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Study of the Reduction of Cobalt(III) Complexes by In Situ NMR Spectroscopy

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Abstract—An approach for monitoring the redox activation of drug delivery in cobalt(III) complexes by in situ NMR spectroscopy is proposed. The reduction of the heteroleptic cobalt(III) complexes containing the 6,7-dihydroxycoumarin molecule applied as a model drug is studied using the proposed approach. The replacement of the bipyridine ligand in the cobalt(III) complex by phenanthroline considerably increases the redox-activated release rate of the drug.

Keywords: in situ NMR spectroscopy, dihydroxycoumarin, cobalt complexes, redox-activated drug delivery
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INTRODUCTION

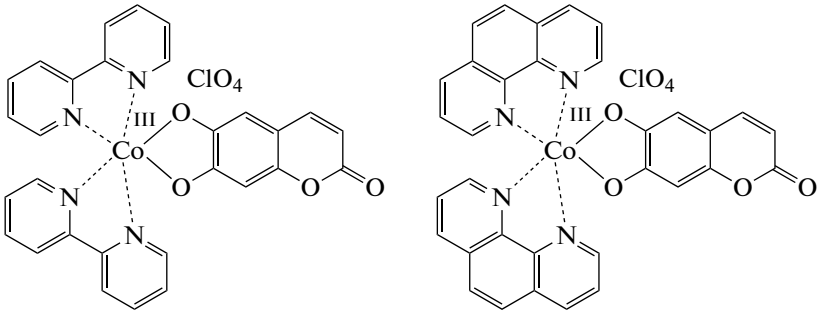
Chemo- and radiotherapy frequently used in clinical practice significantly lose efficiency during the treatment of so-called "solid" tumors, because they contain regions with a low oxygen level [1]. However, the differentiation of cancerous and healthy tissues by the oxygen level made it possible to develop a new strategy of selective chemotherapy: the use of chemical compounds, whose inhibition ability is activated under the conditions of hypoxia of solid tumors (redox-activated drugs).

"Molecular platforms" based on biogenic metal complexes are actively considered in the recent time as one of the methods diminishing a negative effect of anticancer drugs on the human organism. The molecular platforms make it possible to perform targeted drug delivery to tumor cells [2, 3]. Among diverse variants of similar platforms, redox-active compounds of biogenic metals, for example, cobalt, are of special interest [4, 5]. The cobalt(III) ion can coordinate and inactivate cytotoxic ligands to form inert complexes that can circulate in the human organism via blood vessels without damaging healthy tissues. The concentration of biogenic reducing agents, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced glutathione and ascorbate increases under the hypoxia conditions and leads to the activation of these complexes upon the reduction of the cobalt(III) to cobalt(II) ion [6]. This is accompanied by the dissociation of the complexes with the release of the drug acting as an organic ligand. Selectivity of this preparation in tissues with a low oxygen level is provided by the fast backward oxidation of the cobalt(II) to cobalt(III) ion in healthy tissues with a normal oxygen concentration [7]. A series of the cobalt(III) complexes with some drugs and their precursors that demonstrated a high potential of this approach have been prepared to date. Among them are the cobalt(III) complexes with the compounds of the class of alkylating anticancer drugs, which are analogs of nitrous yperite [8, 9], inhibitors of matrix metalloproteinase of a wide range of marimastat action [10], inhibitors of the receptor of the epidermal growth factor [11], esculetin (coumarin derivative with a potential anticancer activity) [5], and phenylalanine (model compound of anticancer drug melphalan) [12].

In spite of encouraging results obtained to the moment, numerous restraints should be surmounted to transform the developed strategy of redox activation into the stage of clinical tests. One of the main problems is that many results obtained in vitro were not reproduced in vivo. Therefore, the properties of the cobalt complexes should further be optimized for using as a molecular platform for redox-activated drug delivery.

This problem can successfully be solved by the development of a method for studying in situ redox-activation processes of drugs in the cobalt complexes under the conditions close to biological ones. Optical absorption spectroscopy is used most frequently to study reduction processes [5]. The main drawback of the method is the overlapping of absorption bands of the starting complexes and reaction products, which significantly impedes an analysis.

In this work, we proposed an approach that makes it possible to monitor the reduction of the cobalt(III) complexes by in situ NMR spectroscopy. The cobalt(III) complexes $[Co(Bipy)_2(coumarin)]ClO_4$ (**I**) and $[Co(Phen)_2(coumarin)]ClO_4$ (**II**) [5, 13] containing bipyridine or phenanthroline and 6,7-dihydroxycoumarin dianion as ligands (Scheme 1) were chosen as objects of the study. Complexes **I** and **II** were chosen because of their ability to liberate coumarin upon the reduction of the cobalt(III) ion by biogenic reducing agents, which predetermines their promising biological properties. For example, the cytotoxicity of complex **I** toward colon cancer cells under the hypoxia conditions was demonstrated [5], and complex **II** was studied in vitro as a drug for anticancer photodynamic therapy [13].



