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Antioxidant and trace metal determinations in human lavage fluids

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We use this protocol and it's working

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Abstract

Low molecular weight antioxidants (including ascorbate, urate and glutathione), metal transport and chelation proteins (including transferrin and ferritin) and metals (including Fe and Cu) concentrations, including a measure of the catalytically active metal pool, can be determined in respiratory tract lining fluid (RTLF) collected from bronchoalveolar lavage from the diseased patients and healthy controls.

Guidelines

The determination of antioxidant concentrations and their associated oxidative products in biological samples is not a trivial task, as the process of obtaining, handling, and processing samples both prior to and post storage, during the actual assay procedures, has the potential to result in erroneous oxidation. It is therefore essential that sample processing is rapid, samples are kept chilled, away from the light and potential metal contamination. Whilst much focus in the literature has focused on the antioxidants themselves, it is also the case that many of the oxidation products themselves are transient, being subject to further oxidation and hydrolysis. The methods described below have been optimised to ensure the rapid and reliable quantification of antioxidants and their oxidation products in complex biological substrates.

Materials

All chemicals used in step 2 and subsequent analyses were obtained from Sigma Aldrich Chemical Company Ltd (Poole, UK) unless otherwise stated.

Before start

Collection and preparation of bronchoalveolar lavage fluid samples for antioxidant analysis:

Bronchoscopy was performed on an outpatient basis, following an overnight fast, using a flexible video bronchoscope (Olympus BF IT240, Tokyo, Japan) inserted through the mouth with the subject in the supine position.

Pre-medication with atropine (1 mg) was given subcutaneously 30 minutes prior to bronchoscopy to reduce airway mucus secretion, with Lidocain (5% and 1%) sprayed onto the airways to achieve topical anaesthesia.

Collection and preparation of bronchoalveolar lavage fluid samples for antioxidant analysis:

- 1 Bronchoalveolar lavage (BAL) was performed by the instillation and immediate aspiration of 3 consecutive aliquots of 60 mL sodium chloride (NaCl) saline pH 7.3 at 37° C into the lingular or middle lobe.

These recovered aspirates were kept as separate aliquots on wet ice.

BAL fluids were passed through a nylon filter (pore diameter 100 µM) to remove the mucus and the cellular content was isolated by centrifugation at 400 rpm for 15 minutes at 4°C.

The obtained pellets were then resuspended in phosphate buffer solution (PBS) to a concentration of 106 cells/ml. Total and differential cell counts were carried out on cyto-centrifuge preparations stained with May-Grünwald Giemsa, with a 400 total cell count per slide.

The resultant supernatants for BAL were then pooled and treated as follows:

An aliquot of lavage fluid (450 µL) was treated with 50 µL of 50% metaphosphoric acid (MPA), vortexed for 30 seconds and centrifuged at 13,000 rpm for 5 minutes (4°C) to remove protein. The resultant supernatant was then stored at -80°C within 30 minutes of BAL collection until required for analysis.

Samples for GSH and GSSG determination were treated with the metal chelator deferoxamine mesylate (DES – Sigma) and the synthetic antioxidant butylated hydroxytoluene (BHT – sigma) both at 2 mM, 5 µl of each to 490 µl of lavage, prior to storage at -80°C.

The remaining lavage was untreated but immediately aliquoted and stored at -80°C. These samples were used for mediator analysis (specific proteins), determination of metal concentrations and for the ascorbate depletion assay, the later as an indicator of the presence of catalytically active metals in the samples.

Antioxidant and oxidative damage marker analyses

- 2 Ascorbate (AA) and urate (UA) were measured simultaneously by reversed-phase HPLC with electrochemical detection (Iriyama, Yoshiura et al. 1984). [doi: 10.1016/0003-](https://doi.org/10.1016/0003-)

2697(84)90451-2

Pre-acidified and deproteinated samples stored at -80°C in 5% MPA were thawed on wet ice.

Lipid extraction was achieved by transferring 400 μl aliquots of these acidified samples to eppendorfs containing 100 μl of 5% MPA and 200 μl of heptane chilled to 4°C , followed by vortexing for 60 seconds and centrifugation at 13,000 rpm for 5 minutes (4°C).

The resultant lower layer was then carefully decanted into amber HPLC vials for analysis. All sample processing was performed on wet ice, with care taken to always protect the samples from light between processing steps.

