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Short communication

Bromide–sulfur interchange: Ion chromatographic determination of total reduced thiol levels in plasma

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Abstract

Plasma thiol concentration has long been recognised as a potential indicator for assessing the severity of oxidative stress processes within physiological systems. While such measurements are normally restricted to research studies, this communication has sought to develop and characterise a novel approach through which this parameter could be exploited within routine clinical settings. The protocol is based on the rapid derivatisation of reduced thiol functionalities (protein and monomolecular moieties) through the homogenous reaction of a naphthoquinone bromide derivative. Bromide released in the reaction can be easily quantified through ion chromatography (Isocractic Dionex DX-120 incorporating an IonPac® AS14 anion exchange column and a 25 μL sample loop with conductivity detector. Mobile phase consisted sodium carbonate/bicarbonate (3.5 mM/1 mM) at a flow rate of 1.5 mL/min). Method selectivity and sensitivity has been critically evaluated. The technique covers the range 15 μM–3.5 mM PSH with a detection limit of 9 μM PSH and analysis time of 5 min. The efficacy of the approach for the analysis of human plasma from five volunteers was assessed (ranging from 49 to 72 μM with an intra assay variation of less than 5% in all cases). The responses were validated through comparison with the standard Ellman colorimetric technique.

Keywords: Albumin; Plasma; Thiol; Bromide; Ion chromatography; Oxidative stress

1. Introduction

Plasma thiols (PSH) such as glutathione, cysteine and albumin are known to play a crucial role in the prevention of free radical induced oxidative damage. There are, however, occasions where the increased production of the latter can overwhelm such defences and have a significant impact on physiological well-being [1,2]. The potential diagnostic value of measuring total PSH concentration has long been recognised with the depletion in concentration of the reduced thiol proffered as a generic indicator for the onset of various clinical complications. These have been associated with diabetes [3–5], various forms of cancer [6,7], kidney function [8,9] and pre-eclampsia [10,11]. There is little doubt that PSH could, in the appropriate context, provide a semi-quantitative handle through which to identify the onset and monitor the progress of illnesses exacerbated by oxidative stress processes. Clinical acceptance and exploitation of the PSH measurement has largely been restricted to research studies. Transforming the parameter from research tool to a robust indicator that can positively influence patient management, however, can only be achieved through widespread adoption and more extensive trials within clinical contexts. It is to this end that the present communication has sought to assess the efficacy of a novel ion chromatographic (IC) approach that could facilitate the rapid measurement of total PSH within plasma samples.

The detection route proposed in the present instance is detailed in Scheme 1 and involves the nucleophilic substitution of a bromide derivatised quinone indicator (**I**). In the presence of reduced thiols (**II**), it could be anticipated that a relatively rapid reaction would occur leading to the formation of the corresponding quinone–thiol conjugate (**III**) and release of bromide ion. The latter would act as an indirect measure of total PSH and, as IC is a widely established technique within most bioanalytical environments, it should allow rapid technology transfer from

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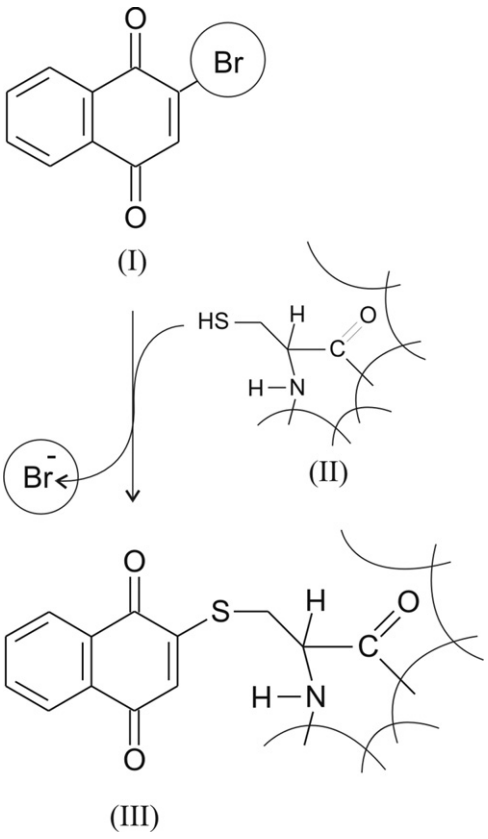