$[Co(Bipy)_2(coumarin)]ClO_4$ $[Co(Phen)_2(coumarin)]ClO_4$

Scheme 1.

EXPERIMENTAL

Complexes **I** and **II** were synthesized using published procedures [5, 13]. The cobalt(III) complexes $[Co(L)_2Cl_2]Cl$ (L = Bipy, Phen) synthesized by the oxidation of the corresponding cobalt(II) complexes by gaseous chlorine were used as precursors [14]. Chlorine was obtained by the reaction of potassium permanganate with concentrated hydrochloric acid and dried by passing through concentrated sulfuric acid [15]. 1,10-Phenanthroline (99%, Sigma-Aldrich), 2,2'-bipyridine (99%, Sigma-Aldrich), cobalt(II) chloride (98%, anhydrous, Sigma-Aldrich), 6,7-dihydroxycoumarin (98%, Sigma-Aldrich), lithium perchlorate (98%, Alfa Aesar), and triethylamine (99%, Sigma-Aldrich) were used as received.

General synthesis procedure for complexes I and II. A solution of 6,7-dihydroxycoumarin (0.5 mmol, 89 mg) and triethylamine (1 mmol, 101.2 mg, 139 μL) in methanol (10 mL) was added to a solution of $[Co(L)_2Cl_2]Cl$ (L = Bipy, Phen) (0.5 mmol) in methanol (15 mL). The resulting mixture was refluxed for 3 h and then cooled to room temperature. A solution of lithium perchlorate (1.25 mmol, 133 mg) in methanol (5 mL) was added to the mixture, and the obtained solution was cooled for 30 min with stirring in a water bath for the crystallization of the target complexes. The formed green precipitate was filtered off, washed with isopropanol and diethyl ether, and dried under reduced pressure.

The yield of compound **I** was 258 mg (80%). ^{1}H NMR (300 MHz; D_2O; δ, ppm): 5.92 (d, J = 9.3 Hz, 1H, CHC*H*COO), 6.50 (s, 1H, C*H*), 6.70 (s, 1H, C*H*), 7.42–7.45 (m, 4H, C*H*), 7.61 (d, J = 9.4 Hz, 1H, C*H*CHCOO), 7.74–7.80 (m, 2H, C*H*), 8.19–8.25 (m, 2H, C*H*), 8.36–8.42 (m, 2H, C*H*), 8.52 (d, J = 8.0 Hz, 2H, C*H*), 8.62 (d, J = 8.1 Hz, 2H, C*H*), 8.69 (d, J = 5.7 Hz, 1H, C*H*), 8.76 (d, J = 5.7 Hz, 1H, C*H*). Mass spectrum (ESI), m/z: $[Co(Bipy)_2(coumarin)]^+$, calculated 547.08, found 547.1.

The yield of compound **II** was 290 mg (83%). ^{1}H NMR (300 MHz; CD_3CN; δ, ppm): 5.83 (d, J = 9.4 Hz, 1H, CHC*H*COO), 6.46 (s, 1H, C*H*), 6.57 (s, 1H, C*H*), 7.49 (d, J = 9.4 Hz, 1H, C*H*CHCOO), 7.63–7.71 (m, 4H, C*H*), 8.20–8.21 (m, 4H, C*H*), 8.36–8.39 (m, 2H, C*H*), 8.72–8.76 (m, 2H, C*H*), 9.01 (d, J = 8.2 Hz, 2H, C*H*), 9.10 (d, J = 5.3 Hz, 1H, C*H*), 9.17 (d, J = 5.2 Hz, 1H, C*H*). Mass spectrum (ESI), m/z: $[Co(Phen)_2(coumarin)]^+$, calculated 595.08, found 595.0.

X-ray diffraction (XRD). The XRD study of single crystals of complex **I** obtained by the diffusion of diethyl ether vapors to its solution in acetonitrile was carried out on a Bruker Quest D8 CMOS diffractometer (MoK_α radiation, graphite monochromator, ω scan mode). The structure was solved using the ShelXT program [16] and refined by full-matrix least squares using the Olex2 program [17] in the anisotropic approximation for F^2_{hkl}. The positions of the hydrogen atoms were calculated geometrically and refined in the isotropic approximation by the riding model. Selected crystallographic data and structure refinement parameters are given in Table 1.

