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UPLC–MS/MS measurement of *S*-nitrosoglutathione (GSNO) in human plasma solves the *S*-nitrosothiol concentration enigma☆

Dimitrios Tsikas*, Mario Schmidt, Anke Böhmer, Alexander A. Zoerner, Frank-Mathias Gutzki, Jens Jordan

Institute of Clinical Pharmacology, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany

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ABSTRACT

We developed and validated a fast UPLC–MS/MS method with positive electrospray ionization (ESI+) for the quantitative determination of *S*-nitrosoglutathione (GSNO) in human plasma. We used a published protocol for the inactivation of plasma γ-glutamyltransferase (γGT) activity by using the γGT transition inhibitor serine/borate and the chelator EDTA for the stabilization of GSNO, and *N*-ethylmaleimide (NEM) to block SH groups and to avoid *S*-transnitrosylation reactions which may diminish GSNO concentration. *S*-[^{15}N]Nitrosoglutathione (GS^{15}NO) served as internal standard. Fresh blood was treated with NEM/serine/borate/EDTA, plasma spiked with GS^{15}NO (50 nM) was ultrafiltered (cut-off 10 kDa) and 10 μL aliquots of the ultrafiltrate were analyzed by UPLC–MS/MS. Five HILIC columns and an Acquity UPLC BH amide column were tested. The mobile phase was acetonitrile–water (70:30, v/v), contained 20 mM ammonium formate, had a pH value of 7, and was pumped isocratically (0.5 mL/min). The Nucleoshell column allowed better LC performance and higher MS sensitivity. The retention time of GSNO was about 1.1 min. Quantification was performed by selected-reaction monitoring the mass transition *m/z* 337 ([M+H]$^+$) → *m/z* 307 ([M+H—^{14}NO]$^{\bullet+}$) for GSNO (i.e., GS^{14}NO) and *m/z* 338 ([M+H]$^+$) → *m/z* 307 ([M+H—^{15}NO]$^{\bullet+}$) for GS^{15}NO. NEM/serine/borate/EDTA was found to stabilize GSNO in human plasma. The method was validated in human plasma (range, 0–300 nM) using 50 nM GS^{15}NO. Accuracy and precision were in generally acceptable ranges. A considerable matrix effect was observed, which was however outweighed by the internal standard GS^{15}NO. In freshly prepared plasma from heparinized blood donated by 10 healthy subjects, no endogenous GSNO was determined above 2.8 nM, the limit of quantitation (LOQ) of the method. This study challenges previously reported GSNO plasma concentrations being far above the present method LOQ value and predicts that the concentration of low-molecular-mass and high-molecular-mass *S*-nitrosothiols are in the upper pM- and lower nM-range, respectively.

1. Introduction

S-Nitrosothiols or organic thionitrites with the general formula R—S—N=O (RSNO) are reaction products of organic thiols (R—S—H; RSH), notably cysteinyl thiols, and higher oxides of nitric oxide (NO) such as dinitrogen trioxide (N_2O_3). Despite its radical nature, NO is relatively stable in heme-free aqueous solutions. Three of the most reactive NO-species towards RSH are nitrous acid (H—O—N=O; HONO, pK_a 3.3), the nitrosyl cation ($^+$NO), and N_2O_3. Under acidic conditions, HONO is readily formed from nitrite, the autoxidation product of NO. From an analytical standpoint, RSNO are "problem children" of the NO family [1–3]. RSNO's *S*-nitroso group is thermally labile and chemically very reactive towards thiols, transition metal ions such as Cu^{2+} and reducing agents such as ascorbic acid. On the other hand, the *S*-nitroso group is readily formed under acidic conditions from HONO and RSH. Both nitrite and RSH are ubiquitous in biological systems. Together, these mechanisms give rise to abundant artefactual formation during sample preparation and analysis. RSNO lack self-fluorescence. The molar absorptivity coefficient of the *S*-nitroso group, the most characteristic functional group of RSNO, is quite low ($\varepsilon \approx 0.8\ mM^{-1} \times cm^{-1}$ around 334 nm) and does not allow sensitive quantification below about 1 μM by HPLC-UV [4]. Hence, physiological RSNO are commonly measured indirectly, for instance by converting the *S*-nitroso group to NO or nitrite for detection (for instance Refs. [5–16]). For recent reviews on RSNO analysis see Refs. [17–19]).

