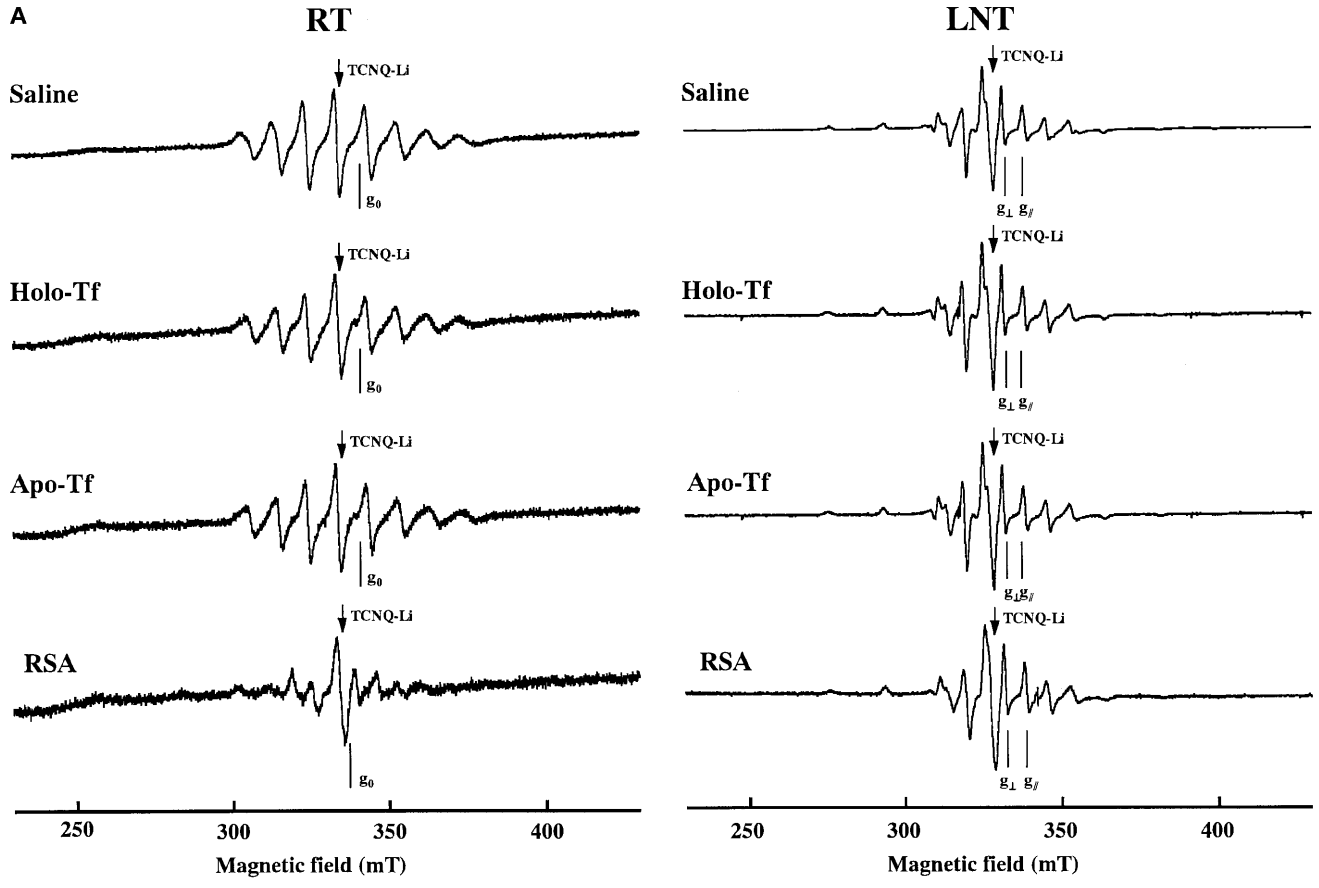
On the basis of these results, we propose here a new halogenated $VO(IPA)_2$ complex that contains the $VO(N_2O_2)$ coordination mode and shows a long-term effect for the treatment of IDDM. This complex has also another advantage. When iodine of the ligand is radio-labeled with ^{123}I, ^{125}I, or ^{131}I, the metabolism, organ distribution, and binding-sites in cells as well as the action mode of the complex can be examined by measuring their radioactivity levels [37, 38].

