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Reactions of Nifurtimox with Critical Sulfhydryl-containing Biomolecules: their Potential Toxicological Relevance

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Key words: Chagas' disease; Nifurtimox; sulfhydryl compounds; nitrite anion; adverse effects; chemotherapeutic effects.

Nifurtimox (Nfx) is a drug used in the treatment of Chagas' disease, an endemic parasitic disease from Latin American countries. It produces undesirable side-effects in patients, frequently forcing the treatment to be stopped. Its toxic mechanism is not fully understood. In this work we describe purely chemical reactions of Nfx with relevant cellular sulfhydryl (SH) compounds. The compounds tested were glutathione (GSH), cysteine (RSH), lipoic acid (LA) and coenzyme A (CoA). All reacted with Nfx to give nitrite (NO_2^-). The relative reaction rates were CoA>LA>GSH>RSH. In studies with GSH and RSH the formation of nitrite was accompanied by decreases in Nfx concentration and increases in the formation of a reaction product revealed by HPLC. We failed to show the presence of liver cytosolic GST (GSH transferase activity)-mediated formation of NO_2^- from Nfx. These NO_2^--releasing processes occurred under *in vivo* conditions in Nfx-treated Sprague-Dawley male rats (240–260 g body weight) at a dose of 100 mg Nfx kg^{-1} p.o. In urine samples NO_2^- excretion was accompanied by unchanged drug and two unidentified more polar metabolites detectable by HPLC. The Nfx reactions with critical SH from molecules such as GSH, RSH, LA and CoA, and potentially others containing SH residues (e.g. enzymes or structural proteins), might have toxicological relevance not only for the Nfx side-effects but also for the chemotherapeutic effects on *Trypanosoma cruzi*. In addition, Nfx reactions with GSH might be crucial in Nfx detoxification. Copyright © 2004 John Wiley & Sons, Ltd.

INTRODUCTION

Nifurtimox (Nfx) is a drug used in the treatment of the acute phase of Chagas' disease (American trypanosomiasis), an endemic parasitic disease suffered by tropical and subtropical countries of Latin America (Pinto Días, 1984; Stoppani, 1999; Rodríguez Coura & de Castro, 2002). It is estimated that 16–18 million people are infected by *Trypanosoma cruzi* and that 100 million (i.e. 25% of the population of Latin America) are at risk of acquiring the infection (WHO, 2000). In addition, chagasic patients are migrating northwards to the USA and even eastwards to Europe: nowadays, around 100 000 infected individuals are living in the USA, most of them immigrated from Mexico and Central America (Pinto Días, 1992).

Acute infections in children (the frequent victims of the insect bites) can be fatal (2–8% mortality from the central nervous system effect of the disease) and consequently drug treatment is mandatory (Pinto Días, 1984). Notwithstanding, the two drugs available for treatment, Benznidazole and particularly Nifurtimox, have important toxic side-effects that frequently force treatment to stop, which is an important drawback to their use. The mechanisms of these side-effects were not fully understood, particularly in the case of Nfx (Docampo & Moreno, 1985; Castro & Toranzo, 1988; Castro, 2000). Most studies available on the toxicology of Nfx correlate the occurrence of Nfx-induced deleterious effects with the nitroreductive biotransformation of this nitroheterocyclic compound (Gorla & Castro, 1985; Bernacchi *et al.*, 1986; de Castro *et al.*, 1989, 1990; Carrizo *et al.*, 2000; Montalto de Mecca *et al.*, 2001, 2002). However, the hypothesis was advanced that peroxynitrite formation from Nfx resulting from the interaction of nitric oxide and superoxide generated during biotransformation of the Nfx might play a role in Nfx toxicity (Carrizo *et al.*, 2000; Montalto de Mecca *et al.*, 2002). In the present studies, the potential reactions of Nfx with relevant sulfhydryl (SH)-containing biomolecules is explored.