A Gilson 234 auto-sampler was used to inject 20 μl aliquots of each sample for analysis on a 5 μm C18 column (4.6×150 mm) from Phenomenex, eluted with a 0.2 mM K_2HPO_4 - H_3PO_4 mobile phase containing 0.25 mM octanesulphonic acid (pH 2.1) at a flow-rate of 1.5 ml/min. An E&EG amperometric electrochemical detector was used for detection with the voltage set at 400 mV and a current sensitivity of 0.2 μA . Ascorbate and urate concentrations were determined against appropriate standards (AA range: 0-12.5 μM ; UA: 0-25 μM).

Mobile phase = 0.2M KH_2PO_4 / 0.25mM octane sulphonic acid, pH2.11 (54.44g KH_2PO_4 + 0.108g octane sulphonic acid / 2litres – titrated with orthophosphoric acid to pH2.1, filtered with vacuum filtration system & 0.45 μm nitrocellulose filter.

Vitamin C (dehydroascorbate (DHA) + ascorbate) was also measured by pre-treating the 400 μl aliquots of acidified sample with 50 μl of 50 mM Tris(2carboxyethyl)phosphine (TCEP) in 5% MPA (Molecular Probes, Eugene, Oregon USA), plus 50 μl of 5% MPA for 15 minutes and then performing the lipid extraction with the addition of 200 μl of heptane and HPLC analysis as described above.

The DHA concentration was then calculated by subtracting the measured ascorbate concentration from the total vitamin C concentration.

2.1 Comments on the determination of DHA concentrations.

Representative lavage ascorbate and vitamin C (following sample pretreatment with the reductant dithiothreitol (DTT), an alternative to TCEP in unacidified samples) traces are illustrated in figure 1. Nasal lavage fluid was used for these illustrative chromatograms as

the lavage procedure, described by Mudway et al. (1999)(see DOI provided below), permits rapid sampling of the nasal epithelial lining fluids and therefore allows samples to be processed for analysis within 10–15 minutes, minimising the likelihood of artefactual oxidation of ascorbate. [doi: 10.1183/09031936.99.13614399](https://doi.org/10.1183/09031936.99.13614399)

Nasal lavage was performed on a healthy male subject and the sample was processed for immediate determination of ascorbate and vitamin C content at 400 mV. The fresh lavage contained 0.43 μM of ascorbate and 3.63 μM of vitamin C following sample reduction with DTT (final concentration 5 mM), hence 3.2 μM DHA. The authenticity of this peak was confirmed relative to the retention time of a 3.125 μM ascorbate standard prepared in 5% MPA and by spiking a 400 μL aliquot of the lavage with 50 μL of a 5 μM ascorbate solution prior to the addition of 50 μL of 50% MPA. The subsequent concentration in the sample was 1.39 μM , indicating a 96% recovery of the added ascorbate.

Samples were also incubated in the presence of ascorbate oxidase for 5 minutes at room temperature (5U/mL final concentration), which completely abolished the ascorbate peak. To confirm that the increase in the ascorbate peak following DTT reduction could not be attributed to the appearance of another signal with an identical retention time, we re-ran the sample at 810 mV, a potential at which thiols (cysteine, glutathione and homocysteine) are readily detectable in this assay. At this potential, following sample reduction with DTT, a cysteine peak was apparent, close to, but separate from the ascorbate peak. This peak was unaffected by the ascorbate oxidase treatment.

To confirm that an ascorbate oxidation product other than DHA was not being recycled by DTT we repeated the ascorbate oxidase treatment with the amendment that following the initial 5 minute incubation, the sample was transferred to an incubator at 37°C for a further 10 minutes to promote the hydrolysis of DHA. Following DTT reduction and acidification of this sample no ascorbate was recovered, whilst the cysteine and uric acid peaks seen at 810 mV were unaffected.

