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A High-Throughput Screening Assay of Ascorbate in Brain Samples

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Abstract

Ascorbate is a vital reductant/free radical scavenger in the CNS, whose content defines – to a large extent - the redox status and the antioxidant reserves. Quick, reliable and specific methods for its measurement in brain samples are highly desirable. We have developed a new high-throughput screening assay for measurements of ascorbate using a fluorescence plate-reader. This assay is based on a direct reaction of ascorbate with a nitroxide radical conjugated with a fluorogenic acridine moiety, 4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl radical (AC-TEMPO), yielding fluorescent hydroxylamine product (AC-TEMPO-H). The reaction was monitored over time using fluorescence and electron spin resonance techniques. The appearance of fluorescent AC-TEMPO-H was linear within the range of 3.75–75 μM AscH- in the sample (0.5–10 μM AscH- in the well). Assay was validated with high performance liquid chromatography method. The concentration of ascorbate in murine tissue samples, including brain samples after traumatic brain injury and hemorrhagic shock, was measured.

Keywords

Vitamin C; ascorbic acid; nitroxides; controlled cortical impact; hemorrhagic shock; head trauma

Introduction

Ascorbate (vitamin C) is a vital reductant/free radical scavenger in the brain, cerebrospinal fluid and plasma, whose content defines - to a large extent - the redox status and antioxidant reserves of tissues and biofluids. Additionally, ascorbate acts an important cofactor – one-electron reductant – in the process of norepinephrine biosynthesis by copper-containing dopamine beta hyroxylase (DBH) (Diliberto and Allen, 1981). As a reductant, it can also participate in regulation of activity of heme-peroxidases. Further, the role of ascorbate as a physiologically important donor of electrons may be essential for the recycling of vitamin E

from its phenoxyl radical hence regulation of lipid peroxidation (reviewed in (Bors and Buettner, 1997; Diliberto et al., 1991; Harrison and May, 2009)). Ascorbate is actively transported into both the brain and neurons against a concentration gradient. The high concentrations of ascorbate in cerebrospinal fluid (200–400 μM) and brain (2–10 mM), relative to plasma (in the range of 60–100 μM) (Harrison and May, 2009), emphasize its importance in the central nervous system. Many disease states such as ischemic or traumatic brain injury and radiation exposure have been shown to deplete cellular ascorbate, an effect significantly contributing to propagation of neuronal cell damage (Ates et al., 2007; Bayir et al., 2002; Borisenko et al., 2004; Bors and Buettner, 1997; Exo et al., 2009; Viant et al., 2005). Given the high importance of ascorbate in disease and its potential to be used as a biomarker of oxidative stress, quick, reliable and specific methods for its measurement are highly desirable. Different spectroscopic (including electron paramagnetic resonance (EPR) spectroscopy) and chromatographic assays have been developed to monitor ascorbate levels in tissues. They vary in sensitivity, speed, substrate specificity and other parameters (Bors and Buettner, 1997; Vislisel et al., 2007; Washko et al., 1992). High performance liquid chromatography (HPLC) has been thought to be the most sensitive and specific method for the assessments of ascorbate, but it is the most time-consuming method.

Recently Buettner's group described a sensitive and inexpensive assay for ascorbate measurements using a plate reader (Vislisel et al., 2007). This method is based on oxidation of $AscH^-$ by a nitroxide radical, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), followed by a fluorogenic reaction with o-phenylenediamine. While this protocol represents a useful approach, we have set up a goal to develop a more sensitive and simplified high-throughput assay for measuring ascorbate in biological samples, particularly in the brain samples. Our assay is based on direct reaction of ascorbate with a nitroxide radical conjugated with a fluorogenic acridine moiety, 4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl radical (AC-TEMPO), yielding fluorescent hydroxylamine product (AC-TEMPO-H). Simplicity, specificity, a wide range of measured $AscH^-$ and stability of the assay allows high throughput biochemical testing using a 96 well plate format and a fluorescence plate reader. We demonstrate the utility of the protocol for the analysis of ascorbate content in murine tissues, including brain samples from rats subjected to mild traumatic brain injury (TBI) and combined TBI and hemorrhagic shock (HS) exposure.

