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Technical note

Interference by *S*-nitroso compounds with the measurement of nitrate in methods requiring reduction of nitrate to nitrite by cadmium

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Abstract

Various methods suited for the measurement of nitrate require its reduction to nitrite by cadmium under acidic or alkaline conditions. N^G-Nitroarginine analogs have been shown to interfere with the measurement of nitrate by such assays. In the present work we show by gas chromatography−mass spectrometry that under alkaline reduction conditions the *S*-nitroso compounds *S*-nitrosoglutathione and *S*-nitrosohomocysteine but not *S*-nitroso-*N*-acetylcysteine and *S*-nitroso-*N*-acetylpenicillamine can considerably contribute to nitrate and thus interfere with its measurement. Our results suggest that *S*-nitroso compounds may interfere with the measurement of nitrate in methods requiring cadmium-catalyzed reduction of nitrate to nitrite. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *S*-Nitroso compounds; Nitrate; Nitrite; Cadmium

1. Introduction

The major metabolite of L-arginine-derived nitric oxide (NO) has been shown to be nitrate [1]. Measurement of nitrate in biological fluids is a useful index parameter to assess NO synthase activity in vivo. Various analytical methods have been developed for the quantitation of nitrate in biological fluids (reviewed in [2]). A large number of these methods require reduction of nitrate to nitrite. These methods include the frequently used photometric assay based on the Griess reaction and gas chromatography–mass spectrometry (GC–MS) [2,3]. Reduction of nitrate to nitrite is commonly performed by using cadmium either under acidic or alkaline conditions. In the present study we investigated a possible interference by *S*-nitrosoglutathione (GSNO), a physiological metabolite of NO [4], and other *S*-nitroso compounds in a previously reported GC–MS method for nitrate [3]. In the present work, we show by GC–MS that GSNO can considerably interfere with the measurement of nitrate when it is reduced by cadmium under alkaline conditions. Our results suggest that the physiological GSNO and *S*-nitrosohomocysteine (SNhC) but not the unphysiological *S*-nitroso-*N*-acetylcysteine (SNAC) [5]

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and *S*-nitroso-*N*-acetylpenicillamine (SNAP) can interfere with the measurement of nitrate by all those methods that require reduction of nitrate to nitrite by cadmium.

2. Experimental

2.1. Materials and chemicals

Reduced glutathione (GSH), *N*-acetylcysteine, DL-homocysteine and sodium [^{15}N]nitrate (98 atom% at ^{15}N) were obtained from Sigma (Munich, Germany). Sodium [^{15}N]nitrite (98 atom% at ^{15}N) was bought from Cambridge Isotope Laboratories (Andover, MA, USA). Sodium nitrite, sodium nitrate, toluene, acetic acid, ammonium chloride and cadmium powder (100 mesh) were purchased from Merck (Darmstadt, Germany). 1,2,3,4,5-Pentafluorobenzyl (PFB) bromide, cadmium acetate dihydrate and *N*-acetyl-DL-penicillamime were obtained from Aldrich (Steinheim, Germany). *S*-Nitroso compounds were prepared by mixing aqueous solutions of their thiols (each 10 m*M*; 1 ml) with nitrite (each 10 m*M*; 1 ml) and HCl (each 5 *M*; 0.1 ml) and incubation for 5 min [3]. Purity of all *S*-nitroso compounds was determined as >98% by high-performance liquid chromatography [3]. Chemical identity of GSNO and SNAC was verified by flow injection analysis electrospray ionization mass spectrometry as described elsewhere for SNAC [5]. Characteristic ions were obtained for GSNO at *m*/*z* 695 ($[2M+Na]^+$, 20%), 673 ($[2M+H]^+$, 35%), 613 ($[2M+H-2\times NO]^+$, 15%), 359 ($[M+Na]^+$, 48%), 338 ($[M+H]^+$, 100%) and 307 ($[M+H-NO]^+$, 45%). Dilutions were prepared in 50 m*M* NaH_2PO_4, pH 7, and used immediately. Neither stock solutions of *S*-nitroso compounds nor freshly prepared dilutions of them were found to contain nitrite by the Griess assay and thiols by the method of Ellman [3].

2.2. Reduction and derivatization procedures and GC–MS analysis

Aliquots (1 ml) of solutions of GSNO in phosphate buffer (0, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μ*M*) or freshly obtained human plasma (0, 10, 20, 40, 60, 100 μ*M*) were spiked with [^{15}N]nitrate or [^{15}N]nitrite (each 50 μ*M*). Aliquots (each 100 μl) of buffer and plasma samples were diluted with ammonium chloride buffer (5 wt%, pH 8.8; 900 μl). Also, aliquots (each 100 μl) of buffer were diluted with acetic acid acidified distilled water of pH 4.5. Accurately weighed cadmium powder (10 mg) was added to half of the samples (100 μl) and reduction was performed by shaking for 90 min at room temperature. In the second half of the samples (100 μl) no cadmium was added but the samples were shaken for 90 min at room temperature. Similarly was processed with buffered solutions of SNAC, SNhC and SNAP. To the supernatants (100 μl) were added acetone (400 μl) and PFB bromide (10 μl), and the reaction mixtures were allowed to stand at 50°C for 60 min. Acetone was removed under nitrogen and reaction products were extracted by vortex-mixing with toluene (1 ml) for 1 min. GC–MS was performed on a Hewlett-Packard MS engine 5890A connected directly to a gas chromatograph 5890 series II (Waldbronn, Germany). A fused-silica capillary column Optima 17 (15 m×0.25 mm I.D., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany) was used. Other GC–MS conditions were as described elsewhere in detail [3]. Aliquots (1 μl) were injected in the splitless mode and quantification was performed by selected ion monitoring of *m*/*z* 46 for nitrite and *m*/*z* 47 for [^{15}N]nitrite.