Table 1. Selected crystallographic data and structure refinement parameters for $[Co(Bipy)_2(coumarin)]ClO_4$

Parameter	Value
Empirical formula	$C_{31}H_{23}N_5O_8ClCo$
FW	687.92
T, K	1
Crystal system	Orthorhombic
Space group	*Fddd*
Z	32
a, Å	34.4574(6)
b, Å	45.2707(9)
c, Å	16.4894(3)
α, deg	90
β, deg	90
γ, deg	90
V, Å^3	25722.0(8)
ρ_{calc}, g cm^{-3}	1.421
μ, cm^{-1}	6.74
F(000)	11264
$2\theta_{max}$, deg	52
Number of measured reflections	65190
Number of independent reflections	6339
Number of reflections with $I > 3\sigma(I)$	5323
Number of refined parameters	432
R_1	0.0945
wR_2	0.2045
GOOF	1.166
Residual electron density (max/min), e Å^{-3}	0.515/–0.598

The full set of XRD parameters for complex **I** was deposited with the Cambridge Crystallographic Data Centre (CIF file CCDC no. 2169544; http://www.ccdc.cam.ac.uk/).

In situ NMR spectroscopy. A solution of complexes **I** and **II** (7.6 μmol) in a mixture of D_2O (200 μL) and CD_3CN (400 μL) was placed in an NMR tube, and a solution (10 μL) of ascorbic acid in D_2O (6.25×10^{-7} M) was added. To study reduction in an argon atmosphere, argon was bubbled for 5 min through a solution of the complex using a long glass capillary prior to the addition of ascorbic acid.

The ^{1}H NMR spectra of the prepared mixtures were recorded at room temperature on a Bruker Avance 300 spectrometer with a working frequency for protons of 300.15 MHz. The chemical shifts (δ, ppm) in the spectra were determined relative to the residual signal of the solvent (^{1}H 1.94 ppm for CD_3CN). NMR spectra were recorded at an interval of two minutes for 40 min. The following detection parameters were used: the spectral range was 170 ppm, the detection time was 0.2 s, the relaxation delay duration was 0.6 s, the pulse duration was 9.5 μs, and the acquisition number was 32. The obtained free induction decays for enhancing the signal/noise ratio were processed using exponential weighing with the coefficient to 1. The conversion rate was estimated from the consumption of the starting complexes. The content of the complexes in the mixture (in % of the initial content) were calculated from the ratio of the integral intensity of the signal from the residual CD_3CN protons to the integral intensity of the multiplet signal at 8.60–8.69 ppm for complex **I** and 8.60–8.89 ppm for complex **II** chosen for integration convenience, since this signal is observed during the whole reduction process and is not overlapped with other signals.

Mass spectrometry. The reduction products were analyzed by mass spectrometry on an LCMS-2020 liquid chromatograph coupled with a mass spectrometer (Shimadzu, Japan) equipped with electrospray ioniza-

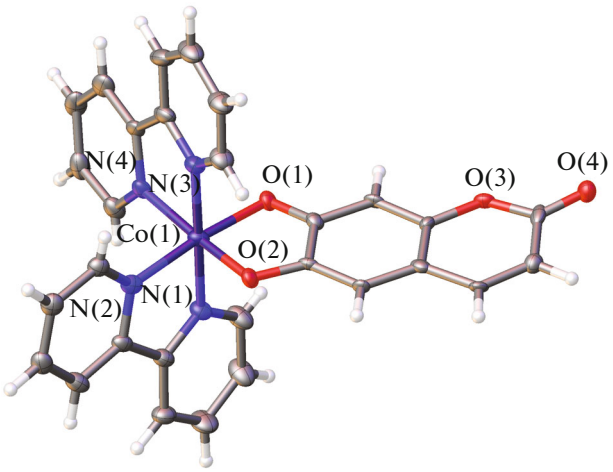


Fig. 1. General view of complex **I** illustrating the coordination environment of the cobalt(III) ion. Hereinafter, perchlorate anions, solvate acetonitrile molecules, and the second component of the disordered 6,7-dihydroxycoumarin dianion are omitted, and non-hydrogen atoms are presented as thermal vibration ellipsoids (p = 20%). Numeration is presented only for metal ions and heteroatoms.

tion and a quadrupole detector (registration of positive and negative ions with m/z in a range of 50–2000). The temperatures of the desolvation line and heating unit were 250 and 400°C, respectively. Nitrogen (99.5%) served as the spraying and drying gas, and acetonitrile (99.9+%, for HPLC, ChemLab) with a flow rate of 0.4 mL/min was the mobile phase. The volume of the analyzed sample was 0.1 μL.

