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ORIGINAL ARTICLE

Nitrite contamination in hypotensive preparations of dinitrosyl iron complexes with glutathione

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Summary
The content of nitrite admixture in preparations of dinitrosyl iron complexes (DNIC) with glutathione synthesized by treatment of aqueous solutions of Fe^{2+} + glutathione with gaseous NO (complex 1) or by mixing solutions of S-nitrosoglutathione (GS-NO) with solutions of Fe^{2+} + glutathione (complex 2) was determined using the Griess method and HPLC as well as from the level of HNO_2 formed upon interaction of gaseous NO with acidified distilled water. In both preparations, DNIC were predominantly represented by the binuclear form (B-DNIC). In complex 1, the appearance of nitrite in DNIC solutions was induced by nitrogen dioxide present in gaseous NO; its interaction with NO gives an adduct, which is further hydrolyzed to nitrite in aqueous solutions. In complex 2, the presence of nitrite admixture could appear in the presence of nitrite non-incorporated into GS-NO synthesized by mixing glutathione and nitrite in acid media. The per cent content of nitrite (with respect to the total content of complex 1) was 6%, whereas in complex 2 it was as low as 0.4%. Such a low level of nitrite contamination in the course of conventional synthesis of DNIC with glutathione does not make any significant contribution to their biomedical (e.g., hypotensive or vasodilator) activity.

Key words: dinitrosyl iron complexes; nitrite; high-performance liquid chromatography; Griess method

Abbreviations:
B-DNIC, binuclear dinitrosyl iron complex;
M-DNIC, mononuclear dinitrosyl iron complex;
EPR, electron paramagnetic resonance;
GS-NO, S-nitrosoglutathione.

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INTRODUCTION

A search for novel nitrogen monoxide (NO) donors endowed with an ability to mimic miscellaneous biomedical effects of this compound in animal and human organisms is currently under way. It was established that the role of NO donors can be played by dinitrosyl iron complexes (DNIC) with thiol-containing ligands as well as nitrite ions. Obviously, the synthesis of DNIC mediated by gaseous NO or S-nitrosothiols is accompanied by contamination of DNIC preparations with nitrite. A question arises as to what extent the biomedical activities of DNIC with

thiol-containing ligands determined thus far, viz., potent vasodilator and hypotensive effects (Lakomkin et al. 2007, Vanin et al. 2007, Mokh et al. 2010, Vanin and Chazov 2011, Chazov et al. 2012), effective blockade of platelet aggregation (Mordvintcev et al. 1986, Arkhipova et al. 2008), increased red blood cell elasticity (Shamova et al. 2011), acceleration of skin wound healing (Shekhter et al. 2007), etc. (Timoshin et al. 2010, Andreev-Andriyevsky et al. 2011, Remizova et al. 2011), are related to the presence of a nitrite admixture able to release significant amounts of NO under hypoxic conditions (Gladwin et al. 2005, van Faassen et al. 2009, Pluta et al. 2011).

In the course of a NO-dependent synthesis of DNIC with thiol-containing ligands, nitrite can appear in DNIC preparations due to the presence, in gaseous NO, of a nitrogen dioxide (NO_2) admixture. Correspondingly (Cotton and Wilkinson 1966), if the NO_2 admixture is present in gaseous NO in negligibly small concentrations, nitrite is formed as a result of NO_2 binding to NO and consequent N_2O_3 hydrolysis:

$$H_2O + N_2O_3 \Leftrightarrow 2H^+ + 2NO_2^- \qquad (1)$$

During the synthesis of DNIC with thiol-containing ligands from S-nitrosothiols, part of nitrite can remain in a S-nitrosothiol solution due to chemical equilibrium established during the synthesis of S-nitrosothiol in an acid medium, which favours nitrite conversion into nitrous acid able to S-nitrosate thiols (Beloso and Williams 1997):

$$HNO_2 + RSH \Leftrightarrow RSNO + H_2O \qquad (2)$$

With a further increase in pH to neutral values, part of HNO_2 remaining in the solution undergoes deprotonation, thus stimulating the appearance of contaminant nitrite in DNIC preparations.

Recently, one of the representatives of thiol-containing ligands, viz., DNIC with glutathione, was used in a industrial-scale synthesis of a novel pharmaceutical product endowed with potent hypotensive activity. The novel preparation (commercial name Oxacom®) has undergone pharmacological testing and clinical trials on healthy volunteers. Its single intravenous dose (5 mg/kg or 0.2 μmoles of DNIC/kg, respectively) caused 20–25% lowering of both systolic and diastolic arterial pressure over a period of 6–8 hours (Vanin and Chazov 2011, Chazov et al. 2012).

The aim of the present study was to determine the nitrite admixture in preparations of DNIC with glutathione synthesized by conventional methods as described above.

MATERIALS AND METHODS

Materials

Ferrosulfate (Fluka, Buchs, Switzerland), reduced glutathione and sodium citrate (Sigma, St. Louis, USA) were used. Gaseous NO was obtained by a reaction of ferrosulfate with sodium nitrite in 0.1 M HCl with subsequent purification of gaseous NO by low-temperature sublimation in an evacuated glass system.