Scheme 1. Naphthoquinone bromide (NQBr) reaction pathway.

research study to routine application. Numerous strategies have been employed for the detection of thiols and the various merits and limitations have been critically reviewed [12–14]. A core aim of method proposed within Scheme 1 is that it will provide greater simplicity than many of the assay systems previously reported.

In principle, the approach should be capable of direct integration within routine electrolyte measurements without compromising the integrity of the latter nor unduly increasing the procedural workload. In doing so, the PSH data should be readily accessible and hence facilitate a greater critical assessment of the parameter's clinical remit. The main analytical issues relating to the use of the system advocated in Scheme 1 are the selectivity, sensitivity of the indicator and the ease with which it can be adopted for routine use. The investigation has sought to address these concerns, assess the transferability of the technique to the analysis of real samples (human plasma) and provide a critical appraisal of efficacy in relation to conventional technologies.

2. Experimental

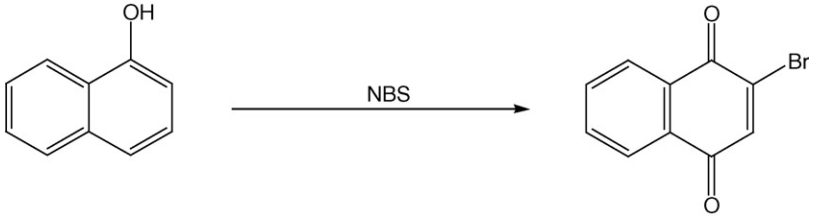
2.1. Methods and materials

All reagents were of the highest grade available and used without further purification. All standards were prepared using UHQ deionised water (Elga, High Wycombe, UK) with a resistivity of not less than 18 $M\Omega^{-1}$ cm. Chromatographic analysis was carried out in isocratic mode with a Dionex (Camberley, UK) DX-120 series ion chromatography system equipped with a Dionex IonPac® AS14 anion exchange column and a 25 μL sample loop. The mobile phase consisted of nitrogen purged sodium carbonate/bicarbonate (3.5 mM/1 mM) at a flow rate of 1.5 mL/min. Detection was achieved through the use of an integrated conductivity cell thermostatted at 35 °C. Absorbance spectra were obtained using a PerkinElmer (Beaconsfield, UK) Lambda 25 spectrometer. NMR spectra were measured on a JEOL (Welwyn Garden City, UK) ECX 400 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Mass spectra were recorded on a Micromass (Elstree, UK) Platform LC–ESI–MS. Flash chromatography was performed on 40–63 silica gel (Merck). IR was recorded on a PerkinElmer Spectrum 100 Series with a Universal ATR Sampling Accessory.

2.2. Clinical samples

A clinical trial involving five volunteers was conducted. The participants were aged between 20 and 45. Blood was collected in heparinised gel permeation vaccutainers and then centrifuged at 3000 rpm (500 × *g*) for 5 min. The plasma was withdrawn and stored at −18°C. The concentration of PSH was assessed prior to storage and after defrosting using an established Ellman's Assay protocol with the calibration and analysis data taken at 412 nm [3,5,6,15].

2.3. Preparation of 2-bromo-1,4-naphthoquinone



A solution of 1-naphthol (0.72 g, 0.50 mmol) in glacial acetic acid (50 mL) was added to a stirring solution of *N*-bromosuccinimide (3.56 g, 20 mmol) in 2:1 water/glacial acetic acid (150 mL) at room temperature over a period of 60 min. The mixture was stirred at room temperature for 12 h followed by heating to 50–60 °C with stirring for 1 h. Subsequently, water (100 mL) was added to the reaction mixture followed by extraction into ethyl acetate (3 × 100 mL). The organic layer was combined and washed with 5 M $NaHCO_3$ solution, dried over Na_2SO_4, filtered and concentrated under vacuum to yield the crude product. Purification using column chromatography on

silica gel with ethyl acetate and hexane (7:3) as eluent yielded the 2-bromo-1,4-naphthoquinone (0.65 g, 54.4%) as a yellow powder.

