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Formation of S-nitroso compounds from sodium nitroprusside, nitric oxide or nitrite and reduced thiols: analysis by capillary isotachophoresis

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Abstract

Sodium nitroprusside (SNP) is a potent, rapidly acting intravenous hypotensive agent. The direct action on blood vessels is thought to be due to its nitroso (NO) group. This suggestion is supported by the discovery of an endothelial relaxing factor, most probably being the radical nitric oxide (NO·) or a S-nitroso compound. In this paper we describe for the first time an analytical capillary isotachophoretic (ITP) method for the determination of SNP, of some potent vasodilating and platelet anti-aggregatory S-nitroso compounds from biological and pharmacological precursors, and of their final metabolites nitrite and nitrate. ITP was applied to study SNP stability in aqueous solutions and chemical formation of S-nitroso compounds by the reaction of reduced thiols such as N-acetyl-L-cysteine, glutathione and N-acetyl-D,L-penicillamine with SNP at pH 7.4. We found that these thiols react spontaneously with SNP to give the corresponding S-nitroso compounds. This result suggests that the vasodilating and anti-aggregatory properties of SNP may be in part due to the formation of S-nitroso compounds from SNP.

1. Introduction

Sodium nitroprusside (SNP; $Na_2[Fe(CN)_5NO]$) is a potent, rapidly acting drug which is used in hypertensive emergencies, heart failure and for controlled hypotension during surgery. The vasorelaxant action of SNP is thought to be produced by the release of the radical nitric oxide (NO·) at or in the vascular smooth muscle cell. The radical NO· activates soluble guanylate cyclase leading to formation of cyclic guanosine monophosphate (cGMP) and thus to relaxation [1,2]. This thought is supported by the identification of an endothelium-derived relaxing factor (EDRF) as NO· or a related species such as S-nitroso-L-cysteine (SNC) [2]. Endothelium-derived NO· (EDNO·) is produced from L-arginine by the action of NO· synthase and has potent vasodilating and anti-aggregatory properties (reviewed in Ref. [2]). Since the discovery of NO· as an EDRF, SNP and other NO-containing compounds, including S-nitroso compounds such as SNC, S-nitroso-N-acetyl-L-cysteine (SNAC) and especially S-nitroso-N-acetyl-penicillamine (SNAP), are frequent-

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ly used in studies on the pharmacological effects of EDRF on blood vessels and platelets [2,3–6].

Due to the increasing interest in NO· and nitrovasodilators in pharmacology, analytical methods permitting simultaneous analysis of SNP, S-nitroso compounds, the corresponding reduced and oxidized thiols, and the final metabolites of NO·, i.e. nitrite and nitrate, are required. In the past, capillary isotachophoresis (ITP) has been demonstrated to be an excellent analytical tool for the analysis of inorganic anions including nitrite, nitrate, the SNP-related $K_3[Fe(CN)_6]$ which has been used as a leading electrolyte [7], and of many pharmaceuticals (see literature cited in Ref. [8]). In two previous publications ITP has been shown to be applicable to the analysis of reduced and oxidized glutathione (GSH), and of some glutathionyl and cysteinyl conjugates [9,10]. In this paper we describe an analytical ITP method for the analysis of SNP, of some S-nitroso compounds, and nitrite and nitrate. Analytical methods for the analysis of SNP published until 1976 have been reviewed in Ref. [11]. The first capillary electrophoretic technique for S-nitroso compounds was published by Stamler and Loscalzo while our work was in progress [12].

2. Experimental

2.1. Chemicals and reagents

SNP, oxidized and reduced GSH, L-cysteine and N-acetyl-L-cysteine (NAC) were purchased from Sigma (Deisenhofen, Germany). The sodium salts of nitrite and nitrate, hydrochloric acid and hexanoic acid were obtained from Merck (Darmstadt, Germany). Hydroxypropylmethylcellulose was purchased from Ega-Chemie (Steinheim, Germany). S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) was from Biomol (Hamburg, Germany). Unless otherwise specified, S-nitroso derivatives of L-cysteine, NAC and GSH were prepared and analyzed by high-performance liquid chromatography (HPLC) and UV spectrometry as described elsewhere [13]. N-Acetyl-D,L-penicillamine (NAP) was purchased from Aldrich (Steinheim, Germany). SNP for infusion (Nipruss) was obtained from Schwarz Pharma (Monheim, Germany). For qualitative and quantitative measurements synthetic SNP and Nipruss were diluted with water immediately prior to use. These and other solutions were stored protected from light in brown silanized flasks which were placed on ice during ITP analysis.