Synthesis of DNIC with glutathione

DNIC with glutathione were synthesized using two approaches, viz., the reaction of bivalent iron and glutathione with gaseous NO and the reaction of bivalent iron and glutathione with S-nitrosoglutathione (GS-NO). In the former case, DNIC was synthesized by treatment of ferrous sulfate and glutathione solutions with gaseous NO at Fe^{2+}:glutathione molar ratios of 1:4 and at NO pressure of 100–150 mm Hg as described in (Vanin et al. 2010). The synthesis was performed in a Thunberg apparatus filled with 100 ml of the gaseous phase and 4.5 ml of the glutathione solution. After dissolution of the ferrous sulfate solution (0.5 ml) (pH 5.5) in distilled water and loading of the glutathione solution (4.5 ml) in 10 mM HEPES pH 7.4 into upper and bottom chambers of the Thunberg apparatus, the tube was evacuated and NO was injected. Both solutions were mixed in the presence of NO and shaken for 5 min after which NO was evacuated from the tube. The reaction of NO with Fe^{2+} in distilled water gave mononitrosyl iron complexes (MNIC) with media ligands (Begel et al. 2011); DNIC with thiol-containing ligands were prepared by a reaction of MNIC with thiols and gaseous NO.

In the second approach, DNIC with glutathione (Oxacom) were obtained using GS-NO as a NO donor with subsequent lyophilization of DNIC solutions in the presence of dextran (M_r = 40 kDa). GS-NO was prepared using the following protocol. At first, 8.3 g of glutathione was dissolved in 96 ml of distilled water. In view of strongly acidic properties of glutathione, the pH of the test solution was brought to 2.5. The addition of 0.51 g of $NaNO_2$ in 5 ml of distilled water to the test solution (glutathione) gave GS-NO. GS-NO concentration (73 mM) was determined from the intensity of the optical absorption band at 340 nm and correlated to the initial concentration of nitrite within the limits of the experimental error (±1 mM), suggesting the involvement of the bulk of nitrite in GS-NO synthesis and total lack of free nitrite in the test solution. Thus, at the glutathione:sodium nitrite ratio of 270:73 mM (3.7:1) in media solutions of GS-NO and, as a consequence, of DNIC with glutathione

formed in its presence, the nitrite admixture was negligibly small, which was corroborated by experimental data.

Subsequent synthesis of DNIC with glutathione was performed in the following way. The GS-NO solution (101 ml) was mixed with 365 mL of an media solution of 26 mM citrate-phosphate buffer (pH 7.4) supplemented with 12.5 mg of dextran (M_r = 40 kDa) [11]. The final pH was adjusted by adding a saturated solution of NaOH after which 34 ml of a ferrous sulfate (750 mg) + sodium citrate (3500 mg) solution in distilled water was added to the test solution (466 ml). Aliquots (3 ml) of the test solution (500 ml) were placed into 10-ml glass tubes and lyophilized at –45 °C (residual moisture ≤P5%). The total mass of the lyophilized samples (Oxacom) was 180 mg including 6.4 mg DNIC (3.5% by weight). After storage in a degassed medium, Oxacom preparations retained their physico-chemical, EPR and optical characteristics and physiological activity, i.e., an ability to induce long-lasting hypotension for at least 1 year. Prior to experiment, lyophilized Oxacom preparations were dissolved in distilled water.

The final concentration of DNIC with glutathione prepared by treatment of ferrous sulfate and glutathione solutions with gaseous NO at the Fe^{2+}:glutathione molar ratios of 1:4 (hereinafter referred to as complex 1) was determined by the optical method (see below) and was equal to 5 mM (as calculated per iron content in DNIC). The concentration of DNIC with glutathione in solutions of lyophilized DNIC with glutathione (hereinafter referred to as complex 2) synthesized in the presence of GS-NO (pharmacological name Oxacom®, Chazov et al. 2012) after dissolution of 180 g of complex 2 in 3 ml of distilled water was also equal to 5 mM (as calculated per iron content in DNIC).

Detection of nitrite admixture in DNIC solutions by the Griess method

Detection of nitrite admixture by the Griess method was performed in solutions of DNIC with glutathione after their dissolution in distilled water to a concentration of 0.5 mM (as calculated per iron content in DNIC) after which Griess reagents and acetic acid (100 μM) were added to 1 ml aliquots of the test solution. Nitrite content was determined from the intensity of the absorption band of the azo dye at 540 nm ($\varepsilon = 10^4$ M^{-1} cm^{-1}) using a calibration curve for nitrite concentrations in aqueous solutions established by the Griess method.

Determination of nitrite admixture in solutions of complex 1 from concentration of nitrous acid

To determine the content of nitrite, which can be formed in solutions of complex 1 by reaction (1), NO was fed into a degassed Thunberg apparatus containing 5 ml of distilled water (pH 1.0) at 150 torr. This reaction gives nitrous acid (HNO_2) whose absorption band had a quintet structure at 354 nm (Riordan et al. 2005). To determine its concentration and thereby nitrite content, the intensity of the optical absorption band of HNO_2 at 354 nm was compared to that of the absorption band of a 1 mM solution of sodium nitrite in 5 ml of distilled water (pH 1.0) also fed into the Thunberg apparatus. The HNO_2 solutions were evacuated after additional degassing of the Thunberg apparatus and their absorption spectra were measured in air.