Materials and methods

Reagents

L-Ascorbic acid (AA), sodium phosphate dibasic, sodium phosphate monobasic, sodium hydroxide, diethylenetriaminepentaacetic acid (DTPA), dimethyl sulfoxide (DMSO), metaphosphoric acid (MPA), bovine serum albumin, urea, ThioGlo-1, glutathione peroxidase and ascorbate oxidase (AO) were all purchased from Sigma Aldrich (St. Louis, MO). 4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl radical (AC-TEMPO) was purchased from Invitrogen (Carlsbad, CA).

Solution preparation

A stock solution of ascorbic acid (AA, 10 mM) was prepared fresh daily using 50 mM sodium phosphate buffer with 100μM DPTA, (pH=7.4) and diluted appropriately to achieve concentrations of the standards (3.75–75 μM). AC-TEMPO was diluted to a stock concentration of 10mM in DMSO, divided into aliquots and stored in amber colored tubes at −80°C until use. A working solution of AC-TEMPO was prepared for each experiment by diluting the stock solution in phosphate buffer.

Sample preparation

Tissue samples were homogenized using a Tissue Tearor (BioCold Scientific, Fenton MO) in a buffer (tissue weight to volume ratio of 1:20) consisting of sodium chloride (0.1 mol/L final concentration), Tris-chloride (0.01 mol/L final concentration), and Triton X (1%). The homogenates were centrifuged at 1000 g for 10 minutes. The supernatants were then divided into aliquots for measurement of protein, ascorbate and GSH. Metaphosphoric acid (MPA) was added to the aliquot that will be used for ascorbate measurements, such that the final concentration was 5% (v/v) to stabilize ascorbate (Bradley et al., 1973; Comstock et al., 1995) and samples were stored at −80°C until use. At the time of the measurement, the samples were thawed on ice and vortexed for 10 sec every 2 min at least 5 times. Following vortexing the samples were centrifuged at 12,000g for 5 min at 4°C. The supernatant was collected into a microcentrifuge tube and the pH was adjusted to 7.0 using 10M NaOH individually in each sample using pH paper. The volume of base required for the adjustment was recorded.

Fluorescence spectroscopy

For measurements of AC-TEMPO fluorescence, a Shimadzu spectrofluorimeter RF-5301PC set at an excitation wavelength 361 nm, an excitation slit of 3 nm, and an emission slit of 3 nm was used. Time courses of fluorescence intensity were detected using an emission wavelength 440 nm.

Plate reader assay

Each sample was divided into two aliquots, each containing 0.1–0.6 mg/ml protein. To ensure the selectivity of the assay towards ascorbate one of the aliquots (50 μL) was treated with 1 U of ascorbate oxidase (AO) for 40 min at room temperature, the other aliquot was set aside on ice. When all samples were ready, they (20 μl) were transferred onto 96-well plate along with ascorbate standards (0–1.5 nmols). After this, 130 μl AC-TEMPO stock aliquotes (23.1 μM in phosphate buffer) were added immediately using a multi-channel pipette to each well and samples were incubated for 40 min at room temperature in the dark and analyzed reader (PerkinElmer Life Sciences, Boston, MA). Fluorescence was measured using a 390±15 nm filter for excitation and 460±35 nm filter for emission. Fluorescence readings from AO-treated sample were subtracted from non-treated and normalized to protein (corrected for prior dilutions with MPA and NaOH). The precision of the proposed method was tested at two levels: 30 and 45 μM ascorbate (20 μL was taken each time for analysis); each run in triplicate with single or up to three independent runs per day over 14 days.

Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance (EPR) measurements were performed on a JEOL RE1X spectrometer at room temperature with 100 kHz modulation (JEOL, Kyoto, Japan) in gas permeable Teflon tubings (0.8 mm internal diameter, 0.013 mm thickness) obtained from Alpha Wire Corporation (Elizabeth, NJ). The tubing was filled with 60 μl of sample, folded twice, and placed in an open 3.0 mm internal diameter EPR quartz tube. AC-TEMPO spectra were recorded under the following conditions: 3357 G, center field; 25 G, sweep width; 0.5 G, field modulation; 10 mW, microwave power; 0.1 s, time constant; 1 min, time scan. EPR spectra of ascorbate radicals were recorded at 3350 G, center field; 50 G, sweep width; 10 mW, microwave power; 0.5 G, field modulation; 10^3, receiver gain; 0.1 s, time constant; 1 min, scan time. Simulation of TEMPO and ascrobyl radical spectra was performed using WinSim software (NIESH).

High performance liquid chromatography assay of ascorbate

Samples, prepared the same way as those for the plate reader assay, were used for high performance liquid chromatography (HPLC) measurements. A ZORBAX Eclipse XDBC18 column (5-μm particle size, 4.6×150 mm Agilent Technologies, Palo Alto, CA, USA) and a mobile phase of 1:24 methanol–water adjusted to pH 3.0 by acetic acid at a flow rate of 1.0 ml/min were used. A Shimadzu LC-10A HPLC system was used with an LC-600 pump and SPD-10A UV detector (Shimadzu, Kyoto, Japan) at 264 nm. Under these conditions, the retention time for ascorbate was 2.0 min (Kagan et al., 1994; Bayir et al., 2002).

Glutathione

Glutathione (GSH) was determined using ThioGlo-1, a maleimide reagent (Sigma-Aldrich, St. Louis, MO), which produces a highly fluorescent adduct upon its reaction with sulfhydryls (SH) groups. Content of total low-molecular-weight thiols was estimated by an immediate fluorescence response registered upon addition of ThioGlo-1 to tissue homogenate (10–35 μg of protein). An aliquot of the same sample was treated with glutathione (GSH) peroxidase (1 U) and cumene hydroperoxide (100 μmol/L) for 30 min. A fluorescence measurement system, CytoFluor 2350 (Millipore, Bedford, MA), was used for the assay using excitation filter A (360 ± 40 nm) and emission filter B (530 ± 25 nm). The difference between the fluorescence response of the original sample and the GSH peroxidase-treated sample corresponds to the amount of reduced GSH.

Animal experiment

The Institutional Animal Care and Use Committee of the University of Pittsburgh School of Medicine approved this study. Male C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME), 12–15 weeks of age and weighing 22–29 grams, were housed under controlled environmental conditions and allowed ad libitum food and water until the study began. Controlled cortical impact (CCI) and HS + CCI were performed as described earlier (Exo et al., 2009). Briefly, a mild CCI was performed with a pneumatic impactor (Bimba, Monee, IL) with a 3-mm flat-tip impounder that was deployed at a velocity of 5 m/sec and a depth of 1 mm. Brain temperature was maintained at 38±0.5° C throughout the experiment. To achieve HS, 2ml/100g of blood was removed from femoral venous catheter. This hemorrhage volume resulted in a decrease in mean arterial blood pressure to 35–40 mmHg. Mice remained in the HS phase for 90 min. After the HS phase, the mice were randomized to one of two treatment groups, including resuscitation with Hextend (Hospira, Inc., Lake Forest, IL) or polynitroxylated albumin (PNA, SynZyme Technologies, Irvine, CA) targeting a mean arterial pressure (MAP) of >50 mm Hg. In separate studies, adult male rats weighing 350–400 grams were utilized for comparative analysis of ascorbate levels measured using HPLC assay and AC-TEMPO method in rat tissues.

Data analysis

Data are expressed as means ± SD. Changes in variables for different assays were analyzed by either Student's t test (single comparisons) or one-way ANOVA for multiple comparisons. Differences among means were considered to be significant at $p < 0.05$.