2.2. Isotachophoretic conditions

ITP analyses were performed on an LKB Tachophor Model 2127 (Bromma, Sweden) fitted with a polytetrafluoroethylene capillary (25 cm × 0.5 mm I.D.). The leading electrolyte consisted of 10 mmol/l hydrochloric acid, the pH of which was adjusted to 4.0 by the addition of β-alanine, and 0.25% (w/w) hydroxypropylmethylcellulose. The terminating electrolyte consisted of 10 mmol/l hexanoic acid. Both electrolytes were prepared daily using double-distilled water. Analyses were carried out at room temperature at a constant driving current of 25 μA. The zones were detected by UV absorbance (254 nm filter) and conductivity detectors and recorded with an LKB 2120 line recorder at a chart speed of 0.5 mm/s. The terminating electrolyte passed the detectors at a potential of about 4 kV. The total analysis time was of the order of 30 min. The zones were measured by both UV and conductivity signals. Specific zone lengths were determined from calibration curves by injecting up to 20 nmol of each compound separately. Reciprocal reference unit (RRU) values were determined by injecting at least ten times each compound separately. The RRU values were calculated relative to the terminating ion from the relative step heights of the conductivity signals, as described by Everaerts et al. [14].

3. Results

The RRU values and the specific zone lengths of the investigated compounds are listed in Table 1. The calibration curves on which the data in

Table 1
Reciprocal reference unit (RRU) values and specific zone lengths of the investigated compounds

No.	Compound	RRU (mean ± S.D.)	Specific zone length (s/nmol)
1	Nitrate	72.7 ± 12.4	1.80
2	Nitrite	30.5 ± 8.2	1.80
3	Nitroprusside	23.3 ± 0.8	4.07
4	N-Acetyl-L-cysteine, oxidized	4.09 ± 0.14	n.d.
5	S-Nitroso-N-acetyl-L-cysteine	3.68 ± 0.25	n.d.
6	S-Nitroso-N-acetyl-D,L-penicillamine	2.94 ± 0.09	2.23
7	N-Acetyl-D,L-penicillamine, reduced	2.56 ± 0.50	2.30
8	N-Acetyl-L-cysteine, reduced	2.43 ± 0.11	3.27
9	Glutathione, oxidized	2.01 ± 0.01	5.10
10	Glutathione, reduced	1.57 ± 0.01	4.45
11	S-Nitroso-L-cysteine	n.m.	n.m.

n.d. = not determined because of the lack of reference compounds; n.m. = not measurable (for details see text).

this table are based were all linear up to 20 nmol for each compound with coefficients of correlation better than 0.975. The method allowed accurate analysis of 0.2 nmol of nitroprusside (NP) (UV signal) and 0.5 nmol of SNAP (conductivity signal). Under the experimental conditions used SNC could not be detected at all due to its cationic form at the pH value of the leading electrolyte. The inter-assay relative standard deviation calculated from the determination of the RRU values was 3.3% for NP ($n = 18$) and 3.5% for SNAP ($n = 9$) from separate analyses of 20 nmol of each compound carried out within a period of 12 months.