RESULTS AND DISCUSSION

The chosen cobalt(III) ([Co(Bipy)$_2$(coumarin)]ClO_4 (**I**)) and cobalt(II) ([Co(Phen)$_2$(coumarin)]ClO_4 (**II**)) complexes were synthesized using described procedures [5, 13] by the reaction of 6,7-dihydroxycoumarin with $[Co(L)_2Cl_2]Cl$ (L = Bipy, Phen) in the presence of triethylamine and lithium perchlorate. They were isolated in the individual state and characterized by NMR spectroscopy. The structure of complex **I** was also confirmed by the XRD study (Fig. 1) of its crystalline solvate with acetonitrile used as one of the solvents for crystallization. According to thus obtained data, the cobalt(III) ion exists in the low-spin state, which is unambiguously indicated by the Co–N bond lengths <1.96 Å [18]. Its coordination environment formed by four nitrogen atoms of the bipyridine ligands (Co–N 1.917(5)–1.944(5) Å) and two oxygen atoms of the 6,7-dihydroxycoumarin dianion (Co–O 1.797(16)–1.961(15) Å) is close to an octahedron. This can quantitatively be confirmed by "symmetry measures" [19] describing the deviation of the coordination polyhedron CoX_6 (X = O, N) from an ideal octahedron. The lower this value, the better the description of the polyhedron shape by the corresponding polyhedron. In complex **II**, the corresponding value estimated from the XRD data using the Shape 2.1 program [19] is only 0.376. For comparison, the symmetry measure describing the deviation of the coordination polyhedron from an ideal trigonal prism takes an appreciably higher value equal to 15.400.

Two types of aromatic ligands in the complexes resulted in the formation of numerous stacking interactions in the crystals. The stacking interactions are formed by the bipyridine ligands with each other and

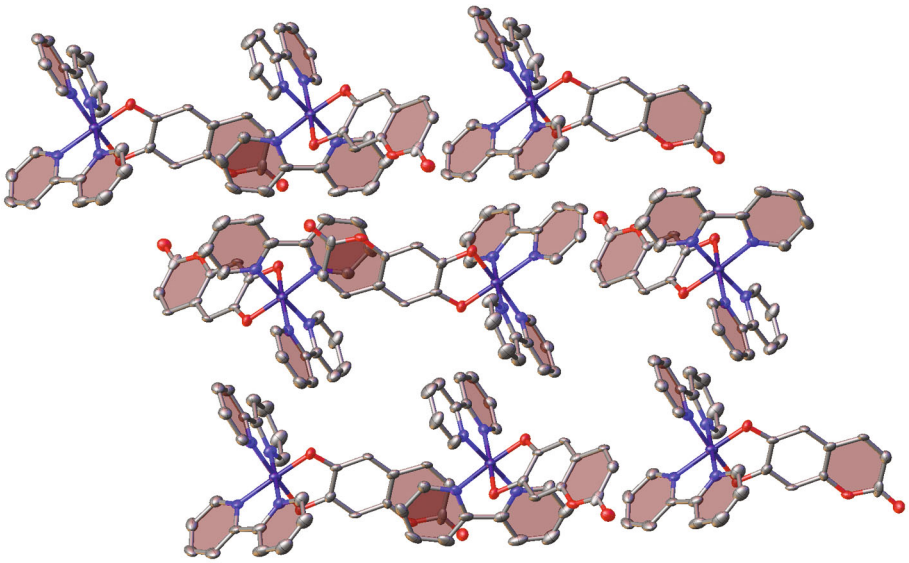
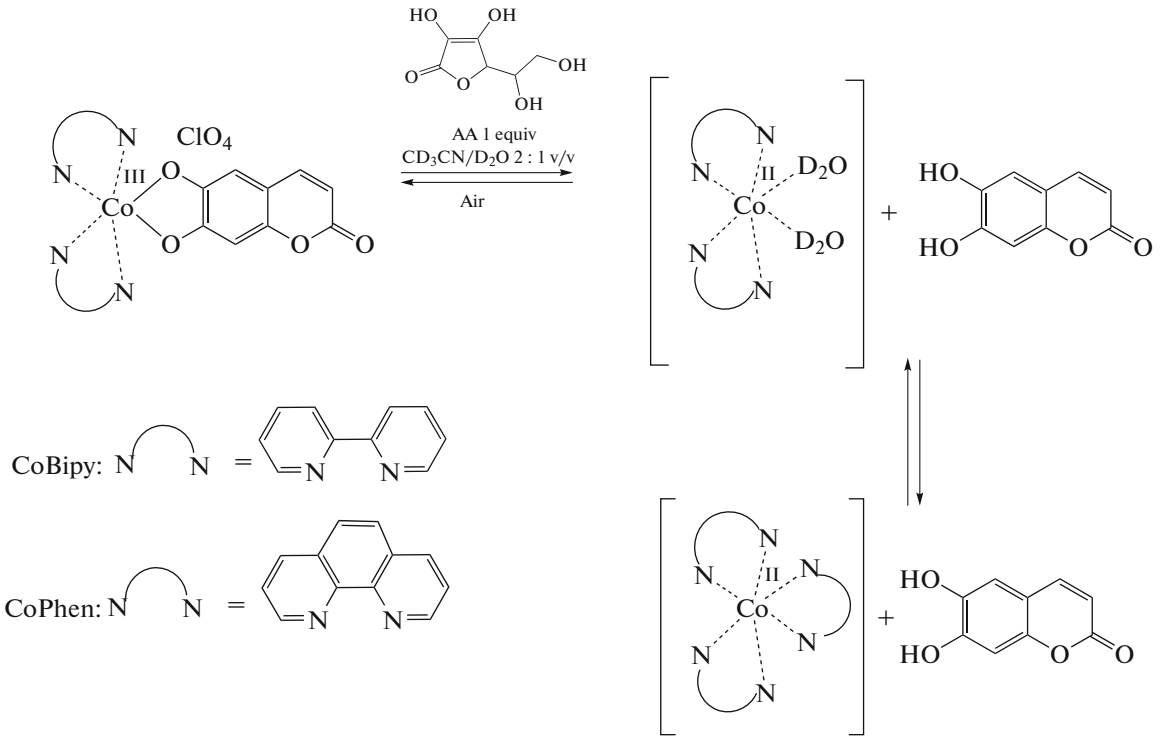