MATERIALS AND METHODS

Chemicals

Nifurtimox — 4-[(5-nitrofurfurylidene) amino]-3-methylthiomorpholine-1, 1-dioxide (Nfx) — was a gift from Bayer, Leverkusen, Germany (see Fig. 1 for chemical

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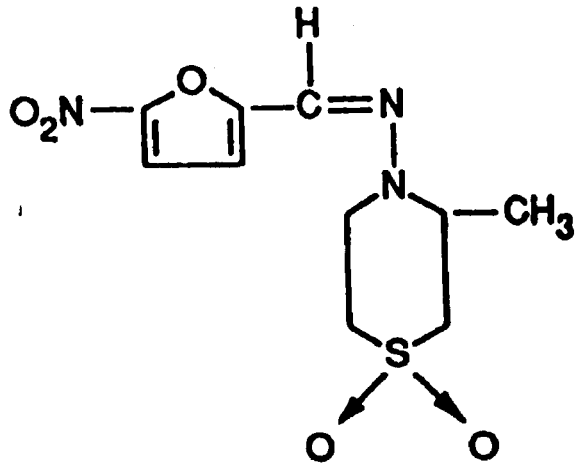

Figure 1. Chemical structure of Nifurtimox.

structure of the test chemical). Nitrofurazone (5-nitro-2-furaldehyde semicarbazone, Nfur) and sulfhydryl-containing compounds were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals employed were of analytical grade.

Animals

Sprague-Dawley male rats of 240–260 g body weight were bred in our laboratory. Rats were maintained in a controlled room on a 12-h lightdark cycle (light phase 07:00–19:00). The temperature in the animal room was 23 ± 2 °C and the relative humidity was between 35 and 65%. Animals fasted for 12–14 h, with free access to water. They were sacrificed by decapitation, bled and their livers were rapidly excised and processed. In the experiments using animals treated with Nfx, the drug was given p.o. as a homogeneous suspension in 1% carboxymethylcellulose (40 mg ml^{-1}) at a dose of 100 mg kg^{-1} body wt. The suspension was prepared in a Potter–Elvehjem homogenizer.

Cytosolic glutathione *S*-transferase (GST)

Livers were homogenized in a Teflon–glass Potter–Elvehjem homogenizer with 4 vols of 0.25 M sucrose and 0.01 M sodium phosphate buffer (pH 7.4). The homogenates were centrifuged at 100 000 *g* for 1 h and the cytosolic supernatant was recovered. The latter was dialyzed overnight against 2 l of 20 mM TRIS·HCL buffer (pH 7.4) containing 0.25 M sucrose and 5 mM ethylenediaminetetraacetic acid (EDTA). All procedures were performed at 0–4 °C (Masana *et al.*, 1984). The dialyzed supernatant was used to establish GST activity.

Enzymatic and chemical determinations

The Nfx GST activity was determined by measuring the release of the nitro group as inorganic nitrite (NO_2^-) according to Asaoka and Takahashi (1983). All incubations were run in 20-ml flasks with agitation at 150 oscillations min^{-1} in a water bath at 37 °C. The incubation mixtures (final volume 2.5 ml) contained the following: 20 mM potassium phosphate buffer (pH 7.4); 250 µl of 10 mM Nfx in *N,N*-dimethylformamide (DMF); 5 mM glutathione, reduced form (GSH); and the cytosolic supernatant (final concentration 7–8 mg protein ml^{-1}). Reactions proceeded for 1 h and placing the flasks on ice interrupted the processes. An assay mixture without enzyme was used as a control. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

The chemical reactions between Nfx and different sulfhydryl-containing biomolecules were also studied under an oxygen-free nitrogen atmosphere. These mixtures contained the following: 20 mM potassium phosphate buffer (pH 7.4); 250 µl of 10 mM nitrofurazone in *N,N*-dimethylformamide (DMF) or Nfx in methanol; and sulfhydryl-containing substances such as GSH, L-cysteine hydrochloride (RSH), DL-α-lipoic acid, reduced form (LA) and coenzyme A (CoASH) (all final concentrations 5 mM).