HPLC detection of nitrite admixture in DNIC preparations

HPLC detection of nitrite and other components of the reaction mixture included reversed phase chromatography (Jourd'heuil et al. 2003) on a 64-mm silica gel column C-18 (Ø 7 μM, length 5.5 cm) modified with an anionic detergent (cetyltrimethylammonium chloride, CTAC) initiating the appearance of positive charges on the HPLC column providing the binding of negatively charged groups of tested compounds to the column. The modification procedure included a 20–30-min passage of 1.0 mM H_2O-methanol solutions of CTAC through a HPLC column at a flow rate of 0.5 ml/min until a chemical equilibrium estimated from the baseline on the printer of a UV detector was established at 210 nm. After modification, the column was washed with a mobile phase (5 mM phosphate buffer pH 5.8 + 0.1 M sodium chloride) for 20 min at a flow rate of 0.1 ml/min. Prior to HPLC, the temperature of the mobile phase (4 °C) was increased to 20 °C. The tested sample (50 μM B-DNIC) was dissolved in a mobile phase (5 mM phosphate buffer, pH 5.8) and passed through a membrane filter with a pore diameter of 0.45 μM with subsequent evacuation of the resulting solution with a forevacuum pump. The filtration procedure enabled complete evacuation of dextran from the DNIC-glutathione solution prepared by using GS-NO (complex 2). HPLC was performed on a Millichrome-5 chromatograph (Medicant, Russia) at a flow rate of 100 μl/min (sample volume – 25 μl) and optical detection at 210 nm.

EPR and optical measurements of DNIC preparations

EPR spectra of DNIC preparations were measured (77K) in a quartz Dewar vessel filled with liquid nitrogen using a modified Radiopan EPR spectrophotometer (Poland) (microwave power – 5 mW; modulation amplitude – 0.2 mT). Concentrations of DNIC and other paramagnetic centers were determined by using double integration of EPR signals. A solution of M-DNIC with glutathione of known concentration synthesized at the Fe^{2+}:glutathione molar ratio 1:100 in 10 mM HEPES (pH 11.0) was used as a reference sample.

Optical measurements were performed in open cuvettes of a UV-2501PC spectrophotometer (Shimadzu Europa GmbH, Germany) with an optical path of 10 mm at ambient temperature.

Statistical evaluations

The data are presented as experimental original recordings or as means ± SEM.

RESULTS

Characterization of DNIC

The main distinguishing features of DNIC with glutathione, which reflect their intactness and concentration, are optical absorption spectra and the electron paramagnetic resonance (EPR) signal at $g_{\perp} = 2.04$, $g_{||} = 2.014$, $g_{aver.} = 2.03$ (Vanin et al. 2010). In this study, all absorption spectra of aqueous solutions of DNIC with glutathione (complexes 1 and 2) displayed intense adsorption bands at 310 and 359 nm, a shoulder at 430 nm and a weak adsorption band at 768 nm (Fig. 1, top, left panel) characteristic of diamagnetic binuclear DNIC – B-DNIC (formula $\{(RS)_2Fe_2(NO)_4\}$) (Vanin et al. 2010). All DNIC solutions had a distinctly orange color. Considering that the extinction coefficients (ε) for the 310 and 359 nm bands (as calculated per one iron atom) of B-DNIC are equal to 4600 and 3700 M^{-1} cm^{-1}, respectively (Vanin et al. 2010) (judging from band intensities (Fig. 1), more than 90% of iron present in the preparations was incorporated into the complexes. This value is consistent with the concentration of the paramagnetic mononuclear DNIC (formula $\{(RS)_2Fe(NO)_2\}$, viz., M-DNIC with an EPR signal at $g_{\perp} = 2.04$, $g_{||} = 2.014$, $g_{aver.} = 2.03$ (Vanin et al. 2010). The EPR signal at $g_{aver.} = 2.03$ was also characteristic of both preparations of DNIC with glutathione tested in this study (complexes 1 and 2) (Fig. 1, bottom, left panel). For both complexes, the intensity of this EPR signal corresponded to the incorporation of 5–7% of the total pool of DNIC iron into M-DNIC with glutathione. These data suggest that in all preparations of DNIC with glutathione tested in this study the latter were predominantly represented by diamagnetic B-DNIC (~2.5 mM) irrespective of their glutathione content. Correspondingly, the ratios between the B-DNIC and free glutathione amounts for complexes 1 and 2 should be equal to 1:6 and 1:16, respectively.

To our knowledge, the ratio between M- and B-DNIC with glutathione in aqueous solutions at neutral ("physiological") values of pH has not yet been established. Our own data demonstrate that at neutral pH DNIC with glutathione are virtually completely represented by B-DNIC.