Fig. 2. Fragments of the crystal packing of complex **I** illustrating the formation of 2D layers due to the stacking interactions of the bipyridine ligands with each other and with 6,7-dihydroxycoumarin dianions (shown by pink).

with the 6,7-dihydroxycoumarin dianions. As a result, 2D layers parallel to the crystallographic *ac* plane with the distances between the centroids of the corresponding aromatic rings and angles between them in the ranges 3.535(4)–4.120(4) Å and 1.3(5)°–6.0(3)°, respectively, are observed in the crystal of compound **I** (Fig. 2).

To study the reduction of cobalt(III) complexes **I** and **II** by in situ NMR spectroscopy, ascorbic acid (1 equiv) as a solution in deuterated water was added to a tube containing a solution of the complex in an acetonitrile-d_3–deuterated water (2 : 1) mixture, and the ^{1}H NMR spectra were recorded. The choice of a mixture of solvents is due to the solubility of complex **II** in water inappropriate for the fast registration of ^{1}H NMR spectra and simultaneously a low solubility of the reducing agent (ascorbic acid) in acetonitrile. Prior to the reduction in an inert atmosphere, a solution of the complex was bubbled with argon for 5 min before adding ascorbic acid (AA). The assumed reduction products are shown in Scheme 2.



Scheme 2.

The ^{1}H NMR spectra presented in Fig. 3 demonstrate the dynamics of the reduction of complex **II** by ascorbic acid. The diamagnetic (from 0 to 10 ppm) and paramagnetic (from 15 to 120 ppm) ranges can be distinguished in the spectra. The first range contains signals of the starting complex, ascorbic acid, its oxidation product, and free 6,7-dihydroxycoumarin. The second range exhibits signals of the formed cobalt(II) complexes. It is seen that, as the reaction occurs, the signal intensity in the diamagnetic range decreases and that in the paramagnetic range, on the contrary, increases. The number of signals observed in the paramagnetic spectral range remains unchanged during reduction in spite of a potential possibility of forming several cobalt(II) complexes (Scheme 2). The number of signals, their chemical shifts, and integral intensity correspond to the $[Co(Phen)_3]^{2+}$ complex, which is additionally confirmed by the mass spectrometric analysis of the reduction products. The mass spectrum of the reaction mixture shown in Fig. 4 contains intense signals with *m/z* 299.7, 517.9, and 453.9 attributed to the $[Co(Phen)_3]^{2+}$, $[Co(Phen)_2(ClO_4)]^+$, and $[Co(Phen)_2Cl]^+$ ions. The appearance of the adducts with the chloride anion in the mass spectrum can be due to the incomplete replacement of the chloride by perchlorate ion during the synthesis of $[Co(Phen)_2(coumarin)]ClO_4$ from $[Co(Phen)_2Cl_2]Cl$. The mass spectrum of negative ions (Fig. 5) exhibits signals with *m/z* 177, 220.8, and 277 corresponding to dihydroxycoumarin ions, their solvates with one acetonitrile molecule, and adducts with the perchlorate anion, which additionally confirms the release of the model drug during reduction.