Results and discussion

EPR was employed as an independent supportive method to prove the underlying mechanism of the assay. In the dual nitroxide-fluorophore probe, 4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl radical (AC-TEMPO), the paramagnetic radical nitroxide moiety suppresses the fluorescence of the chromophore (acridine). In the absence of ascorbate, AC-TEMPO displayed a characteristic triplet ESR spectrum with g = 2 and superfine splitting constant (aN) of 16 G (Fig. 1A, insert). Upon addition $AscH^-$ to AC-

TEMPO, the superposition of two radicals: Asc• and TEMPO• was observed (Fig. 1B insert, red - simulated spectra). The reaction of ascorbate with TEMPO leads to the reduction of latter yielding ESR silent hydroxylamine-TEMPO (TEMPOH). The reaction rate of $AscH^-$ with AC-TEMPO measured experimentally by disappearance of nitroxide triplet signal was 3.1 $M^{-1}s^{-1}$ which is in agreement with published data (Vislisel et al., 2007). No fluorescence was detected from AC-TEMPO in the absence of ascorbate (Fig. 1C, D). The fluorescence signal was detected when the nitroxide was reduced to diamagnetic hydroxylamine (AC-TEMPOH) by addition of ascorbate (Fig. 1C, D) or sodium dithionite (data not shown). The magnitude of fluorescence intensity was proportional to the concentration of $AscH^-$ in the sample (Fig. 1C, D). Simultaneous ESR measurements also showed that disappearance of the initial characteristic triplet of ACTEMPO (Fig. 1A) was proportional to $AscH^-$ added in the sample over time (Fig. 1A–B). Under our experimental conditions (20–50 μM AC-TEMPO and $AscH^-$ < AC-TEMPO), accumulation of fluorogenic AC-TEMPO-H reached saturation at 40 min (Fig. 1C); the saturation occurred faster at lower ascorbate concentrations. At this time, the dependence of fluorescence *vs* $AscH^-$ was linear (Fig. 1D).

We also tested whether sample preparation procedure affects accuracy of the method. Freshly prepared ascorbate standards and the ones prepared the same way as the tissue samples were used for calibration (Fig. 1E). Two calibration curves were identical. After optimization of the reaction conditions, the maximal sensitivity - the lowest detectable level of $AscH^-$ of 75 pmol/well or 3.75 μM in sample - was achieved at an ACTEMPO concentration of 20 μM.

Clinical and Laboratory Standards Institute (CLSI) document EP15-A2 (EP15-A2, 2006) was used as a guide for determining the precision of the proposed method. We used a spreadsheet for assisting with the calculations as described by D. Chester in The Clinical Biochemist Reviews (available from the Australian Association of Clinical Biochemists web-site (Chester, 2008)). The precision of the proposed method was tested at two levels: 30 and 45 μM ascorbate. The within-run imprecision of proposed method was in the range of 2.6%; the total imprecision was ~7.6% (Table 3).

To better characterize fluorescence features of the chromophore, we employed fluorescence spectroscopy in a wide range (370–620 nm) as well as different pH (pH=2.9 – 12.1, Fig. 1F), ionic strengths, incubation time and confirmed the typical spectra with excitation and emission maxima corresponding to those published in the literature (Soh et al., 2001). Based on this detailed information, the fluorescence plate reader parameters were setup.

To test the most probable sources of interference, we examined the effects of several physiologically relevant reductants with ascorbate determinations using ACTEMPO. These results are shown in Table 2 as rates of AC-TEMPO reduction (expressed as pmols per minute) by ascorbate alone or by ascorbate in the presence of GSH, cysteine, dopamine, urea and bovine serum albumin. Additionally, typical examples of time-course of accumulation of fluorescent AC-TEMPOH upon reaction with ascorbate in the presence of several concentrations of dopamine are shown in Fig. 1G. Our results showed that these compounds in concentrations exceeding those present in our samples did not affect AC-TEMPO-based ascorbate determinations (Table 2). High concentrations of GSH (5 mM) lead to a 15% increase in effective rate of ascorbate oxidation. Addition of BSA accelerated reaction rate, probably due to absorption of both ascorbate and fluorescent probe to BSA (Lozinsky et al., 2001). Further, to ensure specificity of the response, we chose to employ treatment of the samples with ascorbate oxidase - to completely and selectively remove ascorbate from them - and then subtracted these readings from non-treated samples