From the compounds investigated, nitrate, nitrite and NP have the highest mobilities in the present ITP system. A mixture consisting of 20 nmol of each of these compounds was completely separated by ITP (data not shown). Fig. 1a shows an isotachopherogram from the direct analysis of a reaction mixture from bubbling NO· gas into a methanolic solution of NAP. Based on Table 1 and on Fig. 1b the isotachopherogram in Fig. 1a shows the formation of SNAP, of its oxidation products nitrate, nitrite, and most probably of oxidized NAP (zone 12; RRU = 2.39), and unreacted NAP. ITP analysis of methanolic solutions of the commercially available SNAP also showed the presence of nitrate and nitrite but not of oxidized NAP (Fig. 1b). HPLC analysis of the same reaction mixture and of a methanolic solution of the reference SNAP did not show any nitrite. Therefore, nitrite and nitrate are most likely formed from SNAP in the reservoir of the leading electrolyte. This is supported by the observation from HPLC analyses that when SNC, SNAC, GSNO and SNAP are diluted in buffers of pH 4 and higher they are converted mainly to nitrite and the corresponding thiol.

Evidence for the formation of S-nitroso compounds by the reaction of SNP with thiols is demonstrated by ITP using NAP and the commercially available SNAP as a reference (Fig. 1c). Interestingly, ITP analysis of this reaction mixture did not show formation of nitrite or nitrate. Also, incubation of SNP with NAC resulted in the rapid formation of a UV-absorbing species migrating immediately behind SNP with a RRU value of 3.68 (Fig. 2). The appearance of the same zone after bubbling NO· gas in an oxygen-free solution of NAC strongly suggests the formation of SNAC by the reaction of SNP with NAC. Similarly, reaction of GSH with SNP resulted in the formation of S-nitroso-GSH (GSNO). This finding was validated by the ITP analysis of GSNO which was prepared by the reaction of GSH with nitrite under acidic con-