A rise in pH of B-DNIC with glutathione from 7.4 to 10.5–11.0 caused a drastic change in their optical absorption spectra manifested in the appearance of one band (390 nm) characteristic of M-DNIC instead of the 310 and 359 nm bands (Fig. 1, top, right panel) (Vanin et al. 2010). Under these conditions, the intensity of the EPR signal ($g_{aver.} = 2.03$) characteristic of this particular form of DNIC increased 15–20-fold as a result of which the whole bulk of DNIC iron was incorporated into M-DNIC (Fig. 1, bottom, right panel). Additional evidence in favour of such a transformation of DNIC was derived from calculations of M-DNIC concentration from the intensity of the absorption bands at 390 nm ($\varepsilon = 4600$ M^{-1} cm^{-1}) (90–95% of B-DNIC content as calculated per one iron atom). With a further decrease of pH to 7.4 accompanied by reverse transformation, viz., conversion of M-DNIC into B-DNIC, the EPR signal at $g_{aver.} = 2.03$ decreased 15–20-fold.

The conversion of B-DNIC into M-DNIC was induced by a drastic increase in the concentration of glutathione molecules ionized at thiols. Their interaction with B-DNIC (formula $\{(RS)_2Fe_2(NO)_4\}$) was accompanied by the rupture of the bridge glutathione ligand and synthesis of M-DNIC (formula $\{(RS)_2Fe(NO)_2\}$). At pH 7.4, the low content of ionized glutathione molecules could initiate the formation of only B-DNIC.

Detection of nitrite admixture in DNIC preparations by the Griess method

The content of nitrite in preparations of complexes 1 and 2 detected by the Griess method was equal to 15±2 and 1±0.2 μM, respectively (data from five independent measurements); for comparison, that of both complexes calculated per one iron atom was 0.5 mM. Expressed on a per cent basis, they were equal to 3 and 0.2% nitrite per 0.5 mM DNIC. Considering that in solutions M- and B-DNIC are predominantly represented by the binuclear form

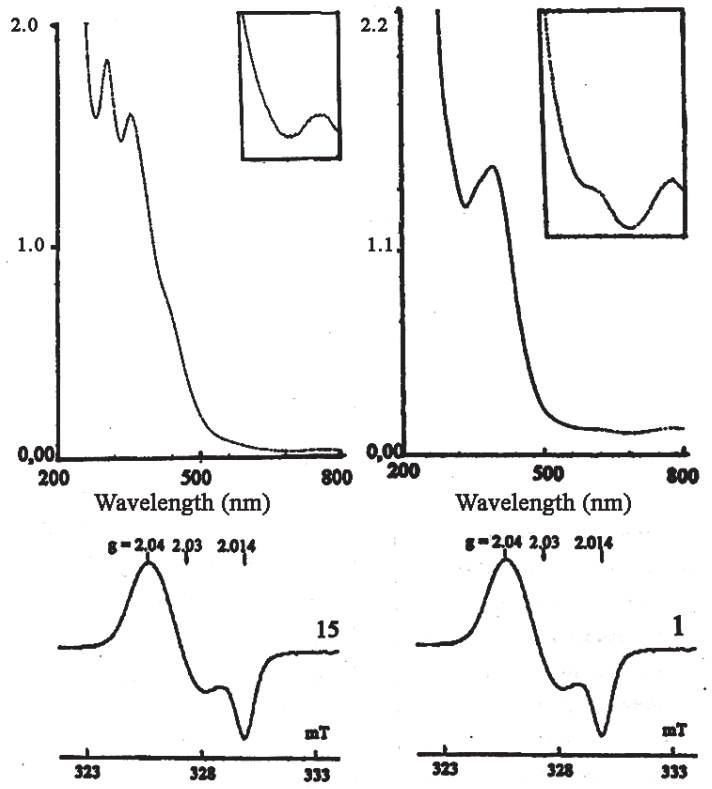

Fig. 1. **Top: absorption spectra of a 0.4 mM solution of DNIC with glutathione recorded at pH 7.4** (left panel – predominance of the B-DNIC spectrum) **and pH 10.6** (right panel – predominance of the M-DNIC spectrum). Framed are the absorption bands recorded in the range from 600 to 800 nm at higher amplification of the spectrophotometer. **Bottom:** EPR signals ($g_{aver.}$ = 2.03 recorded in solutions of DNIC with glutathione at pH 7.4 (left) and 10.6 (right). The relative amplifications of the radiospectrometer are shown at right side.

(B-DNIC), the average values of this ratio obtained 1–2 min after addition of Griess reagents to DNIC solutions and recalculated per 0.25 mM B-DNIC were 6 and 0.4%. During the next 10–15 min, the content of nitrite determined by the Griess method increased 1.5–2-fold. In the absence of such an increase with time (from 1–2 to 10–15 min) during determination of nitrite content in aqueous solutions containing only nitrite, we assumed that the time-dependent increase in nitrite content in DNIC solutions might be due to decomposition of DNIC in acid media in the presence of Griess reagents and subsequent oxidation of NO released from the complexes to NO_2. This finding led us to assume that the 6 and 0.4% content of nitrite admixture in complexes 1 and 2 and determined by the Griess method during the first few minutes of the reaction represent genuine values. This speculation is consistent with the results of a determination of nitrite admixture by alternative methods (see below).