Figure 1:

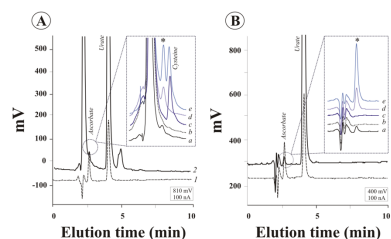


Figure 1: Typical chromatograms derived from freshly prepared nasal lavage fluid obtained from a healthy male subject. Panel 'A' illustrates a set of traces at 810 mV, with thiols (cysteine and reduced glutathione) and panel 'B' at 400 mV, without thiols. The ascorbate and urate peaks are highlighted. The trace labelled '1' in both diagrams corresponds to a standard containing ascorbate and uric acid at 3.125 and 6.25 μ M respectively, prepared in 5% MPA. Trace '2' corresponds to the acidified (5% MPA final concentration) nasal lavage fluid sample. The inset panels illustrate a magnification of the circled section of the main trace with, 'a', corresponding to the acidified lavage sample; 'b', the sample treated with ascorbate oxidase; 'c', the sample treated as outlined previously, but subsequently incubated at room temperature for 10 minutes prior to sample reduction with 0.1% DTT (final concentration); 'd', the lavage fluid spiked with ascorbic acid; and 'e', the lavage fluid immediately reduced with DTT, prior to sample acidification. The position of the ascorbate peak is marked with an asterisk.

Determination of total glutathione

3 Glutathione determinations:

Total glutathione concentrations were measured using the GSSG-reductase DTNB recycling method modified for use on a plate reader by Baker et al. (Baker, Cerniglia et al. 1990). [doi: 10.1016/0003-2697\(90\)90208-q](https://doi.org/10.1016/0003-2697(90)90208-q)

This is based on a kinetic assay in which glutathione causes a continuous reduction of 5,5'-dithiobis(2nitrobenzoic acid) (DTNB) to TNB. Glutathione reductase and NADPH subsequently recycle the oxidised glutathione (GSSG) formed to regenerate reduced glutathione (GSH). The product, TNB, is assayed by measuring the absorbance at 405 nm, its production being proportional to the total glutathione present, and is expressed as GSH equivalents: total glutathione = GSH + (2 x GSSG).

Determination of total glutathione:

Materials:

100mM sodium phosphate buffer containing 1mM EDTA pH7.5

To prepare buffer: 100mM Na₂HPO₄·12H₂O (35.81g/dm³)

100mM NaH₂PO₄ (3g/250cm³)

Put about 900mls of dibasic phosphate solution into 1litre beaker. Add 0.372g EDTA (ethylenediaminetetraacetic acid.disodium salt) to the phosphate solution; adjust to

pH7.5 using the two PO₄ solutions until a volume of 1 litre is reached.

1mM DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) (9.905mg/50mls PO₄ buffer), protect from light with foil and put on to stir.

3.3mM GSSG (oxidized glutathione) – this is prepared from a 1 in 100 dilution of a 330mM GSSG stock (20.2mg/100mls PO₄ buffer) - keep both on ice.

1mM NADPH (8.3mg/10mls PO₄ buffer) - make fresh each day & keep on ice.

Glutathione reductase (G3664) enzyme (activity will vary from batch to batch – check before use)

2-vinyl pyridine

DTNB reaction mix (prepare immediately before use, and protect from light): 5.85mls PO₄ buffer, 2.8mls DTNB, 3.75mls NADPH, The buffer + DTNB + NADPH is mixed with the 5mL Gilson pipette about 4 times. 18.7 Units* of GSSH reductase enzyme is added and the substrate remixed.

Standards: The GSSG standards are prepared in 1.5mL Greiner tubes as outlined below: The 3.3mM GSSG stock = 3.3nmol/ml = 165pmol/50ml. This 165pmol GSSG/50ml is equivalent to 330pmol GSH/50ml (the top standard).

Std no	1	2	3	4	5	6	7	8	9	10	11	12
μL stock GSSG	0	91	182	273	364	455	546	636	727	818	909	1000
μL PO ₄ buffer	1000	909	818	727	636	546	455	364	273	182	91	0
GSSG pmols 50μL	0	30	60	90	120	150	180	210	240	270	300	330

Assay procedure:

For the determination of total glutathione (GSx): 50 μl aliquots of the DES/BHT-treated lavage samples and standards are added in duplicate to a 96-well ELISA microplate (Greiner Bio-one, Stonehouse, UK) and 100 μl of DTNB reaction mix added to achieve final concentrations of 0.15 mM DTNB, 0.2 mM NADPH and 1 U glutathione reductase (from baker's yeast) in phosphate buffer (100 mM sodium phosphate containing 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5) in each well.