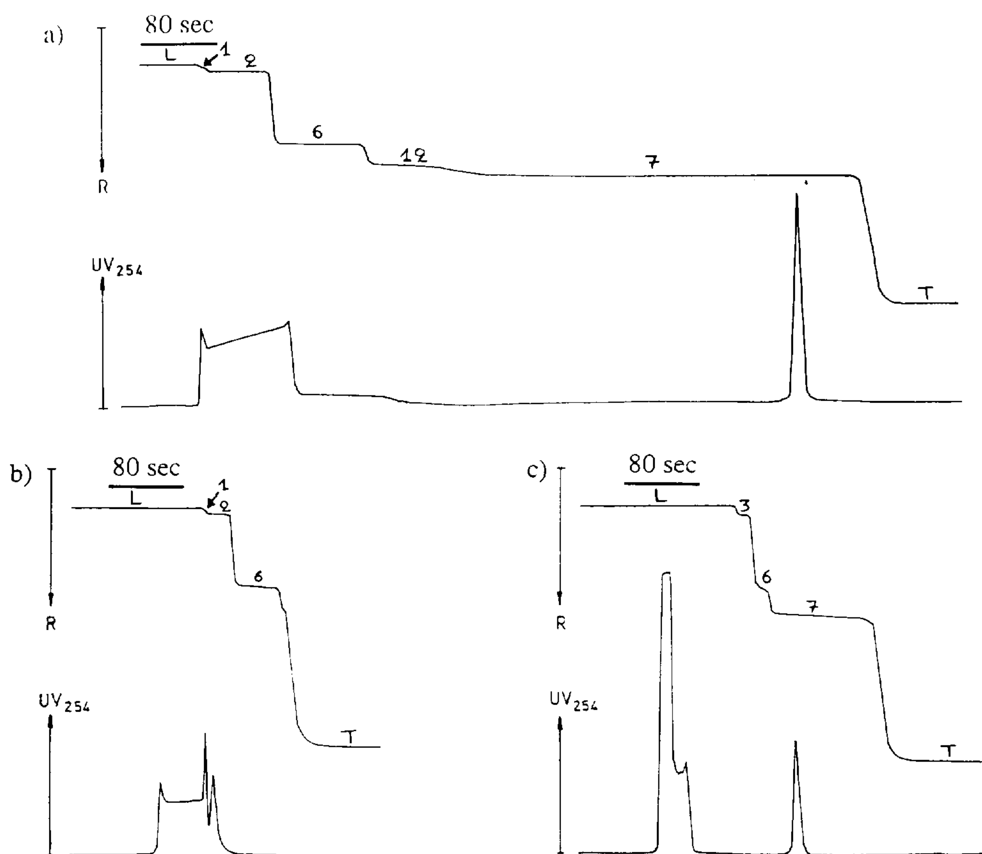

Fig. 1. (a) Isotachopherogram from the reaction mixture of N-acetyl-D,L-penicillamine (100 mmol/l) in dry methanol and nitric oxide gas. (b) Analysis of 20 nmol of commercially available S-nitroso-N-acetyl-D,L-penicillamine. (c) Isotachopherogram from the analysis of a reaction mixture of sodium nitroprusside (1 mmol/l) and N-acetyl-D,L-penicillamine (10 mmol/l) in Tris buffer (0.1 mol/l, pH 7.4) in the dark after 3 h of incubation. L = leading ion; T = terminating ion; for identification of other zones see Table 1. Zone 12 in (a) is most probably due to oxidized N-acetyl-D,L-penicillamine. R = resistance.

ditions (data not shown). Analysis by ITP of an incubation mixture of SNP and L-cysteine showed only a decrease in the concentration of SNP but no formation of an additional zone. A similar result was also obtained by analysis of SNC formed by the reaction of L-cysteine with nitrite in acidic solution. Significant amounts of SNAP, SNAC and GSNO were formed from SNP and the corresponding thiol when molar ratios of thiol to SNP of 10:1 and higher were used. Our results are in agreement with a report on the formation of S-nitroso compounds by the reaction of SNP with reduced thiols [15].

SNP was found by us to be very stable in aqueous solutions when protected from sun light (Fig. 3a). After incubation of SNP in double-distilled water or buffers (pH 7.4) for up to four weeks at room temperature in the dark (brown flasks) no degradation of SNP was observed. SNP was also found to be stable in aqueous solutions for at least 9 h when these solutions, placed in a cuvette, were irradiated with UV light with a wavelength of 254 nm (Fig. 3b). The color of these solutions remained unchanged with time. The color of SNP solutions exposed indirectly to sun light changed to yellow. Changes in SNP resulting from exposure to sun light could only be detected by the UV detector

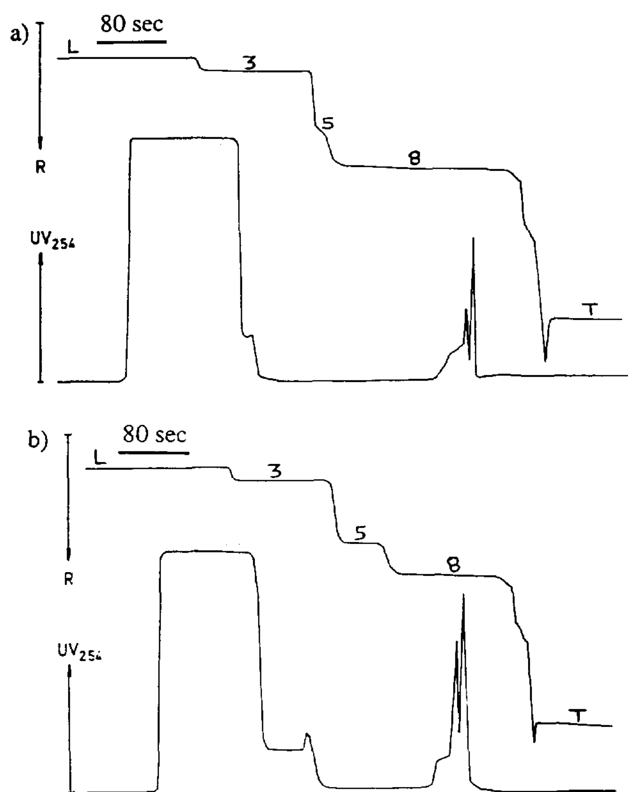

Fig. 2. Incubation of sodium nitroprusside (5 mmol/l) and N-acetyl-L-cysteine (10 mmol/l) in Tris buffer (0.1 mol/l, pH 7.4) at room temperature in the dark for (a) "zero" minutes, and (b) for 24 h. L = leading ion; T = terminating ion; for identification of other zones see Table 1. R = resistance.