3. Results

Fig. 1 shows that GSNO in buffer contributed to nitrate in a concentration-dependent manner when reduction was performed both under alkaline and acidic conditions. In the concentration range investigated, the contribution of GSNO to nitrate was considerably greater under alkaline than under acidic conditions. In the absence of cadmium GSNO contributed to nitrite under alkaline conditions more than under acidic conditions most likely due to higher instability of GSNO under alkaline conditions. GSNO added to human plasma contributed both to nitrite and to nitrate. Regression analysis between nitrite (y_1) or nitrate (y_2) measured in and GSNO added (x) to human plasma revealed straight lines with the regression equations: $y_1 = -2.99 + 0.449x$

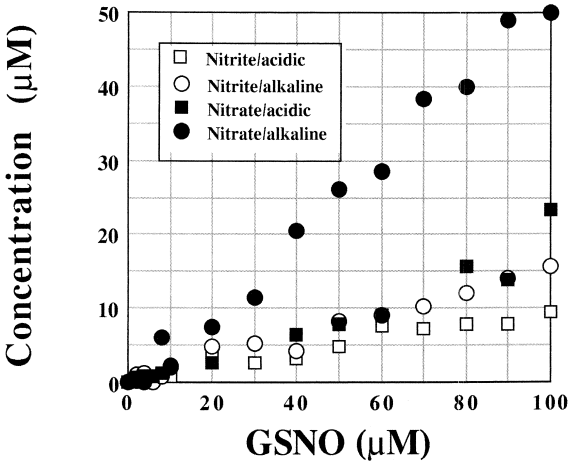

Fig. 1. Contribution of *S*-nitrosoglutathione (GSNO) to nitrite and nitrate under alkaline and acidic conditions for reduction of nitrate to nitrite by cadmium. Nitrate and nitrite were quantitated by GC–MS [3] using ^{15}N-labeled nitrate and nitrite (each 50 μ*M*) as internal standards. Points represent data from single analyses.

(r^2=0.931) for nitrite and y_2=16.6+0.814*x* (r^2=0.922) for nitrate. Similar results were also obtained for SNhC in buffer (y_1=−0.13+0.256*x* (r^2=0.997) and y_2=1.17+0.828*x* (r^2=0.944)). Our results clearly show that in buffer and in human plasma the *S*-nitroso groups of GSNO and SNhC were partly converted by cadmium to nitrite under both reduction conditions. Whether and in to what extent the *S*-nitroso group of GSNO and SNhC were converted to further compounds or remained unchanged under these conditions was not investigated. The exogenous SNAC did not contribute to nitrate under alkaline conditions in the same concentration range. Regression analysis between nitrite (y_1) or nitrate (y_2) measured in and SNAC added (*x*) to buffer revealed straight lines with the regression equations: y_1=−1.38+0.544*x* (r^2=0.997) for nitrite and y_2=1.38+0.505*x* (r^2=0.912) for nitrate. Similar results were also obtained for SNAP (not shown). Cadmium acetate (0–1000 μ*M*) had no effect on nitrite formation from GSNO and SNhC after incubation in buffer (pH 7) for 10 min. Accurate weighing of cadmium powder was necessary because the cadmium preparation used was found to contain nitrate/nitrite as impurity. Linear regression analysis between nitrate measured (*y*, μ*M*) under the alkaline reduction conditions in the absence of GSNO and cadmium powder used (*x*, mg) resulted in a straight line with the regression equation y=1.189+0.034*x* (r^2=0.999).

4. Discussion

N^G-Nitroarginine analogs used as NO synthase inhibitors were found to interfere in the Griess assay, chemiluminescence and GC–MS when reduction of nitrate to nitrite was performed by cadmium [6,7]. In the absence of cadmium N^G-nitroarginine analogs were found not to contribute to nitrite when measured by GC–MS [6]. In the present work we identified the physiological GSNO and SNhC as further potential interfering compounds in the measurement of nitrate by GC–MS. By contrast, the unphysiological *S*-nitroso compounds SNAC and SNAP were found not to contribute to nitrate when reduction with cadmium was performed under alkaline conditions. The distinct behavior of the investigated *S*-nitroso compounds suggests that the structure of their parent moieties may influence the cadmium-dependent susceptibility of the *S*-nitroso group to form nitrite. It can be expected that other *S*-nitroso compounds from endogenous and exogenous sources would also interfere with the nitrate measurement by methods requiring reduction of nitrate to nitrite by cadmium. In contrast to Hg^{2+} [2], Cd^{2+} ions at a concentration of up to 1 m*M* were not able to significantly catalyze nitrite formation from an *S*-nitroso group. Apparently, Cd^{2+} ions that can be formed from oxidation of cadmium during the reduction process are not responsible for the conversion of the *S*-nitroso group to nitrite. The exact mechanism of the cadmium-catalyzed destruction of the *S*-nitroso-group to nitrite remains to be established.

Physiological *S*-nitroso compounds have been reported to occur in human plasma at a total concentration of less than 2 μ*M* [8]. The concentration of nitrate in human plasma is of the order of 20–60 μ*M* [3]. Contribution of plasma *S*-nitroso compounds to plasma nitrate will therefore be negligible especially when reduction is performed under acidic conditions. However, it can not be excluded that administered *S*-nitroso compounds, N^G-nitroarginine analogs and other nitroso- or nitro-containing com-

pounds would considerably interfere with the measurement of nitrate. In such situations removal of these compounds prior to the reduction step is recommended.

Our study also suggests accurate weighing of cadmium powder under alkaline conditions in order to minimize the imprecision of the method resulting from the variation of the contribution of cadmium to nitrate.

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