Unlike complex **II**, the reduction of complex **I** is much slower under similar conditions. The conversion rates of complexes **II** and **I** in air and under an argon

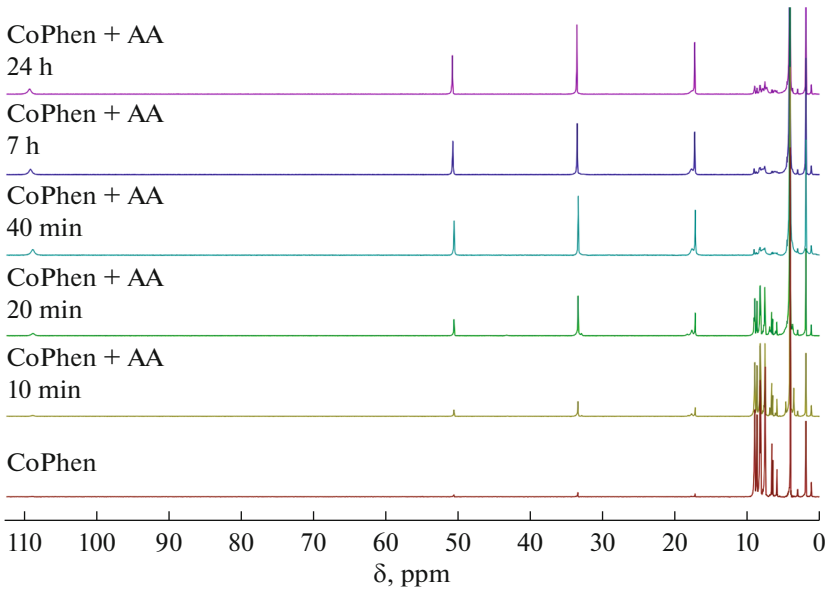


Fig. 3. Dynamics of changing the ^{1}H NMR spectrum in time upon the reduction of complex **II** by ascorbic acid in an argon atmosphere (spectrum was detected in an acetonitrile-d_3–deuterated water (2 : 1) mixture).

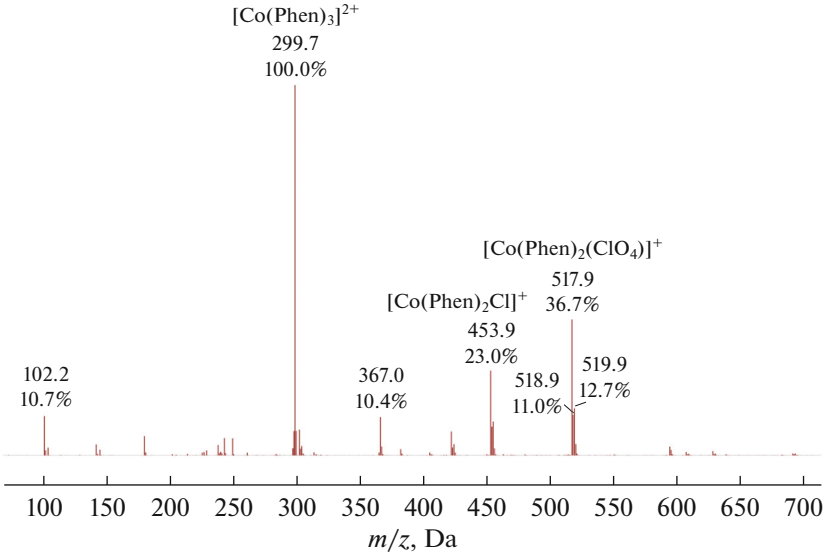


Fig. 4. Mass spectrum of the reduction products of complex **II** by ascorbic acid detected for positive ions.

atmosphere according to the NMR spectroscopy data are compared in Fig. 6. It is seen that the reduction rate of complex **I** by ascorbic acid in the presence of air is extremely low. It should be mentioned that the reversible oxidation occurs in 24 h after the addition of ascorbic acid to complex **II** in the presence of air, and the content of the starting complex increases to 70%, while under an argon atmosphere its content is retained at a level of 4%.

It has previously been shown by mass spectrometry that the reduction of complex **I** affords the $[Co(Bipy)_2(H_2O)]^{2+}$ cation [5]. The paramagnetic range of the NMR spectrum contains eight signals, which can possibly correspond to the $[Co(Bipy)_2(H_2O)]^{2+}$ cation or a mixture of several complexes (Fig. 7). An ion with m/z 405 (Fig. 8) corresponding to the $[Co(Bipy)_2Cl]^{+}$ cation was found in the mass spectrum of the mixture. As already mentioned, the adducts with the chloride ion can be associated with the incomplete replacement of the chloride by perchlorate ion when synthesizing complex **II**. However, the concentrating of the solution and repeated dissolution of the residue in acetonitrile-d_3 result in the appearance of only four signals in the