Table 4 Metallokinetic parameters of vanadyl complexes[a]

	$t_{1/2}$ (min)	V_d (mL/kg)	CL_{tot} (mL/min/kg)	AUC (nmol min/mL)	MRT (min)
$VOSO_4$	5.2±1.2	154±26[b,c]	22.2±5.1[b]	461±100[b] [23.5±5.0 (μg min/mL)]	7.2±1.6[b]
$VO(PA)_2$	$t_{1/2}(\alpha)$=0.4±0.2 $t_{1/2}(\beta)$=8.2±1.1	142±47	15.2±2.9[b,d]	651±130[d] [33.2±6.6 (μg min/mL)]	9.3±1.7[d]
$VO(6MPA)_2$	$t_{1/2}(\alpha)$=0.5±0.1 $t_{1/2}(\beta)$=7.2±2.6	95±10[c]	10.5±2.3[b,c,d]	974±246[b,c,d] [49.7±12.5 (μg min/mL)]	9.6±3.4[e]
$VO(IPA)_2$	$t_{1/2}(\alpha)$=0.5±0.2 $t_{1/2}(\beta)$=31.5±13.7	112±17[b]	2.66±0.77[c]	3850±1110[c] [196.4±56.6 (μg min/mL)]	44.9±19.4[b,d,e]

[a]Rats were treated with the vanadyl complexes [$VOSO_4$ (n=4), $VO(PA)_2$ (n=4), $VO(6MPA)_2$ (n=3), or $VO(IPA)_2$ (n=3)] with 0.5 mg V/kg body weight by intravenous injection under anesthesia

[b]Significantly different at the 5% level of ANOVA
[c]Significantly different at the 1% level of ANOVA
[d]Significantly different at the 5% level of ANOVA
[e]Significantly different at the 5% level of ANOVA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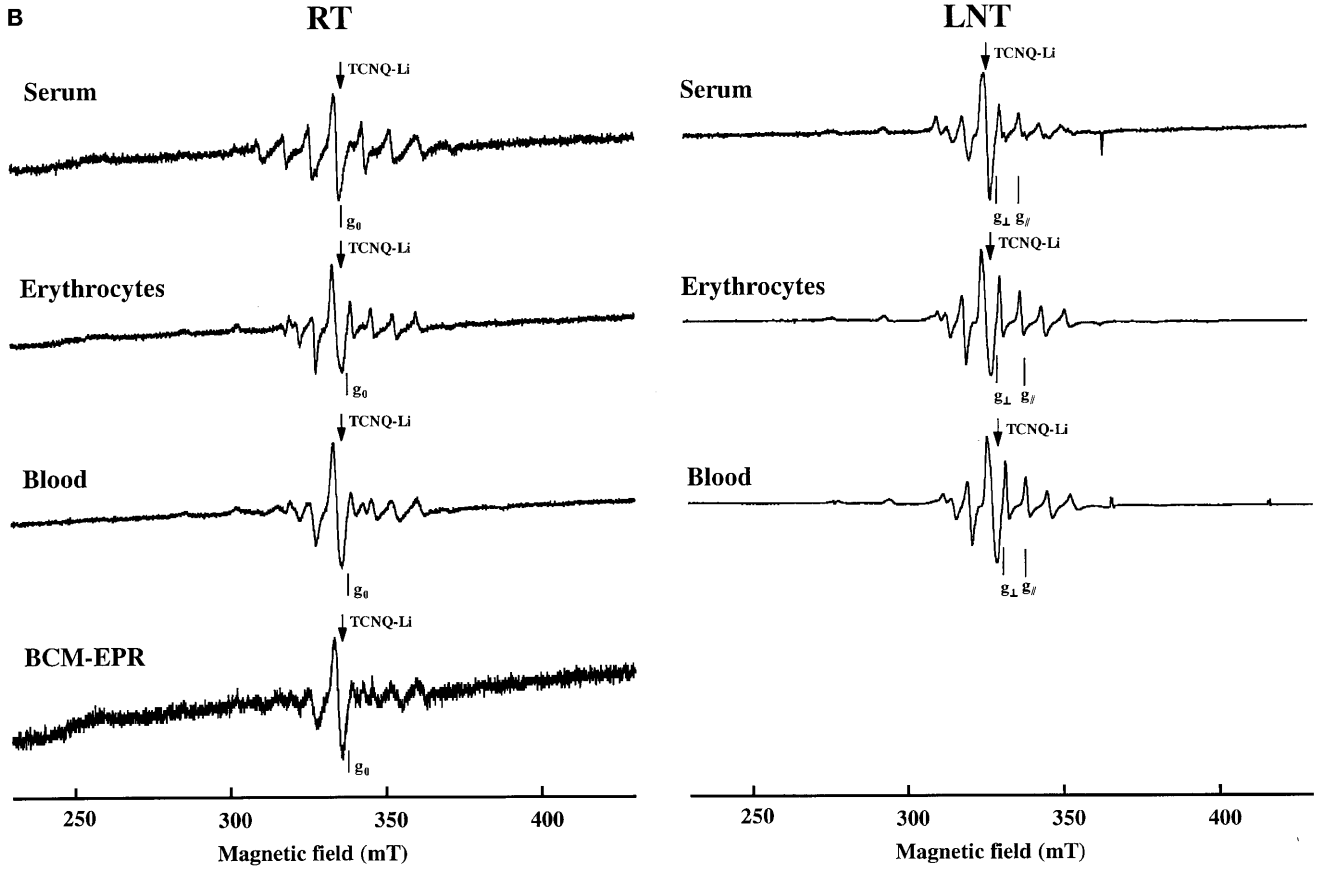
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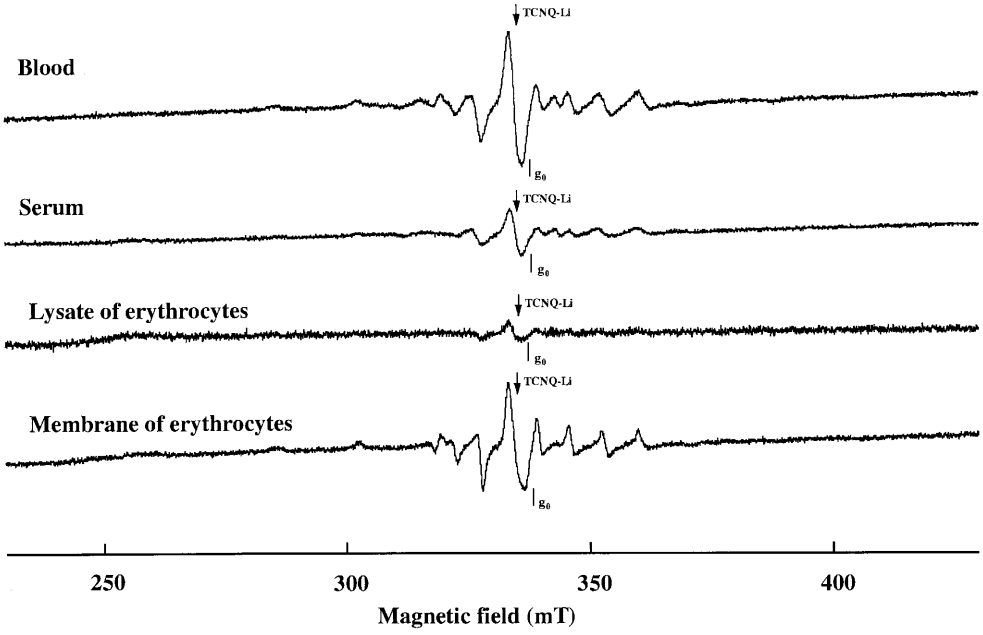