^{1}H NMR ($CDCl_3$): δ 8.19–8.17 (m, 1H, Ar–H), 8.10–8.08 (m, 1H, Ar–H), 7.80–7.77 (m, 2H, Ar–H), 7.52 (s, 1H, Ar–H).

^{13}C NMR ($CDCl_3$): δ 126.9, 127.9, 131.7, 134.2, 134.5, 134.6, 140.2, 140.4, 177.9, 182.5.

IR (ATR): 3055 (Ar–H), 1676 (C=O), 1656 (C=O), 1588 (C=O), 1292 (C=O), 1244 (C=O), 1058 (Ar–Br). ESI–MS (*m/z*) 236 $[M+H]^-$.

2.4. Ion chromatographic assay procedures

A standard solution (5 mM) of 2-bromo-1,4-naphthoquinone (NQBr) was prepared in HPLC grade acetonitrile. Calibration solutions were prepared by dilution from a stock solution of glutathione (10 mM dissolved in electrolyte containing chloride, nitrate and phosphate—each at 100 mg/L). The analysis samples were prepared by adding 325 μL of the NQBr solution to 175 μL of the appropriate standard (0.025–1.0 mM). The solution was gently mixed and then injected into the IC through a 0.45 μm filter (Millipore). An analogous procedure was used for the plasma samples. Upon the introduction of the acetonitrile indicator solution, rapid precipitation of protein was observed. The resulting slurry was then centrifuged at 10,000 rpm (5590 × *g*) for 5 min and the supernatant injected as before.

3. Results and discussion

The spectroscopic profile of 100 μM NQBr (pH 7) is shown in Fig. 1. The native UV chromophore would be readily accessible to conventional LC methodologies and could potentially prove to be a versatile label with the column resolution of the conjugates facilitating the direct speciation of low molecular weight components (i.e. glutathione, cysteine). Bromo functionalised agents such as pentafluorobenzyl bromide (PFBB) have been widely used for this purpose [16–18]. They are, however, relatively non-specific and react with a host of other functionalities [19–21] beyond thiols and rely upon column resolution of the resulting PFB conjugates for quantification of the intended target [16–21]. In all cases, the expunged bromide is simply a by-product of the reaction. In this case, it was hoped that the NQBr would be selective for only the one functionality such that the release of bromide would represent the stoichiometric equivalent of the available SH functionalities. This should simplify the analysis and interpretation of the resulting data.

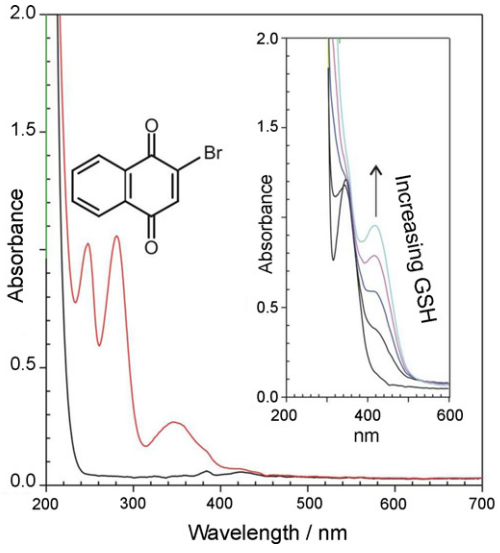


Fig. 1. Absorbance spectrum of NQBr (100 μM, pH 7). *Inset*: Response of 500 μM NQBr to increasing additions (90 μM) of glutathione (GSH).