Twenty years ago, RSNO from low-molecular-mass (LMM) and high-molecular-mass (HMM) RSH have been reported to occur in various biological samples and to exert a variety of physiological functions [20]. Best investigated HMM RSNO include

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* Corresponding author. Tel.: +49 511 532 3984; fax: +49 511 532 2750.
E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).

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S-nitrosoalbumin (ALBSNO) in plasma and *S*-nitrosohemoglobin (HbSNO) in erythrocytes. ALBSNO and HbSNO are considered major storage forms of NO bioactivity in the circulation. Best investigated LMM RSNO include *S*-nitrosocysteine (CySNO), *S*-nitrosoglutathione (GSNO) and *S*-nitrosocysteinylglycine (GlyCySNO). LMM RSNO and the corresponding RSH, notably cysteine, are considered mediators of NO-related bioactivity bearing by ALBSNO and HbSNO [21–23].

Reactions involving transfer of the nitrosyl group $^{+}$NO from RSNO to RSH are characteristic RSNO features. The so called *S*-transnitrosylation reactions proceed rapidly, are reversible, and their equilibrium constants K_{eq} for known *S*-transnitrosylation reactions between physiological RSNO and RSH are close to unity in buffered solutions [4,24]. In theory, as an inevitable consequence of the *S*-transnitrosylation reactions, upon formation of the first RSNO, formation of RSNO from all physiological RSH can be expected.

Over the past 20 years reported RSNO basal values range over four orders of magnitude from "non detectable" to 10,000 nM and even higher for ALBSNO and HbSNO [2,3,18]. A consensus on reference values and intervals for physiological RSNO in human blood has not been reached [25,26]. Indeed, the concentration of physiological RSNO in humans remains controversial [3,18,22,27–29].

The most abundant HMM physiological RSH with respect to their SH functionality are albumin in human plasma (about 400 μM) and haemoglobin in erythrocytes (about 24 mM). The sum of LMM physiological RSH (Cys, GSH, GlyCys) in human plasma is of the order of 20 μM. In human erythrocytes, GSH occurs at concentrations of 2–4 mM and is the most abundant LMM RSH. In theory, ALBSNO should be the most abundant RSNO in human plasma and HbSNO in human erythrocytes. Stamler et al. reported, for the first time, that in six healthy humans the ALBSNO concentration is about 7 μM, while the sum of LMM RSNO, i.e., presumably CySNO, GSNO and GlyCySNO, is 300 nM [20]. These data are in line with $K_{eq} \approx 1$ and the physiological plasma concentrations of Cys, GSH, and GlyCys. Yet, the above-mentioned RSNO concentrations have not been confirmed by others. By means of a stable-isotope GC–MS method we determined a mean concentration of about 200 nM ALBSNO in human plasma [12,15]. This concentration is 35 times lower than that originally reported by Stamler et al. for ALBSNO [20]. We suggested the ALBSNO value of 200 nM as the upper limit for reference values for ALBSNO in human plasma [28]. Based on this value and assuming $K_{eq} \approx 1$ for RSNO/RSH equilibria, it can be estimated that the sum of CySNO, GSNO and GlyCySNO is not greater than 10 nM under physiological conditions, and that the concentration of each of these LMM RSNO is $\leq$3 nM. This estimate seems to be confirmed by data recently published for GSNO in human plasma. Thus, by means of an LC–MS/MS method that uses methimazole as an internal standard for GSNO a mean basal plasma GSNO concentration of 0.33 nM has been measured in healthy untreated subjects [30]. Unfortunately, the analytical performance of the employed LC–MS/MS method has not been reported satisfactorily thus far. The LC–MS/MS method and the reported pM-concentrations of GSNO measured by this technique in plasma of healthy humans remain to be verified [31].