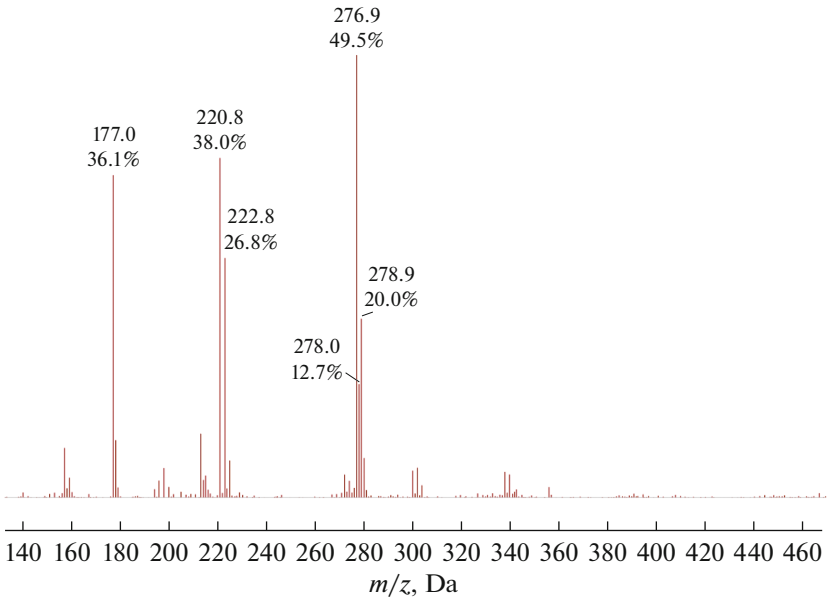


Fig. 5. Mass spectrum of the reduction products of complex **II** by ascorbic acid detected for negative ions.

paramagnetic range of the NMR spectrum corresponding to the protons of the $[Co(Bipy)_3]^{2+}$ cation (Fig. 7) and intense signals of the $[Co(Bipy)_3]^{2+}$, $[Co(Bipy)_2(ClO_4)]^+$, and $[Co(Bipy)_2Cl]^+$ ions in the mass spectrum (Fig. 8).

Thus, we proposed the approach making possible to monitor the redox activation of drugs in the cobalt(III) complexes by in situ NMR spectroscopy. The reduction of the $[Co(Bipy)_2(coumarin)]ClO_4$ (**I**) and $[Co(Phen)_2(coumarin)]ClO_4$ (**II**) complexes containing the 6,7-dihydroxycoumarin anion as one of the ligands was studied using the proposed approach. It turned out that the reduction of complex **II** by ascorbic acid occurred much more rapidly. It was also shown by NMR spectroscopy and mass spectrometry that the cobalt(II) complexes $[Co(Phen)_3]^{2+}A^{2-}$ and $[Co(Bipy)_3]^{2+}A^{2-}$ were the final products of the reduction of the cobalt(III) complexes.

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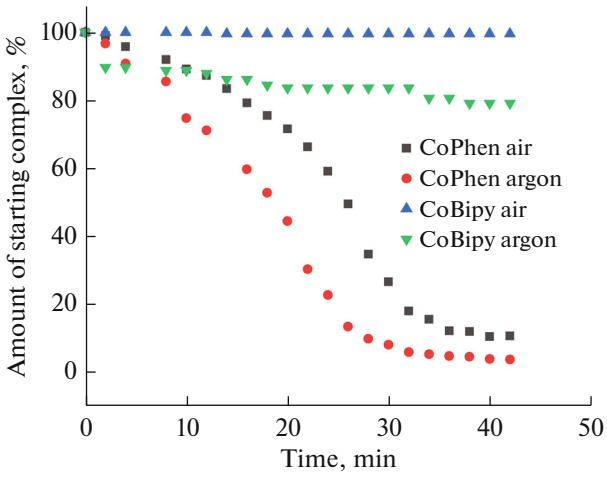

Fig. 6. Comparison of the reduction rates for complexes **II** and **I** in air and under an argon atmosphere according to the NMR spectroscopy data.