HPLC method was used as a reference method to measure ascorbate in rat brain liver and lung (Fig. 1I). The range of measured Asc was 2–45 nmol/mg protein. As presented in Table 1, the variations between these two methods were within 15%. To determine the predictive capacity and accuracy of the measurement with AC-TEMPO, tissue samples were spiked with a known amount of purified ascorbate. Briefly, samples were prepared as described and following pH adjustment a known quantity of ascorbate was added to the sample. The spiked samples were processed exactly the same as non-spiked samples. The predicted values were within 10% of error.

Finally, we utilized this method for analysis of ascorbate in mouse brain after TBI (mild CCI or CCI plus HS) followed by fluid resuscitation with Hextend® or PNA targeting a mean arterial blood pressure of > 50 mm Hg (Fig. 2A). In this model, neuronal death is seen only in combined CCI plus HS, but not in CCI alone. Without fluid resuscitation of HS survival is not possible in the combined CCI plus HS insult (Exo et al., 2009). Hextend® was used since it is the standard resuscitation fluid in combat casualty care. In several animal models, resuscitation with Hextend® has been shown to require less volume and improve cerebrovascular function (Crookes et al., 2004; King et al., 2004). PNA is a novel resuscitation fluid composed of 55 nitroxide moieties covalently linked to albumin that is administered as a 10% solution. Its stable nitroxyl radicals mimic superoxide dismutase (SOD) and detoxify reactive oxygen species (Li et al., 2002). We observed that Hextend® and PNA treated mice displayed significant loss of ascorbate at day 7 after CCI plus HS, perhaps due to the direct reaction of nitroxides of PNA with ascorbate. There was no significant loss of ascorbate after mild CCI alone. It is known that recycling of ascorbate requires reduced glutathione (GSH) (Bradley et al., 1973; Vethanayagam et al., 1999). And, several studies have shown decreased GSH levels after central nervous system insults, particularly after severe TBI (Ates et al., 2007; Bayir et al., 2002; Borisenko et al., 2004). Indeed, in the present study GSH levels were decreased in Hextend® and PNA treated CCI + HS groups (Fig. 2B).

The idea of using dual fluorogenic molecules for ascorbate measurements was first proposed and tested by Lozinsky et al., using TEMPO conjugated with naphthalenesulfonamide (Lozinsky et al., 1999). Later, dansyl-TEMPO (Likhtenstein et al., 2007) or polymethine-cyanine-nitroxides (Sato et al., 2009) were employed for the quantitative measurements of reducing species such as Fe^{2+} and ascorbic acid. Our previous work has established that AC-TEMPO effectively reacts with glutathionyl radical (GS•) yielding AC-TEMPO-H fluorescence (Borisenko et al., 2004). Here we utilized AC-TEMPO as a potentially useful reagent for the development of a simple and sensitive high-throughput fluorometric method for analysis of ascorbate in tissues. No other reagents except ascorbate standards and ascorbate oxidase are necessary. This assay has the ability to measure 96 wells. It is able to determine $AscH^-$ in samples as low as 3.75 μM. A wide variety of samples, be potentially analyzed with great specificity.

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Abbreviations

$AscH^-$	ascorbate anion
Asc•	ascorbate radical

AC-TEMPO	4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl radical
HPLC	high performance liquid chromatography
CCI	controlled cortical impact
PNA	polynitroxylated albumin
HS	hemorrhagic shock
TBI	traumatic brain injury
MPA	metaphosphoric acid
AO	ascorbate oxidase
EPR	electron paramagnetic resonance

References

1. User Verification of Performance for Precision and Trueness; Approved Guideline. 2006. p. 64Chairholder: R. Neill Carey, PhD, FACB
2. Imprecision Spreadsheet provides example calculations for estimating imprecision that conform to CLSI documents EP05-A2 (Precision Performance of Clinical Chemistry Devices) and EP15-A2 (User Verification of Performance for Precision and Trueness).
3. Ates O, Cayli S, Altinoz E, Gurses I, Yucel N, Sener M, Kocak A, Yologlu S. Neuroprotection by resveratrol against traumatic brain injury in rats. Mol Cell Biochem. 2007; 294:137–44. [PubMed: 16924419]
4. Bayir H, Kagan VE, Tyurina YY, Tyurin V, Ruppel RA, Adelson PD, Graham SH, Janesko K, Clark RS, Kochanek PM. Assessment of antioxidant reserves and oxidative stress in cerebrospinal fluid after severe traumatic brain injury in infants and children. Pediatr Res. 2002; 51:571–8. [PubMed: 11978879]
5. Behringer W, Safar P, Kentner R, Wu X, Kagan VE, Radovsky A, Clark RS, Kochanek PM, Subramanian M, Tyurin VA, Tyurina YY, Tisherman SA. Antioxidant Tempol enhances hypothermic cerebral preservation during prolonged cardiac arrest in dogs. J Cereb Blood Flow Metab. 2002; 22:105–17. [PubMed: 11807400]
6. Borisenko GG, Martin I, Zhao Q, Amoscato AA, Tyurina YY, Kagan VE. Glutathione propagates oxidative stress triggered by myeloperoxidase in HL-60 cells. Evidence for glutathionyl radical-induced peroxidation of phospholipids and cytotoxicity. J Biol Chem. 2004; 279:23453–62. [PubMed: 15039448]
7. Bors, W.; Buettner, G. Vitamin C in Health and Disease. Marcel Dekker, Inc.; New York: 1997.
8. Bradley DW, Emery G, Maynard JE. Vitamin C in plasma: a comparative study of the vitamin stabilized with trichloroacetic acid or metaphosphoric acid and the effects of storage at −70 degrees, −20 degrees, 4 degrees, and 25 degrees on the stabilized vitamin. Clin Chim Acta. 1973; 44:47–52. [PubMed: 4707637]
9. Chester D. Evaluating assay precision. Clin Biochem Rev. 2008; (Suppl 1):S23–6. [PubMed: 18852851]
10. Comstock GW, Norkus EP, Hoffman SC, Xu MW, Helzlsouer KJ. Stability of ascorbic acid, carotenoids, retinol, and tocopherols in plasma stored at −70 degrees C for 4 years. Cancer Epidemiol Biomarkers Prev. 1995; 4:505–7. [PubMed: 7549806]
11. Crookes BA, Cohn SM, Bonet H, Burton EA, Nelson J, Majetschak M, Varon AJ, Linden JM, Proctor KG. Building a better fluid for emergency resuscitation of traumatic brain injury. J Trauma. 2004; 57:547–54. [PubMed: 15454801]
12. Diliberto EJ Jr. Allen PL. Mechanism of dopamine-beta-hydroxylation. Semidehydroascorbate as the enzyme oxidation product of ascorbate. J Biol Chem. 1981; 256:3385–93. [PubMed: 6451628]

13. Diliberto EJ Jr. Daniels AJ, Viveros OH. Multicompartmental secretion of ascorbate and its dual role in dopamine beta-hydroxylation. Am J Clin Nutr. 1991; 54:1163S–72S. [PubMed: 1962565]
14. Elsayed NM, Tyurina YY, Tyurin VA, Menshikova EV, Kisin ER, Kagan VE. Antioxidant depletion, lipid peroxidation, and impairment of calcium transport induced by air-blast overpressure in rat lungs. Exp Lung Res. 1996; 22:179–200. [PubMed: 8706635]
15. Exo JL, Shellington DK, Bayir H, Vagni VA, Janesco-Feldman K, Ma L, Hsia CJ, Clark RS, Jenkins LW, Dixon CE, Kochanek PM. Resuscitation of traumatic brain injury and hemorrhagic shock with polynitroxylated albumin, hextend, hypertonic saline, and lactated Ringer's: Effects on acute hemodynamics, survival, and neuronal death in mice. J Neurotrauma. 2009; 26:2403–8. [PubMed: 19691424]
16. Friedman S, Kaufman S. 3,4-dihydroxyphenylethylamine beta-hydroxylase. Physical properties, copper content, and role of copper in the catalytic acttivity. J Biol Chem. 1965; 240:4763–73. [PubMed: 5846992]
17. Harrison FE, May JM. Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. Free Radic Biol Med. 2009; 46:719–30. [PubMed: 19162177]
18. Kagan VE, Kuzmenko AI, Shvedova AA, Kisin ER, Li R, Martin I, Quinn PJ, Tyurin VA, Tyurina YY, Yalowich JC. Direct evidence for recycling of myeloperoxidase-catalyzed phenoxyl radicals of a vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxy chromane, by ascorbate/dihydrolipoate in living HL-60 cells. Biochim Biophys Acta. 2003; 1620:72–84. [PubMed: 12595076]
19. Kagan VE, Yalowich JC, Day BW, Goldman R, Gantchev TG, Stoyanovsky DA. Ascorbate is the primary reductant of the phenoxyl radical of etoposide in the presence of thiols both in cell homogenates and in model systems. Biochemistry. 1994; 33:9651–60. [PubMed: 8068642]
20. King DR, Cohn SM, Proctor KG. Changes in intracranial pressure, coagulation, and neurologic outcome after resuscitation from experimental traumatic brain injury with hetastarch. Surgery. 2004; 136:355–63. [PubMed: 15300202]
21. Li H, Ma L, Hsia CJ, Zweier JL, Kuppusamy P. Polynitroxyl-albumin (PNA) enhances myocardial infarction therapeutic effect of tempol in rat hearts subjected to regional ischemia-reperfusion. Free Radic Biol Med. 2002; 32:712–9. [PubMed: 11937297]
22. Li W, Wu S, Ahmad M, Jiang J, Liu H, Nagayama T, Rose ME, Tyurin VA, Tyurina YY, Borisenko GG, Belikova N, Chen J, Kagan VE, Graham SH. The cyclooxygenase site, but not the peroxidase site of cyclooxygenase-2 is required for neurotoxicity in hypoxic and ischemic injury. J Neurochem. 113:965–77. [PubMed: 20236388]
23. Likhtenstein GI, Ishii K, Nakatsuji S. Dual chromophore-nitroxides: novel molecular probes, photochemical and photophysical models and magnetic materials. Photochem Photobiol. 2007; 83:871–81. [PubMed: 17645658]
24. Lozinsky E, Martin VV, Berezina TA, Shames AI, Weis AL, Likhtenshtein GI. Dual fluorophore-nitroxide probes for analysis of vitamin C in biological liquids. J Biochem Biophys Methods. 1999; 38:29–42. [PubMed: 10078871]
25. Lozinsky E, Novoselsky A, Shames AI, Saphier O, Likhtenshtein GI, Meyerstein D. Effect of albumin on the kinetics of ascorbate oxidation. Biochim Biophys Acta. 2001; 1526:53–60. [PubMed: 11287122]
26. Sato S, Tsunoda M, Suzuki M, Kutsuna M, Takido-uchi K, Shindo M, Mizuguchi H, Obara H, Ohya H. Synthesis and spectral properties of polymethine-cyanine dye-nitroxide radical hybrid compounds for use as fluorescence probes to monitor reducing species and radicals. Spectrochim Acta A Mol Biomol Spectrosc. 2009; 71:2030–9. [PubMed: 18790663]
27. Soh N, Katayama Y, Maeda M. A fluorescent probe for monitoring nitric oxide production using a novel detection concept. Analyst. 2001; 126:564–6. [PubMed: 11394293]
28. Tyurina YY, Tyurin VA, Epperly MW, Greenberger JS, Kagan VE. Oxidative lipidomics of gamma-irradiation-induced intestinal injury. Free Radic Biol Med. 2008; 44:299–314. [PubMed: 18215738]
29. Vethanayagam JG, Green EH, Rose RC, