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

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Keywords: Nitrosothiol; HPLC; S-nitrosoproteins; Quantification

Introduction

NO-related intermediates, including NO^+ like species, can participate in nitro-sating additions to nucleophilic centers of biological molecules. Sulfhydryl-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]. lt 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 nitrosoglutathione (GS-NO) and nitrosocysteine (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), isoamyl nitrite (Wako), ethyllenediaminetetraacetic acid (EDTA; Dojindo Laboratories, Kumamoto, Japan), diethylenetriaminepentaacetic 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 isoamyl 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]:

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

$$[RS\text{-}(Hg)NOj^{2+} + H_2O \rightarrow RSHg^+ + NO_2^- + 2H^+ \quad [5]$$

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 C1B • reversed-phase column (4.6×250 mm;

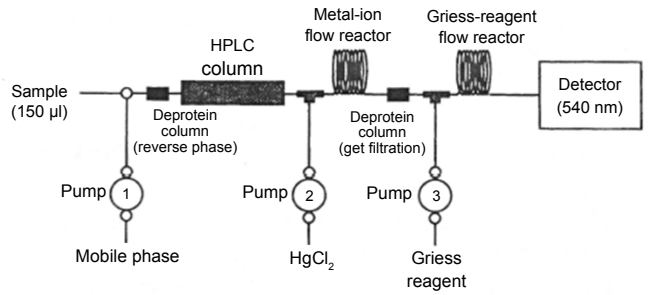

Figure 1: Flow diagram of the HPLC-flow reactor system for measurement of different RS-NOs by use of Hg^{2+}, and Griess reagent flow reactors.

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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 C1B-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 NaCI (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_2O; sulfanilamide (1 .0%) + phosphoric acid (2.0%) in H_2O.

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 f10w reactor. The detection limit for GS-NO was found to be >3 nM.

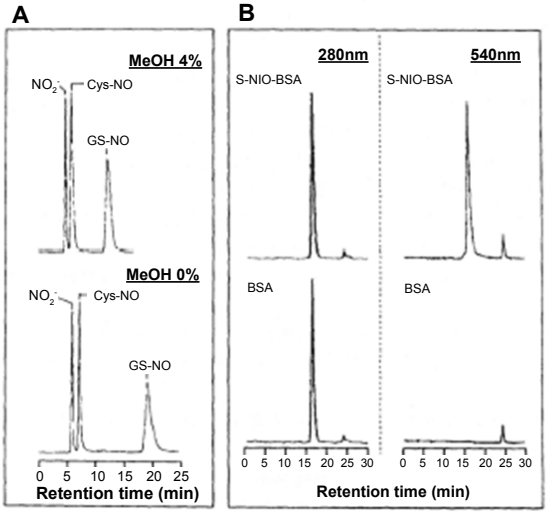
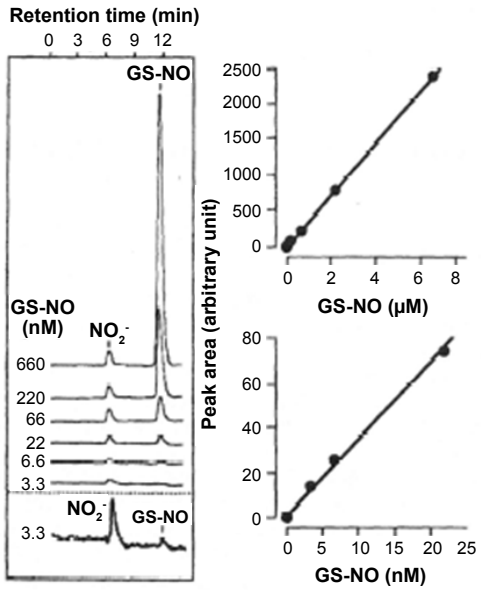


Figure 2: Elution profiles of different RS-NOs for the HPLC/Hg^{2-} flow reactor system.

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



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