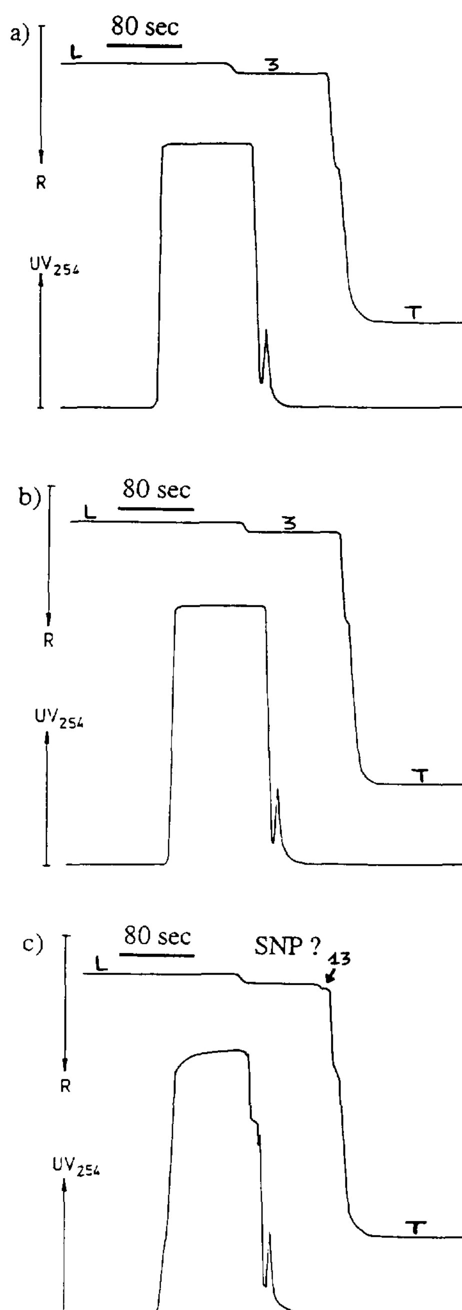

Fig. 3. Isotachopherograms from the analysis of sodium nitroprusside (5 mmol/l) incubated in Tris buffer (0.1 mol/l, pH 7.4). (a) Incubation at room temperature for 48 h in the dark; (b) under irradiation with UV light at 254 nm for 9 h; and (c) in the presence of sun light for 48 h. L = leading ion; T = terminating ion; for identification of other zones see Table 1. Zone 13 in (c) has a RRU value of about 15.9. R = resistance.

with the exception of the compound (zone 13 in Fig. 3c) with a RRU value of 15.9 ± 0.7 (mean ± S.D., $n = 5$) which could also be detected with the conductivity detector. These observations suggest formation of various closely related products with very small differences in mobility but larger differences in absorptivity.

We applied the ITP method for the qualitative and quantitative analysis of SNP in the drug preparation Nipruss. Compared to the chemical SNP (Fig. 4a) we detected in Nipruss (Fig. 4b) besides SNP two unknown compounds: a UV-absorbing minor and a non UV-absorbing major species both migrating immediately behind SNP. The amount of SNP in Nipruss was determined to be 54.36 ± 2.98 mg (mean ± S.D., $n = 6$) ver-