The plate was then transferred to a plate reader (SpectraMAX 190, Molecular Devices) and the immediate rate of change of absorbance at 405 nm followed for two minutes at 30°C, with absorbance measurements made every 10 seconds with mixing between

measurements. Glutathione concentrations in samples were measured by comparison with a set of standards (GSSG: 0–6.6 μM).

Calculations: In the TOTAL GSx assay, the result is given as GSH + 2x GSSG, as one molecule of GSSG will yield two molecules of coloured product after reaction with DTNB.

For the determination of total glutathione (GSSG): Quickly aliquot 130 μl of standard and sample into pre-labelled tubes and add 5 μl of 2-vinyl pyridine. The vinyl pyridine must be used in the fume cupboard and double gloves worn. Samples are then vortex mixed and incubated at room temperature for 1 hour. 50 μl aliquots of vinyl pyridine treated lavage samples and standards are added in duplicate to a 96-well ELISA microplate (Greiner Bio-one, Stonehouse, UK) and 100 μl of DTNB reaction mix added and the rate of rate of change of absorbance at 405 nm as outlined above.

Calculating the GSG concentration:

In the TOTAL GSx assay, the result is given as GSH+ (2x GSSG), as one molecule of GSSG will yield two molecules of coloured product after reaction with DTNB:

GSSG is equivalent 2GSH

Therefore when calculating the GSH concentration the following calculation must be followed:

Total glutathione value - 2X (oxidized glutathione value) = reduced glutathione

Measuring the prooxidant action of metals in biological samples by monitoring ascorbate oxidation over time in the presence and absence of selective metal chelation

4 Background:

Metals in biological samples are typically sequestered into biological chelators, for transport or storage, or associated with low molecular weight ligands. These chelators limit the potential of metals to catalyse damaging oxidation reactions. However, metals in certain ligand forms are still able to participate in redox reactions and several assays have been developed to estimate this catalytically active pool. Here we have exploited the extreme sensitivity of ascorbate to metal catalysed oxidation to estimate the concentration of unchelated metals, predominately Fe and Cu in human lavage fluid, though the method is amenable to other biological samples, including cell cytosol and blood plasma/serum. It is based on a historic assay developed by Steve Aust to evaluate metal contamination in buffers (see DOI provided below), but expanded to include the

use of selective chelators, (Diethylenetriamine pentaacetate (DPTA), and Nitrilotriacetic acid (NTA), to dissect out the relative contribution of Fe and Cu to the observed pro-oxidant activity.

[https://doi.org/10.1016/0003-9861\(89\)90261-0](https://doi.org/10.1016/0003-9861(89)90261-0)

Ascorbate depletion assay:

Ascorbate solution preparation: A concentrated 4mM ascorbate solution is prepared in Chelex-treated water, pH 7.0. The pH of the solution is then adjusted to pH 7.0 with the use of 1M NaOH. The solution is then made up to a final volume of 200 ml with Chelex-treated water (pH 7.0) using a very clean, metal-free volumetric flask. Aliquots of 2.5 ml are placed onto 15 mL centrifuge tubes and stored at -70°C until day of exposure.

Chelex water preparation: Ultra-pure water was employed to decrease background metal contamination when assessing the endogenous pro-oxidant activity of the lavage samples. Each litre of deionised Elga-stat water ($18\ \Omega$) was treated with 30 grams of Chelex 100 resin (iminodiacetic acid-coated polystyrene beads). This solution was prepared in a polycarbonate beaker and after mixing for 24 hours at room temperature, the Chelex 100 resin was removed by vacuum filtration through a $0.45\ \mu\text{m}$ cellulose nitrate membrane. The pH of the purified water was subsequently adjusted to 7 using 1M sodium hydroxide and 1M hydrochloric acid, both previously prepared in ultra-pure water and stored at $4\ ^{\circ}\text{C}$ for a maximum of one month.

Protocol:

- The pro-oxidant activity of the recovered BAL fluid samples (using the untreated lavage samples) was assessed with respect to their capacity to deplete ascorbate (AA) from a single $200\ \mu\text{M}$ antioxidant solution. In this assay, greater ascorbate depletion rates were expected to occur in samples containing unchelated catalytic metals, predominately Fe or Cu.
- A stock AA solution was prepared at a concentration of 4 mM in Chelex treated water and adjusted to pH 7.0.
- An aliquot of each lavage sample ($90\ \mu\text{L}$) was diluted with $5\ \mu\text{L}$ of Chelex-treated water and then incubated with the stock antioxidant solution ($5\ \mu\text{L}$) at $37\ ^{\circ}\text{C}$ for two hours in a plate reader (Spectra Max 190).
- Lavage fluid incubations with AA were performed in triplicate in UV 96-well flat-bottom plates (Greiner bio-one).
- The concentration of AA remaining in each well was quantified by measuring the absorbance at 265 nm every two minutes over the two-hour incubation period. Duplicate blanks and standards ($25 - 200\ \mu\text{M}$ AA) were run in parallel with samples on the 96-well plate, such that a calibration curve was constructed for each two-minute measurement.