Bramanti et al. hypothesized that very low RSNO concentrations may be due to insufficient RSNO stabilization in human plasma upon drawing blood. Indeed, given GlyCySNO's lability, conversion of GSNO to GlyCySNO by circulating γGT could rapidly decrease GSNO concentrations [32]. Bramanti et al. argued that the reason for measuring plasma concentrations of GSNO and other LMM *S*-nitrosothiols in the range of 50–350 nM in their methods is due to inhibition of plasmatic γGT activity by serine/borate and use of stabilizing factors such as NEM to block SH groups and EDTA to complex transition metal ions [33–36]. The GSNO concentrations measured by Bramanti et al. in plasma of healthy humans are about 150–1000 times higher than the GSNO concentrations reported by Taubert et al. for healthy subjects [30]. Thus, RSNO concentrations in human plasma remain uncertain to date.

Over the last years, the LC–MS/MS methodology greatly advanced, especially sensitivity was increased several fold due to progress in chromatography and ionization/transition. In consideration of these technological improvements and of the great deal of information about the chemistry and biochemistry of RSNO available to date, we thought that a UPLC–MS/MS-based method would allow specific, accurate, artefact-free and fast quantification of GSNO in human plasma. This article reports on the development, validation and application of a stable-isotope dilution UPLC–MS/MS method for the quantification of GSNO in human plasma.

2. Experimental

2.1. Chemicals and materials

Sodium nitrite (purity 99.99+%), sodium [^{15}N]nitrite (declared as 99 at% at ^{15}N), glutathione (GSH), glutathione disulfide (GSSG), 2-mercapto-1-methylimidazole (1,3-dihydro-methyl-2H-imidazol-2-thion; methimazole; thiamazole), *N*-ethylmaleimide (NEM) and borax were purchased from Sigma–Aldrich (Steinheim, Germany). Formic acid, ammonia of LC–MS quality, tetra-*n*-butyl ammonium hydrogensulfate (TBAHS), NaCl and K_2HPO_4 were obtained from Merck (Darmstadt, Germany). Ammonium formate of LC–MS quality was purchased from Fluka (Deisenhofen, Germany). Acetonitrile of LC–MS quality, KH_2PO_4, Na_2HPO_4 and NaH_2PO_4 were from Riedel-de-Haën (Seelze-Hannover, Germany). Methanol and other organic solvents were acquired from Mallinckrodt Baker (Griesheim, Germany). Distilled water was daily prepared by using a Milli-Q Synthesis A10 System (Millipore, Billerica, MA, USA). Human γ-glutamyltransferase 1 was obtained from Cell Sciences (Canton, USA). Vivaspin 2 Hydrosart cartridges (2 mL; cut-off, 10 kDa) for ultrafiltration were supplied by Sartorius (Göttingen, Germany). Ultrafiltration was carried out using the ultracentrifuge model Multifuge 3S-R from Heraeus (Hanau, Germany). All glass vials used came from Macherey-Nagel (Düren, Germany). Lithium heparin vacutainer tubes (9 mL) used for blood sampling were obtained from Sarstedt (Nümbrecht, Germany).

2.2. LC columns for LC–MS analysis of GSNO

The following columns were used for the LC–MS/MS analysis of GSNO (see also Table 1). Nucleoshel HILIC and Nucleodur HILIC were from Macherey-Nagel (Düren, Germany). ZIC HILIC was purchased from Merck (Darmstadt, Germany). Kinetex 2.6u HILIC was from Phenomenex (Aschaffenburg, Germany). Aquity UPLC BEH Amide was bought from Waters (Eschborn, Germany).

2.3. Liquid chromatography–mass spectrometry conditions

Analyses were performed on a Waters ACQUITY UPLC–MS/MS system consisting of a solvent delivery device, an autosampler, a column thermostat and the tandem quadrupole mass spectrometer XEVO TQ MS (Waters, Milford, MA, USA).

The mobile phase was acetonitrile–water (70:30, v/v) and contained 20 mM ammonium formate. Mobile phase pH was adjusted to 7.0 by using 10 vol.% aqueous ammonia. Isocratic elution at a flow rate of 0.5 mL/min was performed. LC-columns and autosampler were thermostated at 30 °C and 4 °C, respectively. The sample loop had a volume of 50 μL. Injections (10 μL in quantitative analyses) were performed in the partial loop with needle overfill mode.

Electrospray ionization in the positive (ESI+) mode was used with nitrogen (600 °C, flow rate of 1100 L/h) as the desolvation gas. Capillary and cone voltages of 3.5 kV and 14 kV were found to be optimum and used in quantitative analyses. The