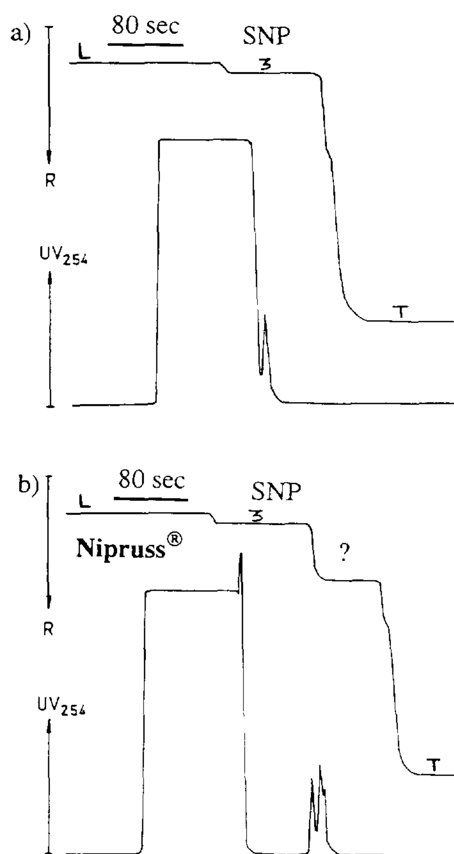

Fig. 4. Isotachopherograms from the analysis of aqueous solutions of sodium nitroprusside: (a) as a chemical (5 mmol/l), and (b) in the pharmaceutical Nipruss (assumed to be 5 mmol/l). Solutions were prepared in double-distilled water and 5 μl of each compound were injected into the tachophor. L = leading ion; T = terminating ion; for identification of other zones see Table 1. R = resistance.

sus an amount of 52.75 mg as given by the manufacturer.

4. Discussion

This paper describes an analytical ITP method for the analysis in aqueous solutions of SNP, some S-nitroso compounds derived from biological and pharmacological precursors and some of their metabolites. Our results demonstrate the excellent applicability of ITP to the analysis of these compounds. SNC could not be analyzed by our anionic ITP method because of its cationic form and instability at the pH of the leading electrolyte. Detection of SNC, however, could be performed by cationic ITP by using a leading electrolyte with a pH of ≤ 2.0 at which SNC is sufficiently stable as found by HPLC (data not shown). The selectivity of the system with respect to S-nitroso compounds could be further improved by UV detection at their characteristic maximum wavelength of ca. 340 nm [13].

Our results show that thiols such as NAC, GSH and NAP are able to react with the NO group of SNP to form the corresponding S-nitroso compounds. The formation of S-nitroso compounds from SNP by the reaction with reduced thiols may be in part responsible for the strong vasodilating and anti-aggregatory properties of SNP [16]. Our study also shows that reduced thiols can directly react with NO· in organic solvents as well as in aqueous solution with a neutral pH to form S-nitroso compounds. Interaction of reduced thiols with SNP in erythrocytes and tissues has been suggested to be responsible for the blood pressure lowering potency of SNP (see references in Ref. [11]). Thus, interaction of thiols with SNP or L-arginine-derived NO· to form circulating S-nitroso compounds may be in part responsible for the vasodilating and anti-aggregatory action of SNP and NO·. An alternative route for the formation of S-nitroso compounds is the reaction of reduced thiols with nitrite in acidic solution with most probably nitrosonium cation (NO^+) as the active species. Formation of S-nitroso compounds may therefore physiologically take place in the gastric fluid.

By using the ITP method SNP was found to be very stable in aqueous solutions when protected from sun light but extremely unstable in its presence. These findings are in agreement with the description in the literature of SNP as an extremely photosensitive ($\lambda > 300$ nm) compound [11]. Direct exposure of aqueous solutions of SNP to sun light has been shown to ultimately yield prussian blue, cyanide, and NO; also formation of pentacyanoaquoferrate(II), which rapidly dimerizes, has been described [11]. Fig. 3c and the co-migration of cyanide and hexacyanoferrat(III) with the leading ion in this ITP

system indicate that this ITP method is not suitable for evaluation of the numerous reaction products of SNP in aqueous solutions.

In conclusion, the results presented here and the recently published work by Stamler and Loscalzo [12] indicate that ITP and capillary zone electrophoresis are suitable techniques for the analysis of S-nitroso compounds and SNP. Although analysis of S-nitroso compounds by HPLC has not been thoroughly investigated [13,17], electrophoretic techniques seem to provide major advantages over HPLC, for example in terms of resolution and efficiency. Thus, ITP offers the opportunity to simultaneously analyse SNP, a large number of S-nitroso compounds, their precursors, as well as their oxidation products including nitrite and nitrate. Besides the biomedical sciences, the most relevant field for application of the ITP method could be the pharmaceutical industry, e.g. in the production and quality control of SNP and S-nitroso compounds.

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