Determination of nitrite admixture in complex 1 from the content of nitrous acid in acid aqueous solutions after their interaction with gaseous NO

Fig. 2 shows the optical absorption spectra of nitrous acid recorded in 5 ml of distilled water (pH 1.0) in the degassed Thunberg apparatus after loading gaseous NO at 150 torr or dissolution of 1 mM sodium nitrite in distilled water. In both cases, we observed a similar absorption band at 354 nm whose quintet structure was characteristic of HNO_2 (Riordan et al. 2005). A comparison of the intensities of these bands allowed us to determine the concentration of HNO_2, formed during its interaction with gaseous NO in acid media by reaction (1). This value was equal to 150±15 μM, i.e., was equimolar to the concentration of nitrite formed from nitrous acid with a rise in pH to neutral values (data from 5 independent measurements).

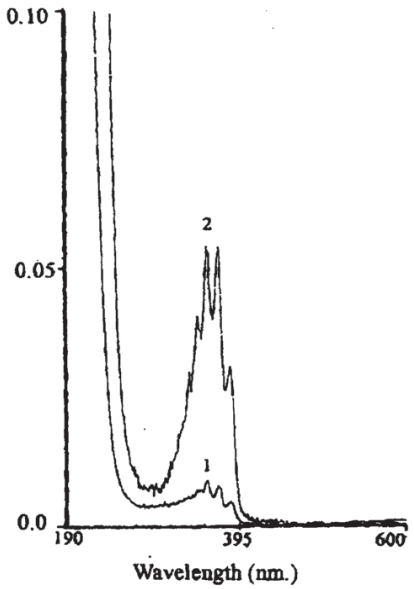

Fig. 2. **The absorption spectrum of HNO_2 appearing in distilled water at pH 1.0** after loading of gaseous NO into the degassed Thunberg apparatus at 150 torr (spectrum 1). Spectrum 2 was induced by absorption of 1 mM solution of $NaNO_2$ in distilled water at pH 1.

As the synthesis of complex 1 was carried out in the presence of gaseous NO at 150 torr (similar to the measurements described previously in this chapter), it may be conjectured that in solutions of complex 1 synthesized in this way, the concentration of contaminant nitrite formed due to the presence of NO_2 admixture in gaseous NO was 150 μM. Calculated on a per cent basis for the 2.5 mM concentration of the binuclear form of complex 1, this value corresponds to 6±0.6% nitrite. The coincidence of this value to that for complex 1 synthesized by the Griess method after mixing DNIC solutions with Griess reagents corroborated our hypothesis on the adequacy of the method employed and the minimally short (1–2 min) assay time.

The content of NO_2 admixture in gaseous NO could be estimated from the concentration of HNO_2 formed upon interaction of the mixture of NO_2 and NO with distilled water at pH 1.0 by reaction (1). In accordance with Avogadro's law, the mass of NO in 100 ml of the Thunberg apparatus at 150 torr is equal to 880 μmoles. As regards the NO_2 admixture fully dissolved in 5 ml of H_2O, its concentration must be equal to $1/2[HNO_2] = 75 \times 10^{-3}$ μmoles/ml × 5 ml = 0.75 μmoles, i.e., 0.08% of the NO content.

Detection of nitrite admixture in DNIC solutions by the HPLC method

Fig. 3 shows HPLC chromatograms of complexes 2 and 1 solutions (a and b, respectively) prepared by 50-fold dilution of the starting solutions (2.5 mM B-DNIC) in 5 mM phosphate buffer pH 5.8 used as a mobile phase component. All chromatograms display two intense elution bands with retention times of 130±5 s and 195±5 s. In addition to two intense elution bands, HPLC chromatograms of the solutions of complex 1 display the presence of a weak band, which appears on HPLC chromatograms as a shoulder or a peak with a retention time of 172±5 s superimposed onto an intense band with a retention time of 195 s.

In order to determine the hypothetical contribution of B- or M-DNIC with glutathione to the aforesaid elution bands, we carried out a series of experiments to demonstrate that none of these DNIC was responsible for the elution bands appearing on the chromatograms. The HPLC procedure was very similar to that used in the synthesis of complex 1, viz., mixing of glutathione and ferrous iron solutions used at the same final concentrations as in the aforesaid synthesis in a deaerated Thunberg apparatus in the absence of NO. The resulting chromatogram resembled that of the solution of complex 1 except that the weak elution band with a retention time of 172 s was absent (Fig. 3c).

It might be expected that the appearance of elution bands characteristic of complexes 1 and 2 solutions is related to the reagents used for their synthesis (reduced glutathione), the side products formed thereupon (oxidized glutathione, nitrite, nitrate or S-nitrosoglutathione) or the compounds formed in oxygen-containing solutions in the course of HLPC (nitrite, nitrate or oxidized glutathione). Indeed, HPLC measurements of 1 mM solutions of reduced and oxidized glutathione, 0.1 mM solutions of nitrite and nitrate or 1 mM solutions of S-nitrosoglutathione established that all these compounds bound to the column with retention times of 130 s and 195 s, reduced and oxidized glutathione), 172 s and 228 s (nitrite and nitrate) and 310 s (S-nitrosoglutathione). The elution profiles of reduced and oxidized glutathione and nitrite are shown in Fig. 3e–g. As can be seen, their positions on the chromatograms correlate to the positions of elution bands of complexes 1 and 2 solutions. Two bands with retention times of 130 and 195 s (Fig. 3a, b) corresponded to reduced and oxidized glutathione, while the weak band (172 s) on HPLC chromatograms of complex 1 solution (Fig. 3b) might be associated with the presence of contaminant nitrite in these DNIC preparations. These findings suggest a good correlation with the

