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Analytical methods

Hemoglobin effects in the Saville assay

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Abstract

There is a great need to establish accurate, sensitive methods for measuring the concentration of nitrosothiols. Although some progress may have been made recently, differing methodologies have lead to reports of basal levels of nitrosothiols in human plasma that differ by three orders of magnitude. The Saville assay has been widely accepted as an accurate method for measuring nitrosothiols, but one that suffers from sensitivity below that of some other methods. Recently, it has been suggested that when hemoglobin is included in reaction mixtures used for the Saville assay, the sensitivity can be increased by an order of magnitude. Here we show that, on the contrary, the presence of sufficient hemoglobin in the Saville assay decreases its sensitivity.

Keywords: Nitric oxide; Nitrosothiols; Saville assay; Hemoglobin

The best or even appropriate methods for measuring nitrogen oxide species, especially nitrosothiols, are the subject of much controversy [1,2]. Common techniques used to measure nitrosothiols include fluorescence, chemiluminescence detection, the Saville assay, and direct measurement by absorbance [3]. Published results using these assays have reported concentrations of nitrosothiols in humans that differ by three orders of magnitude [2]. For example, the amount of *S*-nitrosohemoglobin in human blood has been reported to be as high as 2.5 μM [4] to as low as 1 nM (at the most) [5]. Plasma nitrosothiols in humans also have been reported to be as high as 7 μM [6] and as low as 7 nM [7]. The Saville assay is relatively inexpensive with a detection limit reported around 0.1 μM [3]. Chemiluminescence-based techniques, however, have been reported to detect nitrosothiols at levels at least 1000 times smaller than those for the Saville assay [3]. In order for research on nitric oxide to flourish, commonly accepted procedures for measurement of nitrogen oxide species must be established.

The Saville assay works by displacement of NO^+ from the thiol using mercury which then leads to subsequent reaction in the Saville mixture to form a colored azo dye [3,8]. Recently, it has been suggested that inclusion of hemoglobin (Hb) in the Saville assay mixture increases the assay's sensitivity by an order of magnitude [9]. This increase in sensitivity was attributed to basal absorbance of the Hb of about 0.3–0.4 which would bring the total absorbance in the presence of the dye (which is below 0.1 for low concentrations of nitrosothiols), into a range where absorption spectroscopy is more accurate. In this paper, we examine the rationale behind this approach and present data from measurements of *S*-nitrosoglutathione (GSNO) concentration in the presence and absence of Hb. Contrary to previous work [9] we conclude that inclusion of Hb in the assay actually gives poorer results from when it is not included.

Experimental procedures

All chemicals were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO) unless otherwise indicated. GSNO was prepared as described previously [10]. The initial concentration of GSNO in the stock solution

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used to calculate the expected concentration of diluted samples was determined by the absorption at 338 nm and verified by a chemiluminescence method [11]. Diluted samples of GSNO were measured using the Saville assay as described previously [8,9]. Reagents prepared include solution A (1 mM ammonium sulfamate in 0.5 M HCl), solution B (1% sulfanilamide in 0.5 M HCl), solution C (1% sulfanilamide and 0.2% $HgCl_2$ in 0.5 M HCl), and solution D (0.02% *N*-(1-naphthyl)-ethylenediamine-dihydrochloride). Two sets of samples were prepared: (1) GSNO ranging from 50 nM to 5 μM and (2) GSNO ranging from 50 nM to 5 μM with 150 μM oxyhemoglobin (HbA). For each reaction mixture, 1 ml of two samples was mixed with 1 ml of solution A and incubated for 10 min, followed by addition of 1 ml of solution B to one sample and 1 ml of solution C to the other. After incubation for 5 min, 1 ml of solution D was mixed with both samples, and incubated for another 5 min until formation of the azo dye was complete. The absorbance of each sample was read at 540 nm using a Cary 50 spectrophotometer (1 cm path length) from 700 to 450 nm scanning at a rate of about 5 nm/s. Some additional measurements were performed using a Cary 100 spectrometer scanning at a rate of 10 nm/s. No significant difference was observed when either spectrometer was employed. The final volume of the reaction mixture was 4 ml and all reactions were done at room temperature. The concentration of GSNO was determined by subtracting the absorbance of samples treated with solution B from the absorbance of samples treated with solution C, and calculated using an extinction coefficient of 51 $mM^{-1}cm^{-1}$ at 540 nm. The difference in absorption was calculated both with and without baselining the spectra at 700 nm. Baselining was implemented by subtracting the absorbance at 700 nm to that at all other wavelengths.