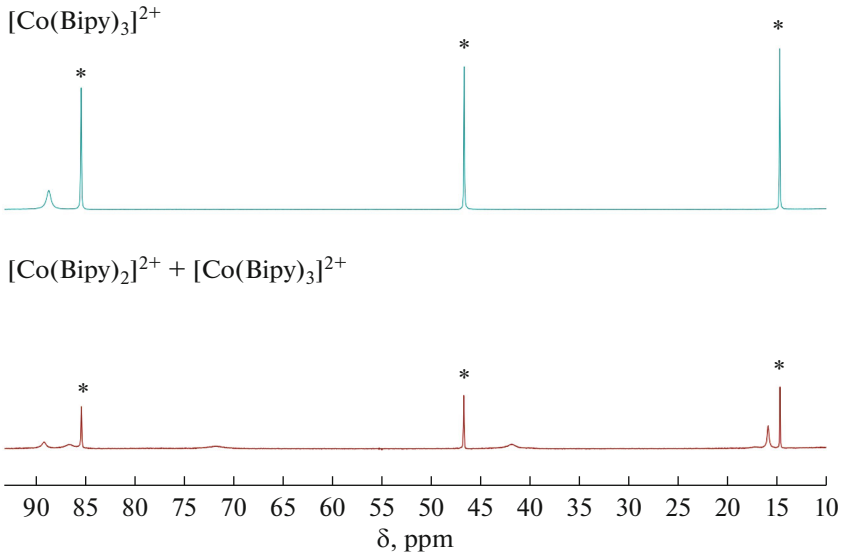

Fig. 7. Comparison of the paramagnetic ranges of the NMR spectra of the reduction products of complex **I** before (bottom spectrum) and after (upper spectrum) concentrating the reaction mixture.

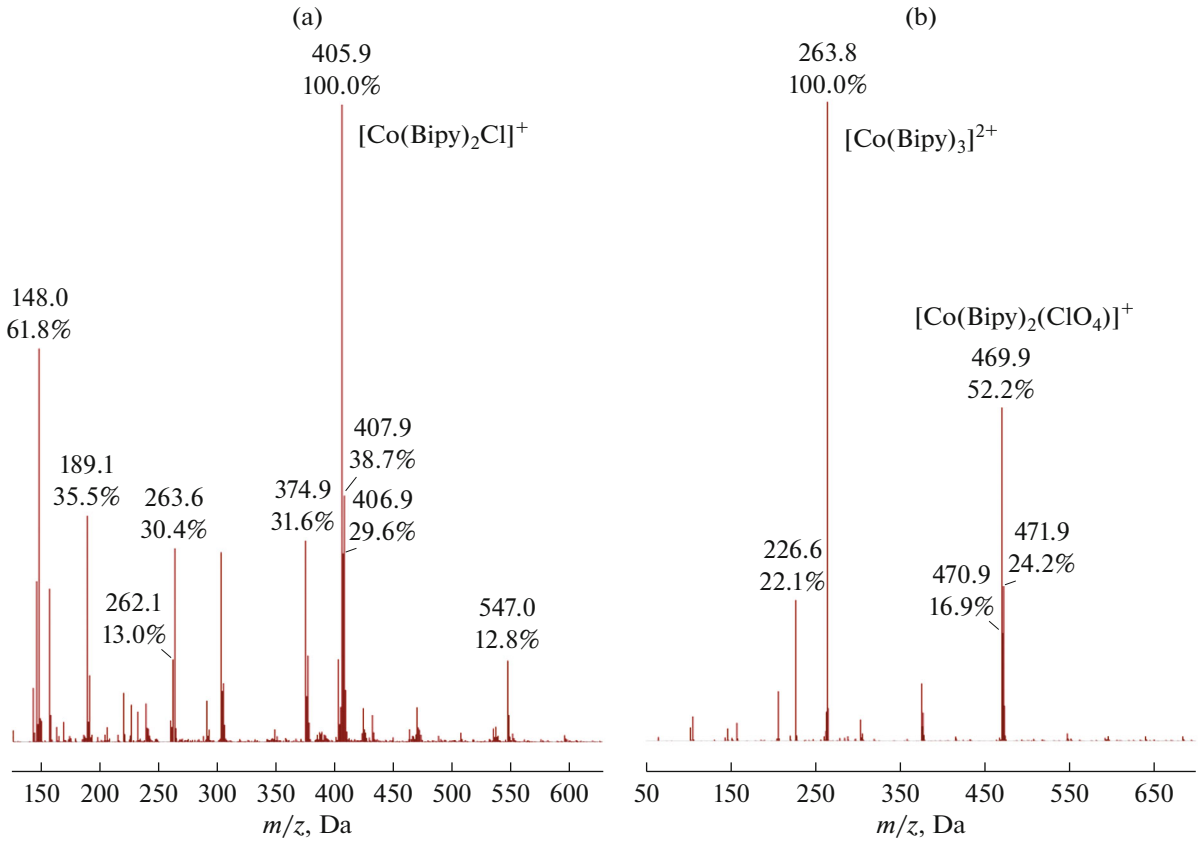

Fig. 8. Comparison of the mass spectra of the reduction products of complex **I** (a) before and (b) after concentrating the reaction mixture.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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