results of HPLC of the complex 2 (50 μM) + nitrite (100 μM) mixture (Fig. 3h). As can be seen, the elution band of the latter is localized between the elution bands of reduced and oxidized glutathione. A comparison of intensities of the elution bands of the mixture components determined from their areas established that mixing did not influence either the intensity of their elution bands or their concentration. The same, viz., the presence in the chromatogram of a weak band with an elution time of 172 s, is true of complex 1 and is suggestive of the presence of contaminant nitrite the concentration of which is of the order of 2–3 μM per 50 μM of DNIC. These values were obtained by comparing the intensity of this band to that of the preparation obtained by mixing glutathione and ferrous iron solutions used at the same final concentrations as in the synthesis of complex 1 in a deaerated Thunberg apparatus in the absence of NO (Fig. 3c) with the subsequent addition of 10 μM nitrite (Fig. 3d).

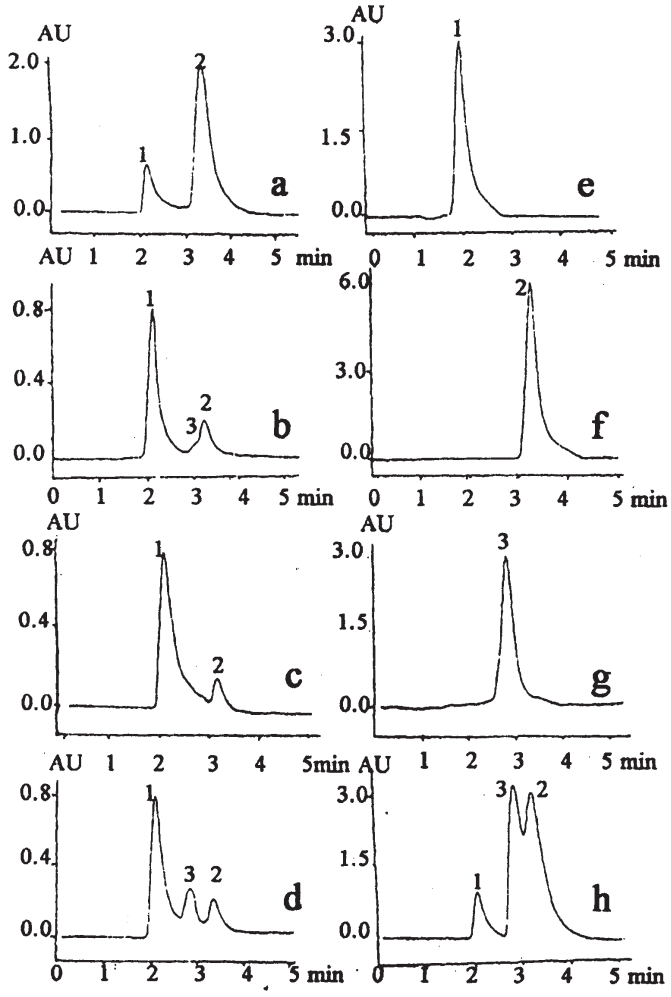

Fig. 3. **HPLC chromatograms of 50 μM solutions of complexes 2 *(a)* and 1 *(b)*; *(c)*** a chromatogram of a solution prepared according to a protocol similar to that used in the synthesis of complex 1, but in the absence of NO; *(d)* the preparation c with subsequent addition of 10 μM nitrite; *(e)* HPLC chromatograms of 1 mM solution of reduced glutathione; *(f)* HPLC chromatograms of 1 mM solutions of oxidized glutathione; *(g)* a HPLC chromatogram of a 0.1 mM solution of nitrite; *(h)* a HPLC chromatogram of 50 μM solutions of complex 2 and its mixtures with 0.1 mM nitrite. In all Figures, the elution bands designated as 1, 2 or 3 correspond to elution bands of reduced and oxidized glutathione and nitrite, respectively.

The use of HPLC allowed us to detect admixture nitrite in solutions of complex 1 at a concentration not exceeding 4–6%, being of the same order of magnitude as that recorded by the Griess method and during determination of HNO_2 formed upon interaction of gaseous NO with acidified distilled water, i.e., 6% in both cases. As regards the detection of nitrite admixture in complex 2, the sensitivity of the method (HPLC) was insufficient for its detection. This finding is consistent with the significant differences between nitrite content in solutions of complexes 1 and 2 determined by the Griess method, viz., 6% and 0.4%, respectively.