Results

Fig. 1 shows examples of absorption spectra collected from GSNO in the presence and absence of Hb. The spectra are for a sample originally containing 0.100 μM GSNO, so that after the fourfold dilution associated with the Saville assay (see Experimental procedures), one expects to measure 0.025 μM GSNO. This is what was obtained in the absence of Hb. On the other hand, analysis of the data collected in the presence of Hb gave a concentration of −0.32 μM. The fact that the absorbance (and calculated concentration) came out negative when Hb was present is probably due to the sensitivity to small errors in pipetting associated with trying to measure a small signal in a large background (see Discussion). Fig. 1 also shows that for a comparable signal (absorbance approximately equal to 0.0015) the noise is larger in the presence of Hb than in its absence.

Fig. 2 shows a summary of results from three to seven trials of the Saville assay on a range of GSNO concentrations. Data are shown when the assay was conducted in the presence or absence of Hb. The best results (shown) for both methods (with and without Hb) were obtained when the absorption spectra were baselined to zero at 700 nm. Some data were collected using more concen-

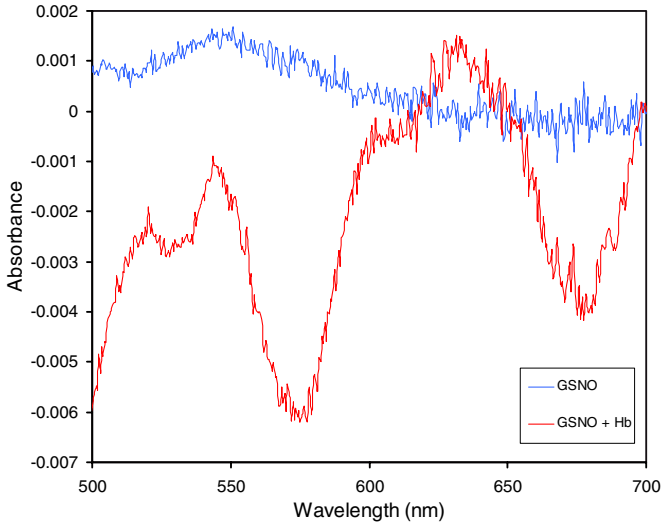

Fig. 1. Absorption spectra from the Saville assay with and without Hb. Difference spectra (those taken without Hg were subtracted from those taken with Hg present) vs. wavelength are shown for a sample initially containing 0.1 μM GSNO with and without 150 μm oxyHbA. Without oxyHbA, the absorbance at 540 nm (after fourfold dilution of the 0.1 μM starting material) was 0.00129 and the GSNO concentration was calculated to be 0.0252 μM. With oxyHbA, the absorbance was measured to be −0.00166 and the concentration was calculated to be −0.0325.

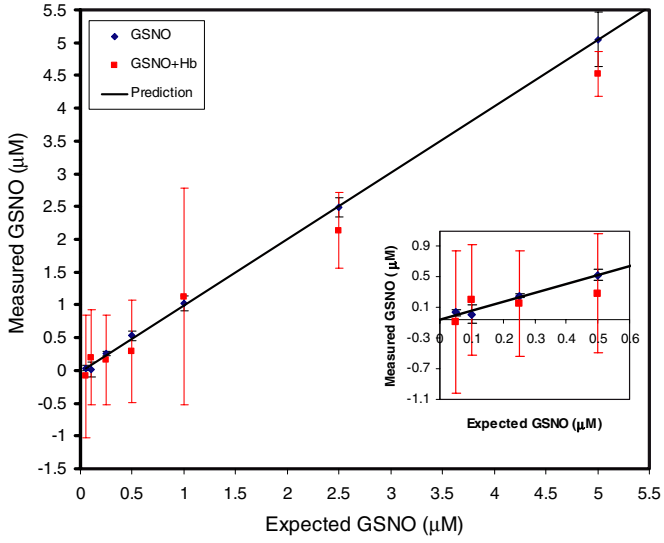

Fig. 2. Summary of measurements of GSNO using the Saville assay with (■) and without (◆) Hb present. The average and standard deviation are shown from three separate sample preparations and assays conducted on different days. The points for expected GSNO of 0.5, 1, 2.5, and 5 μM also include data from four additional experiments carried out on a Cary 100 spectrometer (yielding a total of seven trials). The concentrations shown are for the initial concentration of GSNO (before the fourfold dilution). The predicted amount of GSNO is also shown based on the initial concentration of GSNO (determined by absorbance and the triiodide method) and calculations based on subsequent serial dilutions. The inset shows lower concentrations.