The response of 500 μM NQBr to increasing additions of glutathione (90 μM aliquots) is shown in the inset diagram within Fig. 1. The quinone–thiol conjugate (**III**, Scheme 1) emerges as a new absorption peak at 420 nm. The reaction is rapid (within 30 s) and was found to be stable with no appreciable change (<5%) in absorbance over 24 h. While it could be possible to use the quinone derivative as a simple colorimetric reagent, the performance ($\varepsilon = 2417\ L\ mol^{-1}\ cm^{-1}$) is considerably poorer than that of Ellman's Reagent ($\varepsilon = 13{,}000\ L\ mol^{-1}\ cm^{-1}$) [15]. The NQBr was found to react readily with other reduced thiols (cysteine, homocysteine and bovine serum albumin) with little difference in the absorption characteristics from the glutathione response detailed within Fig. 1. The peak position for the conjugate of cysteine was found to shift to 450 nm, but this is of little diagnostic merit when considering the direct analysis of complex mixtures. There were no interactions with lysine or histidine even at fold excess and this is consistent with previous investigations of functionalised quinone systems used for electrochemical analysis [22]. The influence of pH was also investigated with the NQBr response to glutathione unimpeded across the range pH 3–9. These preliminary results would suggest that under the relatively mild conditions used in the assay (Section 2.3) that NQBr is indeed selective for PSH functionality.

The ion chromatographic response of the NQBr assay procedure towards increasing glutathione (0.06–1.5 mM) is shown in Fig. 2. Four peaks are observed throughout and correspond to the anions that are liable to be found within most typical biofluids: chloride, nitrite, nitrate and phosphate. The introduction of glutathione induces the reaction with NQBr (Scheme 1) and hence the release of bromide. The latter emerges between the nitrite and nitrate signals with clear resolution between the respective peaks. Near identical responses are observed for cysteine and albumin—as expected given that bromide is the principal agent in all cases. There is no opportunity for speciation in contrast to LC methodologies, but the aim here has been focused on the acquisition of a total reduced PSH measurement. The response was linear over the range 15 μM–3.5 mM PSH. The calibration data relating to the bromide peak was found to be: response (μS) = 3.80×10^4 [mol/L PSH] − 0.014 ($N = 8$, $R^2 = 0.9997$). The recoveries for 80 and 500 μM glutathione were 92% and 96%, respectively. The limit of detection was found to be 9 μM (based on 3*sb*) but while insufficient for trace level studies [1,2], it is more than adequate to cover the broad range (300–600 μM) typical for PSH measurements within plasma [3–8].

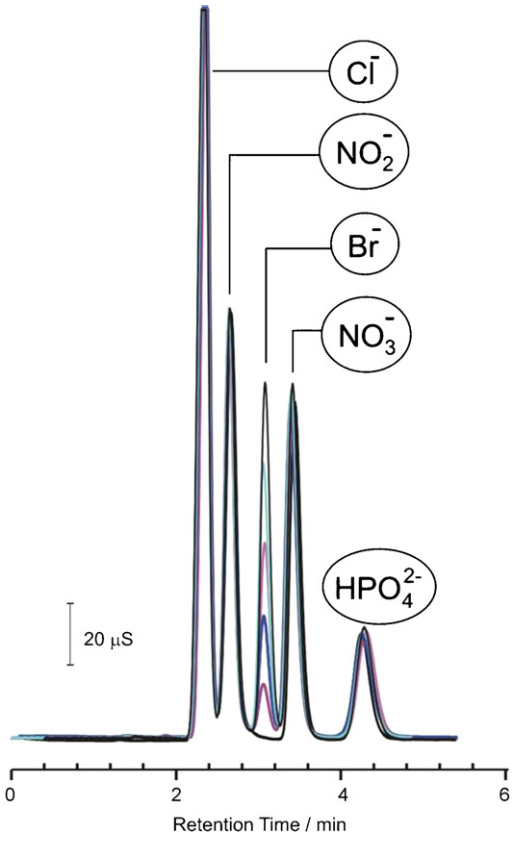


Fig. 2. Ion chromatograms detailing the release of bromide as a consequence of the reaction of NQBr with increasing concentrations of glutathione (250 μM increments).