Detection of reduced and oxidized glutathione in 50 μM solutions of complexes 1 and 2 inspired us to compare their elution bands to elution bands of 1 mM solutions of reduced and oxidized glutathione (Fig. 3e, f) and to establish their content. Our HPLC measurements (Fig. 3a, b) established the presence, in solutions of complexes 1 and 2, of oxidized glutathione at concentrations of the order of 40±10 μM and 300±40 μM, respectively. Because the oxidation of two molecules of reduced glutathione yields one molecule of oxidized glutathione, 80 and 600 μM reduced glutathione underwent oxidation in the solutions of complexes 1 and 2, respectively, at 50-fold dilution of the starting solutions. In accordance with the ratios between the B-DNIC and free glutathione amounts mentioned above (1:6 or 1:16 for complex 1 or 2, respectively) the initial concentrations of free glutathione should be equal to 300 and 800 μM in 50 μM solutions of complex 1 and 2, respectively. A question arises as to what stage of DNIC synthesis or HPLC measurements such significant (~25% of reduced glutathione for complex 1 and ~75% for complex 2 solution) oxidation of glutathione takes place. Most probably, such oxidation takes place during HPLC of oxygen-containing solutions. The presence in DNIC of iron ions, released from the former in small quantities and catalyzing glutathione oxidation by oxygen, is a determining factor. In the absence of DNIC, e.g., during HPLC of reduced glutathione, the elution band of oxidized glutathione was not detected (Fig. 3e), but appeared after addition of ferrous iron to reduced glutathione in the absence of NO treatment and HLPC (Fig. 3c). Glutathione oxidation in the course of synthesis of complex 1 was hardly probable, since such syntheses are run under anaerobic conditions in the presence of gaseous NO. Such oxidation might take place during the synthesis of complex 2, e.g., upon mixing of GS-NO and ferrous iron + glutathione solutions in air. The results of our specially designed experiments are consistent with this hypothesis. After mixing GS-NO and ferrous iron + glutathione solutions in the absence of oxygen, the concentration of oxidized glutathione in the solution of complex 2 diminished to 30% of the initial concentration of reduced glutathione, i.e., to the level characteristic of complex 1. Taking into account additional oxidation of glutathione in the course of HPLC, both processes might be responsible for ~75% oxidation of glutathione in complex 2.

The lack of elution bands of DNIC may seem peculiar at first sight, since the presence in these DNIC of ligands (e.g., glutathione) able to be retained on the HPLC column creates a prerequisite to their binding to the column. At the same time, the lack of binding is suggestive either of complete decomposition of DNIC in the course of HPLC with a concomitant release of free iron ions, NO and glutathione, or the conversion of DNIC with glutathione into DNIC with ligands, which favours the robust binding of newly synthesized DNIC to the HPLC column. Under these conditions, the mobile phase cannot initiate DNIC elution from the column.

According to our data (Vanin et al. 1998). DNICs with two bromine ions were generated in response to incorporation of serum albumin-bound DNIC into reversed micelles formed from cetyltrimethylammoniumbromide (CTAB). The symmetric EPR signal peaking at g = 2.04 and possessing a 7-component hyperfine structure (HFS) elicited by DNIC containing two bromine ions replaced the anisotropic signal ($g_{aver.}$ = 2.03) of protein-bound DNIC at ambient temperature. The appearance of the 7-component HFS was induced by the interaction of the unpaired electron with two bromine ions (nuclear spin of bromine atom I = 3/2), while the formation of DNIC with bromine ions $[(Br)_2Fe(NO)_2]$ might be initiated by the transfer of $Fe(NO)_2$ groups from the protein ligands to Br^- ions. However, these DNIC are poorly soluble in water, since in this medium $[(Br)_2Fe(NO)_2]$ DNIC cannot spontaneously generate an signal EPR with high resolution of HFS. It was hypothesized (Vanin et al. 1998) that DNIC were incorporated into the micellar phase formed by cetyltrimethylammonium molecules because of the intactness of the bond between these molecules and bromine ions. The high solubility of detergent-bound $[(Br)_2Fe(NO)_2]$ complexes in this medium could be responsible for their high mobility and, as a consequence, high resolution of HFS from nuclear bromine in the EPR signal.

These findings provide sufficient grounds for the conclusion that in the course of HPLC of DNIC with glutathione chlorine ions from CTAC to DNIC $Fe(NO)_2$ groups could replace glutathione molecules and initiating the formation of DNIC with chlorine ions. The DNIC formed thereupon are weakly soluble in water (Burbaev 1971, Martini and Tiezzi 1971) and

are localized in the membrane phase formed by CTAC molecules retained on the HPLC column eventually resulting in their complete binding to the column and thereby the lack of elution bands of DNIC with glutathione on the chromatograms.

Indeed, the addition of 200 μM CTAC to a 50 μM solution of complex 1 led to significant distortion of the shape of the EPR signal of complex 1, most probably due to the substitution of glutathione in DNIC for chlorine ions from CTAC (Fig. 4a, b). A comparison of EPR signals of hypothetical DNIC with glutathione + CTAC (Fig. 4b) and DNIC with CTAC obtained by treatment of 200 μM solutions of CTAC + 50 μM Fe^{2+} in dimethylsulfoxide with gaseous NO (Fig. 4c) having identical shapes and manifesting similar positions of their EPR signals in the magnetic field, established that the EPR signal depicted in Fig. 4b was indeed related to DNIC with CTAC.