- The AA concentration in sample wells at each time point was determined against its respective calibration curve and corrected for AA losses by auto-oxidation measured in the blank controls.
- The rate of AA depletion was determined over the two-hour incubation and expressed as $\mu\text{M second}^{-1}$. As the decline of this antioxidant was assumed to follow first order kinetics, only the linear portion of the measurement time course was considered.
- Rates were derived using Microcal Software Limited's OriginLab (version 5.0).
- To determine the influence of metals in the measured rate of AA depletion, samples were incubated with the metal cation chelators DTPA and NTA. Incubations were conducted in a similar procedure as previously described above, however, instead of diluting the lavage samples with 5 μL Chelex-treated water, samples were spiked with 4 mM of with DTPA or EDTA (5 μL).
- Three rates were therefore derived: the ascorbate depletion rate, reflecting the oxidative loss of ascorbate over time in the samples; the ascorbate depletion rate + DTPA; and the ascorbate depletion rate + NTA. These rates provide measures of the overall pro-oxidant capacity of the lavage samples, the fraction attributable to metals (as DTPA is a highly effective chelators and is present in molar excess), and the relative contribution of Fe and Cu, as NTA binds to non-transferrin bound Fe and allows in to redox cycle, whilst inhibiting Cu-dependent catalysis.

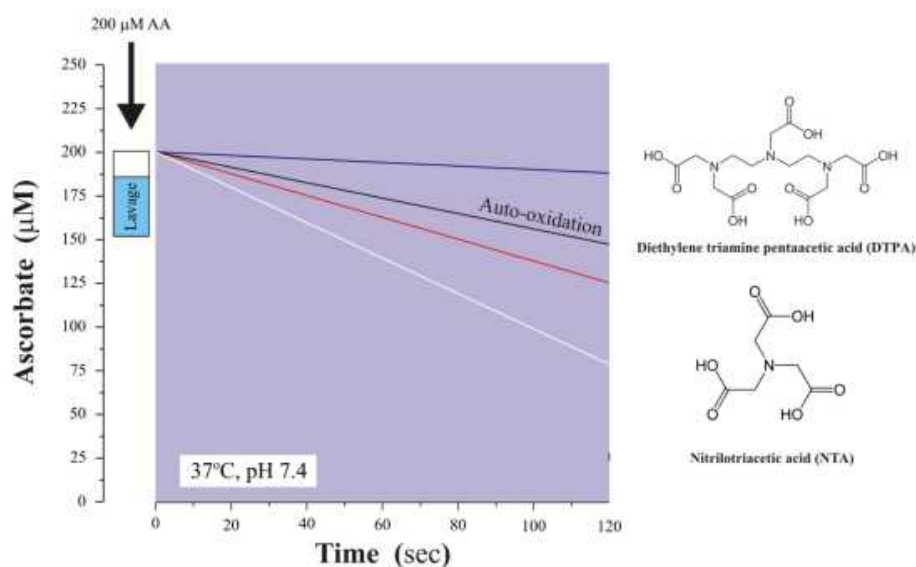


Figure 1: An illustration of the principle of the ascorbate depletion assay. Lavage samples are spiked with ascorbate to achieve a starting concentration of 200 μM . The loss of ascorbate is then monitored at 265 nm every 2 minutes for 2 hours. A typical profile for a lavage sample is shown above using the red line. All incubations are also performed parallel to a water blank, which accounts for the background rate of autooxidation occurring due to trace metal contamination (black line). Addition of the chelator DTPA in excess should completely abolish any metal dependent catalysis, including that contributing to the background oxidation (purple line). Co-incubation, conversely, may act to accelerate the oxidation of ascorbate, specifically in relation to the formation of Fe-NTA, as it is able to mobilise Fe from other biologic chelators in the biological media (white line).