Fig. 8 EPR spectra of $VO(IPA)_2$ (500 μM) dissolved in the fresh blood of rats, its serum separated by centrifugation after $VO(IPA)_2$ dissolved in the blood, and the lysates and membranes of its erythrocytes separated by hemolysis after removing its serum. NaCl solution (0.2%) as the same volume as the removed serum was added to the pellet of erythrocytes to hemolyze the erythrocytes, and their lysates and membranes were separated by centrifugation

Table 5 EPR parameters for $VO(IPA)_2$ in saline, 0.4% transferrin, 4% RSA, serum, blood, or erythrocytes of rats. The values obtained are the mean of duplicate measurements

Vehicle	g_0	$g_{\parallel}$	$g_{\perp}$[a]	A_0 (10^{-4} cm^{-1})	$A_{\parallel}$ (10^{-4} cm^{-1})	$A_{\perp}$ (10^{-4} cm^{-1})[a]
Saline	1.976	1.945	1.991	92.2	166.9	52.7
Holo-transferrin	1.976	1.946	1.991	92.1	167.1	52.8
Apo-transferrin	1.974	1.946	1.991	92.0	166.9	52.6
RSA	1.995	1.949	1.995	89.2	162.5	61.4
Serum	1.993	1.946	1.992	90.9	161.2	61.3
Blood	1.995	1.946	1.989	88.9	159.7	57.3
Erythrocytes	1.995	1.949	1.991	89.1	156.9	56.0
BCM-EPR	1.994	–	–	90.8	–	–

[a] $g_{\perp}$ and $A_{\perp}$ were obtained from the observed data

Fig. 7 **A** EPR spectra of $VO(IPA)_2$ (500 μM) dissolved in saline, 0.4% holo- or apo-Tf solution, and 4% RSA solution. Concentrations of RSA and Tf were prepared to be their physiological levels in rat serum, respectively. EPR spectra were recorded at room temperature (*left side*) and at liquid nitrogen temperature (77 K) (*right side*). **B** EPR spectra of $VO(IPA)_2$ (500 μM) dissolved in serum, erythrocytes, and blood of rats, and monitored using the BCM-EPR method. EPR spectra were recorded at room temperature (*left side*) and at liquid nitrogen temperature (77 K) (*right side*)

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