Nitrosothiol Detection by HPLC Coupled with Flow Reactors of Hg$^{2+}$ and Griess Reagent

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Introduction

NO-related intermediates, including NO$^+$ like species, can participate in nitro-sating additions to nucleophilic centers of biological molecules. Sulphhydryl-containing molecules such as glutathione are particularly susceptible to nitrosation and form nitrosothiol adducts (nitrosothiols; RS-NOs). It seems that these adducts in biological systems play an important role in NO-mediated signalling cascades such as the downregulation of N-methyl-D-aspartate receptor, and the regulation of transcriptional factors [1]; they might also be involved in non-adrenergic and non-cholinergic neuronal responses [2]. It is, therefore, essential to identify nitrosothiols specifically and to quantify them in biological systems.

Herein we describe a sensitive and specific HPLC method coupled with Hg$^{2+}$ and Griess reagent for nanomolar quantification of a wide range of RS-NOs including low-molecular weight RS-NOs such as nitrosogluthathione (GS-NO) and nitrosothiocynte (Cys-NO), and also S-nitrosoproteins, in particular S-nitrosoalbumin and S-nitrosohaemoglobin [3].

Preparation of S-nitrosoproteins

Protocol

**Reagents:** Bovine serum albumin (BSA) (Nacalai Tesque, Osaka, Japan), dithiothreitol (DTT; Wako Pure Chemicals, Osaka, Japan), isooamyl nitrite (Wako), ethylenediaminetetraacetic acid (EDTA; Dojindo Laboratories, Kumamoto, Japan), diethylenetriaminpentacetic acid (DTPA; Dojindo), and 5', 5'-dithiobis[2-nitrobenzoic acid] (DTNB; Nacalai).

**Procedure:** Reduction of BSA

1. Add DTT (100 mM, 10 μL) to BSA (1 mM, 1 mL) in sodium phosphate buffer (pH 7.0, 100 mM).
2. Incubate for 30 min at 37°C.
3. Apply to a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column equilibrated with sodium phosphate buffer (pH 7.0, 10 mM) containing EDTA (1 mM; buffer A), and elute with buffer A.
4. Collect the fractions containing BSA and concentrate the fractions by ultrafiltration.

**Nitrosation of the reduced BSA:**

1. Add isooamyl nitrite (100 mM, 10 μL) to reduced BSA (0.1 mM, 1 mL) in sodium phosphate buffer (pH 7.8, 100 mM) containing DTPA (0.5 mM).
2. Incubate for 30 min at 37°C.
3. Apply to a Sephadex G-25 (Pharmacia) column equilibrated with buffer A, and elute with buffer A.
4. Collect the fractions containing S-NO-BSA and concentrate the fractions by ultrafiltration.

Quantification of Nitrosothiols

**Protocol**

**Reagents:** Nitrosoproteins (e.g., S-NO-BSA) prepared above, GS-NO (Dojindo), Cys-NO (Dojindo), HgCl$_2$, and Griess reagent (naphthylethylenediamine; sulfanilamide (Wako)).

**Principle:** RS-NOs of different molecular size separated by HPLC are converted to NO$^+$ or NO$_2$ in a flow reactor system connected serially to the HPLC (Figure 1), by rapid and quantitative metal-catalysed reaction with Hg$^{2+}$. As reported by Saville, Hg$^{2+}$ decomposes the nitrosothiols stoichiometrically to nitrite according to the equations [4,5]:

\[
\text{RS-NO + Hg}^{2+} \leftrightarrow \text{RS-(Hg)}\text{NO}^{-} \quad [4]
\]

\[
\text{[RS-(Hg)NO]^- + H}_2\text{O} \rightarrow \text{RSHg}^+ + \text{NO}_2 + 2\text{H}^+ \quad [5]
\]

Peak detection is based on the colorimetric assay of nitrite using Griess reagent, which forms a diazo dye having strong absorbance at 540 nm under acidic conditions; the extinction coefficient is 53000 M/cm [6].

**HPLC conditions**

The injection volume is 150 μL.

**Column**

1. Separation: a C18 • reversed phase column (4.6×250 mm;
TSKgel OOS.80Ts (Tosoh, Tokyo) for low molecular weight RS-NOs; a gel filtration column (8×300 mm; Oiol-120; YMC, Kyoto) for the nitrosoproteins.

2. Deproteinating column: small columns (3×10 mm) packed with C18-based resin are placed just before the separation column and just after Hg$^{2+}$ flow reactor coil, in the reversed-phase and gel filtration systems, respectively.

**Mobile phase**

1. Pump 1 for HPLC: sodium acetate buffer (pH 5.5, 10 mM) containing DTPA (0.5 mM) and methanol (0-7%), for low molecular weight RS-NOs (reversed-phase HPLC); sodium acetate buffer (pH 5.5, 10 mM) containing DTPA (0.5 mM) and NaCl (150 mM) for the nitrosoprotein (gel filtration HPLC).

2. Pump 2 for Hg$^{2+}$-flow reactor: HgCl$_2$ (1.75 mM) in sodium acetate buffer (pH 5.5, 10 mM).

3. Pump 3 for Griess reagent flow reactor: naphthylethylenediamine (0.1%) in H$_2$O; sulfanilamide (1.0%) + phosphoric acid (2.0%) in H$_2$O.

**Flow rate:** 0.55 mL/min (pump 1), 0.2 mL/min (pump 2), 0.24 mL/min (pump 3)

**Detector:** visible detector (Eicom, Kyoto) operated at 540 nm.

Typical elution profiles of GS-NO, Cys-NO and S-NO-BSA are shown in Figure 2.

Figure 3 illustrates the correlation between the peak area of GS-NO calculated by the integrator and different concentrations examined by use of the HPLC flow reactor. The detection limit for GS-NO was found to be >3 nM.

**Figure 2:** Elution profiles of different RS-NOs for the HPLC/Hg$^{2+}$ flow reactor system.

A: Low molecular weight RS-NOs (reversed-phase HPLC), B: High molecular weight RS-NOs (gel filtration HPLC)

**References**