A comparison of double integrals of EPR signals of complex 1 (Fig. 4a) and complex 1+ CTAC (Fig. 4b) showed that the concentration of paramagnetic centres formed in complex 1 solution after the addition of CTAC (DNIC with CTAC) exceeded 4-fold the concentration of M-DNIC with glutathione present in complex 1 solution. This discrepancy can be attributed to precipitation of the bulk of poorly soluble DNIC with CTAC from the test solution.

The totality of experimental data led us to conclude that the lack of elution bands during HPLC of DNIC is a result of the transformation of DNIC with glutathione into DNIC with CTAC and tight binding of the latter to the chromatographic column.

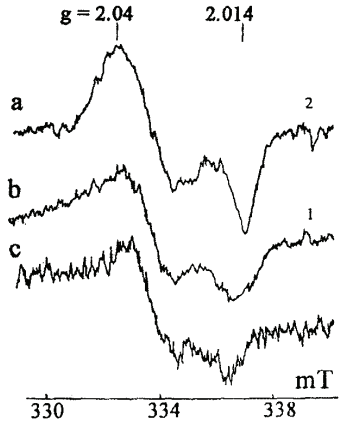

Fig. 4. **The EPR signals of M-DNIC of a 50 μM solution of complex 1** *(a)*; of the same preparation treated with CTAC (200 μM) *(b)*; of DNIC with CTAC synthesized by treatment of the 200 μM CTAC + 50 μM Fe^{2+} mixture with gaseous NO *(c)*. The signals were recorded at 77K. Right side *(a, b)*: amplification of the spectrometer (in arbitrary units).

DISCUSSION

Our experiments showed that both preparations of DNIC synthesized with the use of gaseous NO or GS-NO contain contaminant nitrite at concentrations of the order of 6% (complex 1) and 0.4% (complex 2) with respect to B-DNIC concentration. The relatively high nitrite content in complex 1 can be attributed to the presence of contaminant NO_2 in gaseous NO (0.08% of total NO content). As a matter of fact, contaminant NO_2 can appear in NO as a result of random ingress of air to the Thunberg apparatus and, as a consequence, oxidation of NO to NO_2. However, our studies including multiple treatment of gaseous NO with distilled water at pH 1.0 established that the concentration of nitrous acid formed thereupon was directly proportional to the number of treatments. It follows from these data that the NO_2 admixture is present in gaseous NO itself.

A natural question arises as to whether the amount of contaminant nitrite present in DNIC preparations is sufficient to make a significant contribution to biomedical activity of DNIC. The results of most recent comparative studies into hypotensive and vasodilator activities of DNIC with thiol-containing ligands and nitrite provide strong evidence against this hypothesis. Clinical trials of complex 2 (Oxacom) on healthy volunteers established that Oxacom (0.2 μmoles B-DNIC/kg) effectively (by 20–25% or by 25–30 mm Hg) decreased systolic and diastolic pressure for a period of 6–8 h (Chazov et al. 2012). In rats, a similar hypotensive effect was observed at 5 times higher doses of complex 2 (as calculated per kg of weight); its duration after a single intravenous injection did not exceed 2 h (Lakomkin et al. 2007, Chazov et al. 2012). As regards the hypotensive effect of nitrite on healthy volunteers, its dose dependence was much less pronounced in comparison with DNIC. According to literature data (Pluta et al. 2011), a decrease of arterial pressure by 15 mm Hg was observed at nitrite concentration of about 60 μmoles/kg, which is 300 times higher than the concentration of DNIC in our studies. Noteworthy, the hypotensive effect was recorded in only 2 out of 8 patients and lasted 10 min. In our animal studies, treatment of rats with Oxacom (0.8 μmoles/kg) induced a notable (by 50–60 mm Hg) drop of arterial pressure, while the 100 μmoles/kg nitrite dose caused only a small (10–15 mm Hg) decrease of this parameter in the same animals (Chazov et al. 2012). Taking nitrite content in Oxacom preparations equal to 0.4% of DNIC content as in this study, it becomes clear that this dose is too small and can hardly make any significant contribution to the hypotensive activity of complex 2 (Oxacom). The same concerns the vasorelaxing effect of Oxacom on isolated blood vessels. Previous studies established (Vanin et al. 2007, Mokh et al. 2010) that DNIC with

glutathione (1 μM as calculated per one iron atom in DNIC) induce 70% dilatation of blood vessels, while the vasorelaxing effect of 30 μM nitrite does not exceed 20%. Obviously, nitrite whose content in the Oxacom preparation does not exceed 0.4% of the total DNIC content can in no way be responsible for the vasodilator effect of the latter. As regards complex 1 the content of contaminant nitrite in which may amount to 5% of the total DNIC content, the role of the nitrite admixture in hypotensive and vasodilator activities of DNIC can hardly be significant either. Even if it is granted that treatment of experimental animals with DNIC or its addition to the incubation medium of isolated blood vessels initiates fast decomposition of DNIC with a concomitant release of 2 μM NO from 1 μM DNIC and their further conversion into nitrite, the amount of contaminant nitrite formed thereupon will hardly be enough to produce a vasodilator or hypotensive effect comparable to that of DNIC. It remains to be hoped that future studies into other biomedical activities of DNIC with thiol-containing ligands will provide supporting evidence for the most important result of this study, namely, the presence of nitrite admixture in DNIC preparations hardly has any effect on their physiological activity.

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