Incubation times were 15, 30, 45, 60 and 75 min (only RSH). The NO_2^- was determined as follows: an aliquot (1.5 ml) of the enzymatic or chemical mixture was added to 0.15 ml of acetic anhydride. Then it was left for 5 min at room temperature and mixed with 1.5 ml of 1% (w/v) sulfanilamide in 20% (w/v) HCl followed by the addition of 1.5 ml of 0.02% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride. After diazo-coupling for 20 min at room temperature, 0.15 ml of 1% (w/v) ammonium sulfamate (freshly prepared) was added. Appropriate blanks without Nfx were also performed. When the cytosolic supernatant was present in the incubation, the mixture was centrifuged and left for 5 min at room temperature before the absorbance was measured at 540 nm in a spectrophotometer. Readings were compared against a calibration curve of increasing concentration of $NaNO_2$.

Determination of Nfx concentration by HPLC

The reactions were followed by substrate disappearance. This was calculated by the difference in concentration of Nfx at the beginning and end of the reactions. The content of the drug at different times was determined as follows. An aliquot (250 µl) of the incubation mixture was added to 2 vols (500 µl) of CH_3OH. The incubation mixtures containing the cytosolic fraction were centrifuged at 15 000 *g* for 10 min. The supernatants were filtered through nylon filter membranes (pore size 0.45 µm) prior to HPLC analysis. Samples (1 µl) were analyzed at 40 °C using a Hewlett-Packard Model 1090 Series II liquid chromatograph with a Hewlett-Packard ODS Hypersil column (20 cm × 2.1 mm ID, 5 µm particle size) and diode array detector. The mobile phase was delivered at a constant flow rate of 0.2 ml min^{-1}. The column effluent was monitored at 400 nm, corresponding to a maximum absorbance of Nfx, when solvent consisted of 60% $CH_3OH{\cdot}H_2O$, or at 215 nm when the solvent was 25 mM KH_2PO_4 (pH 2.5). Quantitation of the nitrocompound was by peak-area ratio with reference to a calibration curve obtained using standards treated identically.

Urine metabolite determination of Nfx-treated rats

Rats were housed individually in metabolic cages after administration of 100 mg kg^{-1} of Nfx in 1% carboxymethylcellulose (CMC) p.o. Controls received only CMC p.o. Six animals per group were employed. For NO_2^- determination urine was collected over 24 h, but for Nfx it was collected over 3 and 6 h in an ice bath. The NO_2^- was determined as described above. After filtering through

nylon filter membranes, urine samples (5 µl) were monitored at 400 and 295 nm by HPLC using 60% $CH_3OH{\cdot}H_2O$ as the mobile phase in the same condition as indicated above. Plots of peak areas against concentration were linear over the concentration ranges studied (correlation factor = 0.998).

Statistics

The significance of the difference between two mean values was assessed using Student's *t*-test as described by Gad and Weill (1982).

RESULTS

Cytosolic glutathione *S*-transferase (GST) activity

No detectable activity (as release of NO_2^-) was observed with Nfx as substrate of the GST. Under the experimental conditions used the Nfx concentration was constant during incubation times. Occasionally some samples of liver cytosol exhibited detectable but minor activity.

Chemical reaction of nitrocompounds with some sulfhydryl-containing substances

The release of the nitro group as inorganic nitrite from Nfx in the presence of sulfhydryl-containing substances such as GSH, RSH, LA and CoASH was observed. The reaction between Nfur and GSH is also depicted for comparative purposes. Under the experimental conditions used, NO_2^- formation from both chemicals was time-dependent (Fig. 2).

Nitrite anion formation and Nfx consumption in the presence of GSH or RSH at different times

Nitrite anion formation and simultaneous degradation of Nfx in the presence of GSH or RSH were observed at different periods of time (Tables 1 and 2 and Figs 3 and 4).