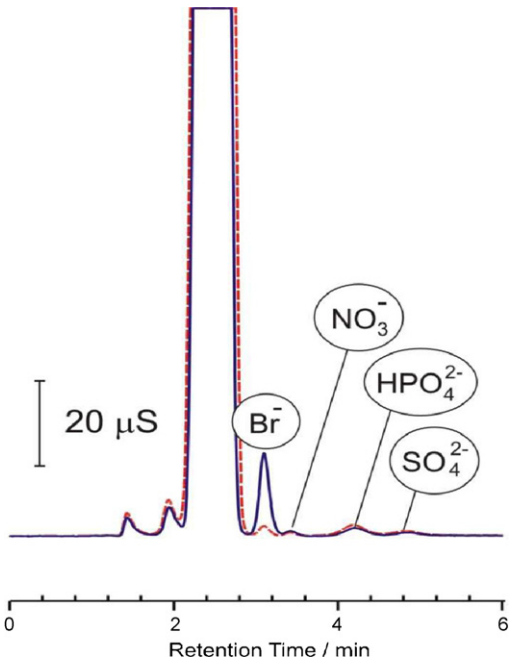


Fig. 3. Ion chromatograms detailing the response obtained to plasma before (dashed line) and after (solid line) reaction with NQBr.

Table 1
Plasma thiol (PSH) concentrations

Gender	Age	NQBr IC (mM)	Ellmans reagent (mM)
M	37	0.362	0.373
F	24	0.365	0.361
M	30	0.334	0.374
F	27	0.406	0.401
F	37	0.384	0.391

Each measurement based on three replicates (R.S.D. < 5%).

4. Clinical study

The ion chromatographic response to plasma before (dotted line) and after (solid line) treatment with the NQBr solution is shown in Fig. 3. The dominant peak, while mostly chloride, is liable to contain a host of components and it was not possible to isolate these in the present study. More importantly, there is clear resolution between the composite chloride peak, bromide and the other anionic electrolytes. Bromide is clearly observable in the untreated plasma with the concentration between different plasma samples found to range from 49 to 72 μM and is consistent with the values found in previous investigations [23–25]. The presence of endogenous bromide does not unduly compromise the validity of the measurement as the appropriate correction can be made through simply running the plasma sample without the NQBr pre-treatment. The results from five plasma samples (after subtraction of endogenous bromide) are detailed in Table 1 along with the results obtained using a standard Ellman's Reagent protocol [3,5,6,15]. The measurements were made in triplicate with the intra assay variation being less than 5% in all cases.

It can be argued that where gross changes in thiol concentration are to be used as a screening system for identifying underlying illness, the relatively constant nature of the endogenous bromide will have little influence and hence only a single measurement would be necessary. This is corroborated by recent studies, where the PSH levels measured in diabetics suffering from vascular complications were 25–33% lower than that of the corresponding control group [3,4]. While similar variations have been observed with pre-eclamptic pregnancies [10,11], the difference can be substantially greater in cancer patients, where in the case of oral squamous cell carcinoma, the levels were decreased by more than 50% when compared with healthy controls [6]. This can represent a depletion of more than 0.25 mM from the respective control group.

5. Conclusions

The potential value of the PSH parameter is two-fold—as an early warning indicator for the onset of underlying oxidative injury and as a marker that could allow the progress of the illness and the subsequent treatment to be assessed in a semi-quantitative manner. The ability to routinely monitor the concentration of PSH by simply modifying an existing IC protocol satisfies the criteria for both options. Importantly, it obviates the need for requesting a separate test set (cf. Ellman's) as it can be accomplished in the normal course of electrolyte measurements without compromising the integrity of the latter. The system proposed here offers a relevant detection range, simplicity of

operation and utilises established technology. While the assay provides an unambiguous signal for bromide within plasma, further investigations would be required to establish its generic application to thiol detection. In its present form—it should, however, provide clinicians with a more facile route through which they can evaluate the use of PSH in different contexts.

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