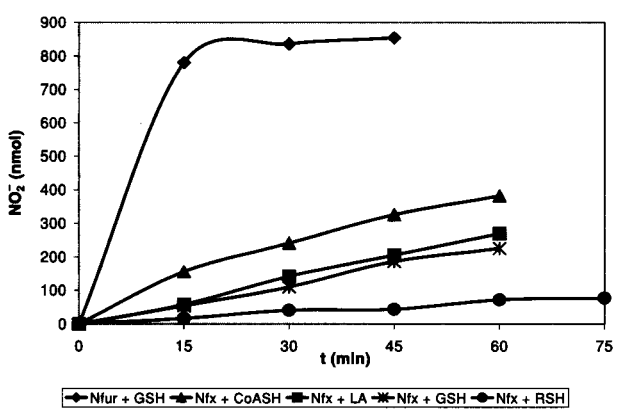

Figure 2. Kinetics of the reaction between nitrofurazone or Nfx with some critical sulfhydryl-containing biomolecules. The incubation mixture contained either 1 mM nitrofurazone (Nfur) plus 5 mM GSH or 1 mM Nfx plus 5 mM GSH or L-cysteine (RSH) or DL-∝-lipoic acid (LA) or coenzyme A (CoASH). The intensity of the reaction was determined by measuring spectrophotometrically the release of the nitro group as inorganic nitrite, as described in the text.

Table 1—NO_2^- formation and Nfx consumption in the presence of GSH at different periods of time

Reaction time (min)	NO_2^- formed (nmol)	Nfx consumed (nmol)
0	0	0
15	54	58
30	110	149
45	187	665
60	226	1010

Reactions were carried out in 20 mM potassium phosphate buffer (pH 7.4). The initial concentration of the reactants were 1 mM Nfx and 5 mM GSH. At times indicated the flasks were placed on ice to interrupt the reaction and NO_2^- or Nfx were determined spectrophotometrically or by HPLC respectively.

Table 2—Formation of NO_2^- and consumption of Nfx in the presence of cysteine at different periods of time

Reaction time (min)	NO_2^- formed (nmol)	Nfx consumed (nmol)
0	0	0
30	41	146
45	45	225
60	73	370
75	77	486

Incubation mixture composition and NO_2^- or Nfx determinations were carried out as discussed in Table 1.

Under the experimental conditions used, NO_2^- generation was quantitatively less significant than Nfx consumption (Tables 1 and 2). The HPLC chromatograms of the reaction between Nfx and GSH showed a new peak (retention time at ca. 4.06 min) whose area increased in a time-dependent manner. The GSH concentration decreased and the oxidized glutathione (GSSG) concentration increased accordingly (Fig. 3). Peak 1 UV spectra did not exhibit any absorption at 400 nm, the wavelength typical of the Nfx nitro group. When Nfx reacted with RSH the HPLC chromatograms also showed an unidentified peak having a close retention time and a similar UV spectral characteristic, although less intense than that mentioned above (Fig. 4). We were unable to detect cystine formation in these experiments because its retention time is within the range of the solvent front.

Urinary excretion of Nfx metabolites

The release of nitrite anion from Nfx was observed *in vivo* in Nfx-p.o.-treated rats (Table 3). In urine samples, NO_2^-

Table 3—Inorganic NO_2^- concentration in the urine of rats after Nfx administration

	Urine volume (ml)	NO_2^- concentration (nmol ml^{-1})
Control	24.5 ± 8.5	87 ± 22
Treated	24.0 ± 5.1	177 ± 35*

Housing and treatment conditions were described in the text. Urine samples were collected over 24 h. Six animals per group were employed. * $P < 0.05$.

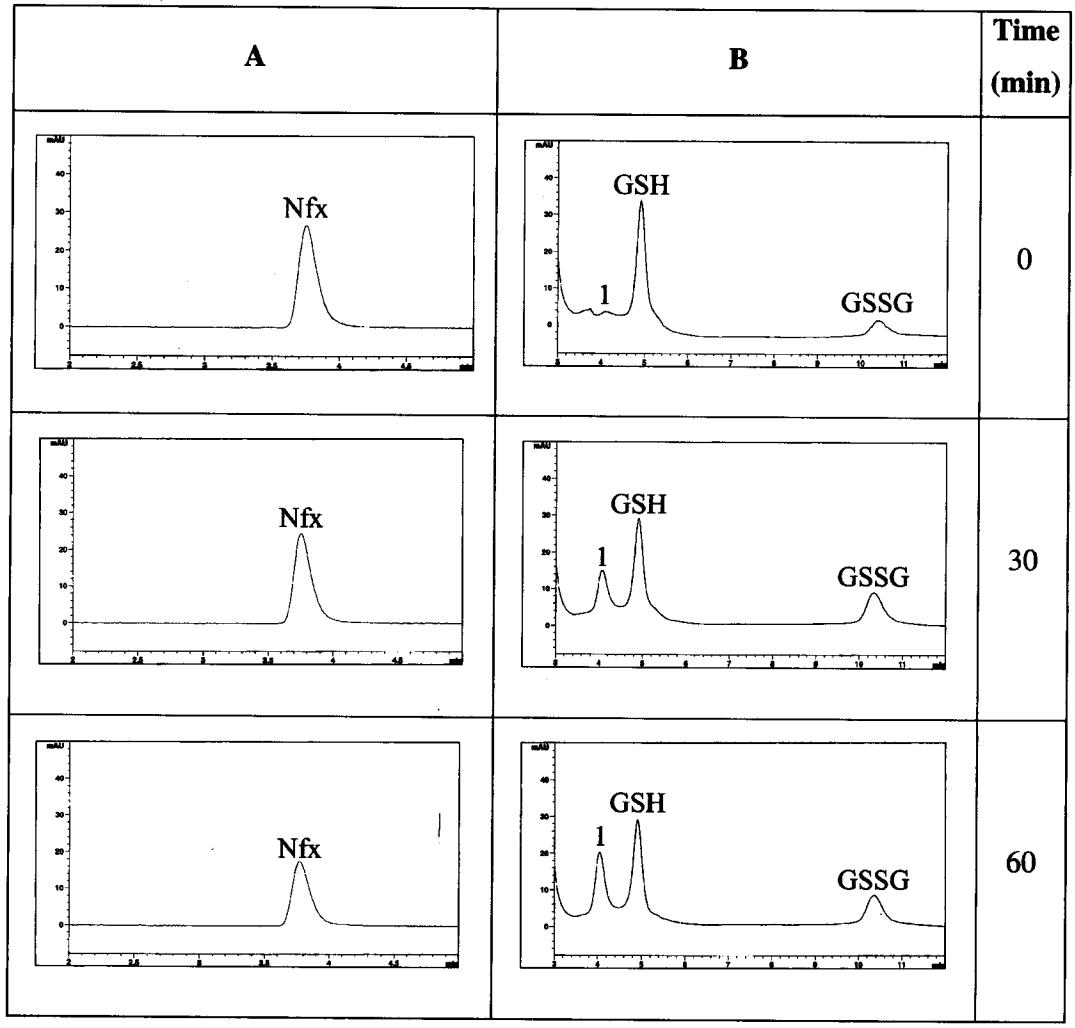

Figure 3. High-performance liquid chromatography analysis of the products formed during the chemical reaction between Nfx and GSH. Chromatograms: (A) 60% CH_3OH-H_2O mobile phase monitored at 400 nm and 1-µl sample volume; (B) 25 mM KH_2PO_4 (pH 2.5) mobile phase monitored at 215 nm and 10-µl sample volume. Peak 1 indicates an unknown reaction product.

Table 4—Urinary excretion of Nfx administered to rats

Time after administration (h)	Urine volume (ml)	Nfx concentration (nmol ml^{-1})	Nfx excreted (%)
3	4.1 ± 3.3	18.9 ± 9.2 (5)	0.08
6	5.8 ± 3.7	20.2 ± 6.7 (6)	0.10

Housing, treatment conditions and Nfx analysis were described in the text. Numbers in parentheses indicate the number of animals employed and values for Nfx excreted are percentages of the total Nfx administrated.

excretion was accompanied by unchanged drug (Table 4) and two more-polar metabolites detectable by HPLC (Fig. 5). The urinary recoveries of the parent drug in Sprague-Dawley rats were 0.08 and 0.10% of the Nfx administrated during the first 3 and 6 h, respectively (Table 4).

DISCUSSION

Our present observations failed to detect any GST-catalyzed biotransformation of Nfx to nitrite in rat cytosolic fraction. However, GSH itself was shown to react chemically to some extent with Nfx to produce nitrite. This reaction was significantly less intense than that occurring with other nitrofuranes, e.g. nitrofurazone (Fig. 2). Other relevant sulfhydryl-containing biomolecules, such as CoA, LA and RSH, also react with Nfx. The relative reaction rates were CoA > LA > GSH > RSH. The Nfx nitrite-forming process in reaction mixtures containing either GSH or RSH was found to be accompanied by the disappearance of Nfx, as shown in Tables 1 and 2. Initially we envisaged the Nfx disappearance process with simultaneous nitrite formation as follows: $RNO_2 + GSH \rightarrow RSG + NO_2H$. The amount of Nfx consumed in the reaction between the sulfhydryl-containing molecules and Nfx was higher than that of the nitrite formed, suggesting that additional reactions are occurring, e.g. Nfx nitroreduction by GSH with the simultaneous formation of GSSG, as detectable in the HPLC experiments (Fig. 3).

There is an indication that these nitrite-forming reactions of Nfx with sulfhydryl-containing groups might occur to some extent under *in vivo* conditions. In effect, nitrite concentrations in 24-h-collected urine from Nfx-treated rats are significantly higher than in controls (Table 3). Under *in vivo* conditions, sulfhydryl groups other than those specifically reported here might additionally be involved

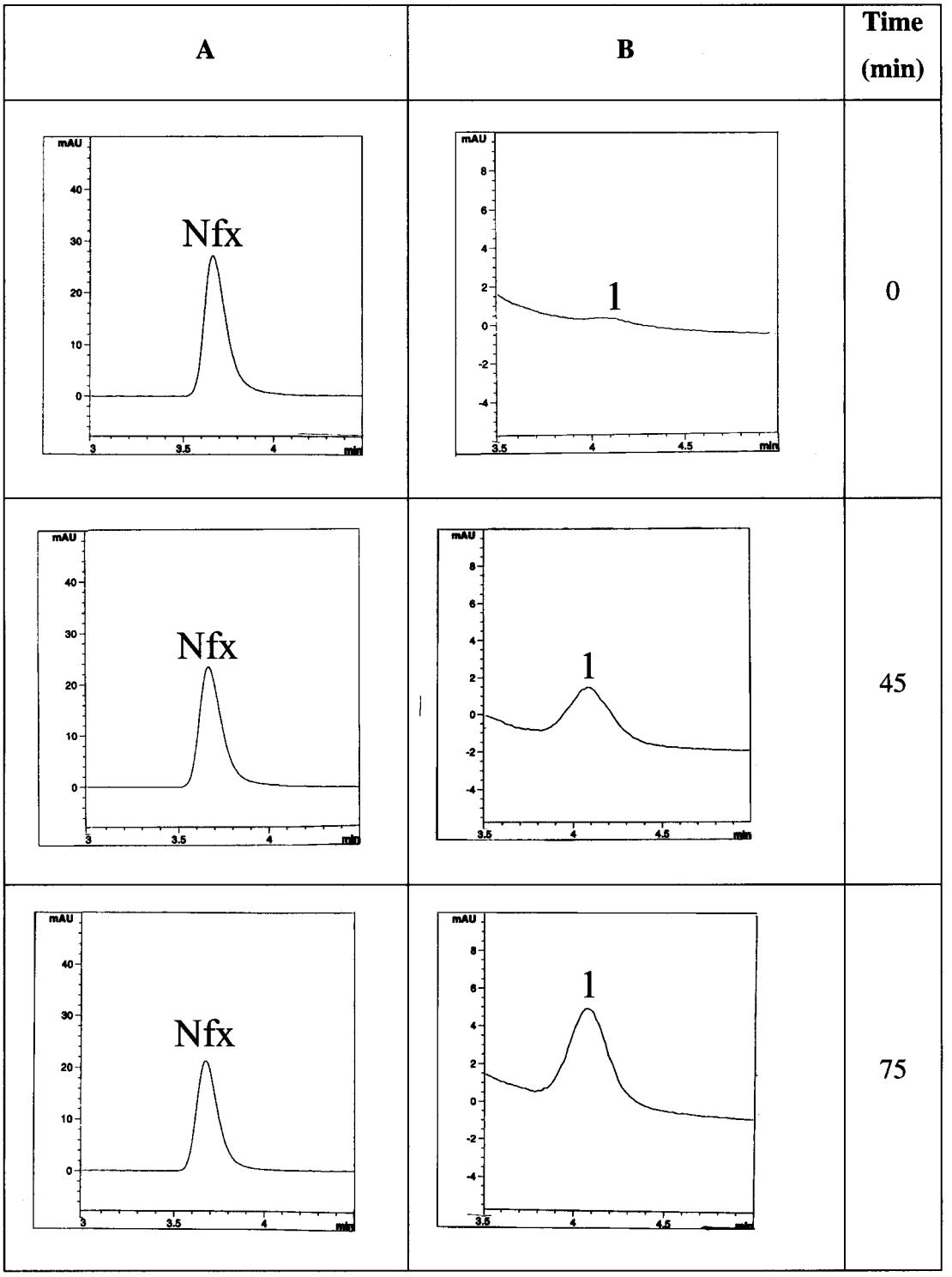

Figure 4. HPLC analysis of the products formed during the chemical reaction between Nfx and L-cysteine (RSH). HPLC conditions were described in Fig. 2. Peak 1, unknown reaction product.

in the nitrite-forming process. Of particular significance would be the case of sulfhydrylic enzymes containing critical SH groups linked to their function, typical examples being glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase, succinic dehydrogenase, pyruvate oxidase, hexokinase, creatine kinase, δ-aminolevulic acid dehydrogenase, DNA polymerase and O^6-alkylguanine-DNA alkyl transferase (Boyer, 1959; Johnstone, 1963; Peters, 1963; Dixon & Webb, 1979; Catalano & Kutchka, 1995; Lin *et al.*, 2002).

Nifurtimox might also harm essential cofactors such as lipoic acid (Moini *et al.*, 2002) or relevant structural proteins where SH groups are important, e.g. in membranes to modulate transport phenomena or membrane deformation and phospholipid asymmetry (Stryer, 1995).

Similarly, the potential reaction of Nfx with CoA might lead to the alteration of some or many of the processes in which it is involved, such as fatty acids, cholesterol or steroid hormones synthesis, the formation of ketone bodies, detoxification via acetylation of many xenobiotics

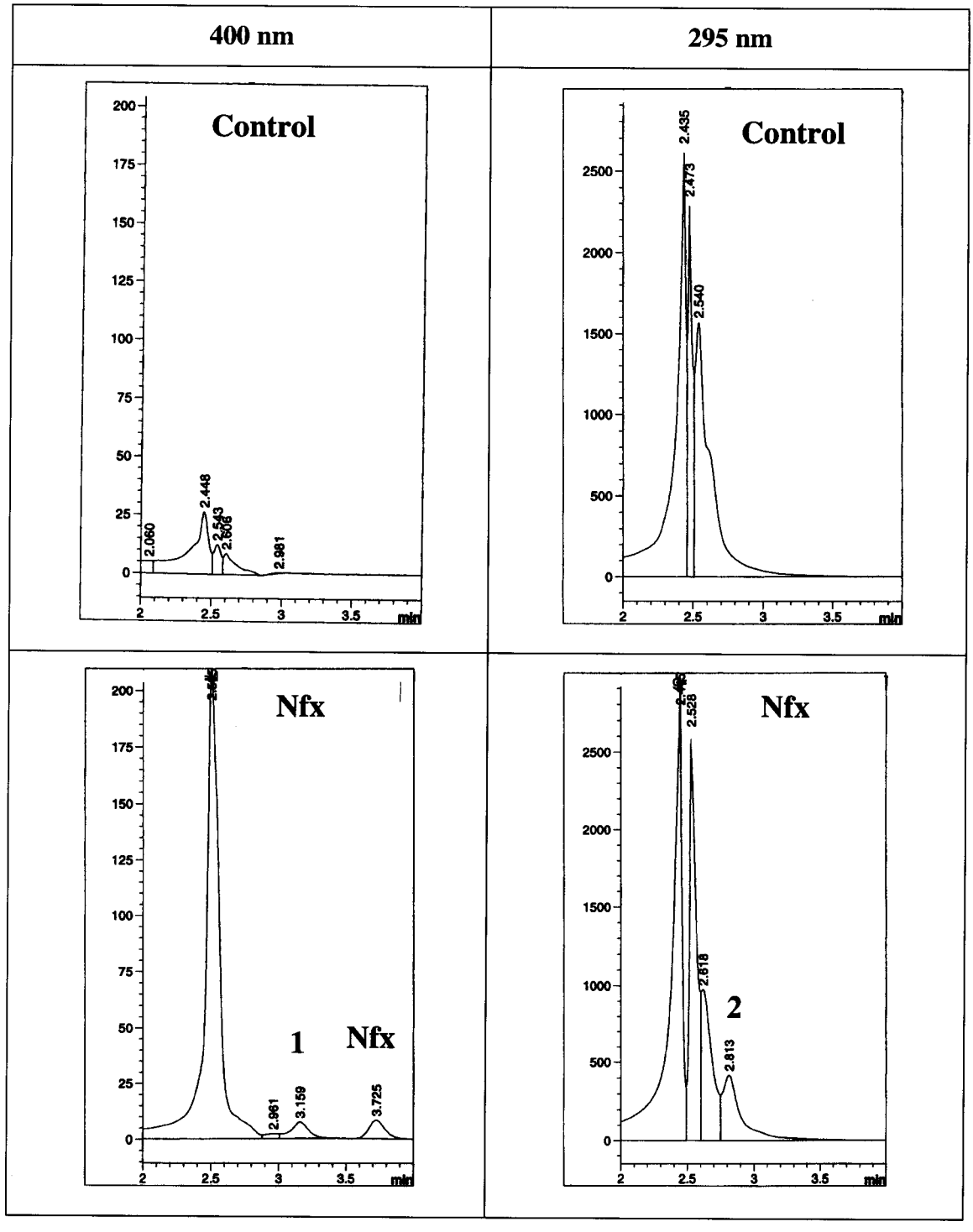

Figure 5. HPLC chromatograms of urine from Nfx treated rats at different wavelengths. Peaks 1 and 2 are urine metabolites of Nfx. Urine was collected and then analyzed by HPLC as described in Methods.

or functioning of the citric acid cycle (Stryer, 1995). Whether some of these enzymes or CoA-requiring processes may be altered during Nfx administration remains to be established. The present observations should be taken only as a possibility to explain some toxic deleterious effects potentially resulting from these types of reactions.

The interactions between Nfx and critical SH-containing biomolecules might also play a role in the chemotherapeutic effects of the drug, which are linked to its trypanosomicide action against the ethiological agent of Chagas disease, *Trypanosoma cruzi*. In effect, it is well known that several crucial biomolecules or enzymes for the parasite's survival contain an SH group, e.g. trypanothione and ovothiol (Ariyanayagam & Fairlamb, 2001).

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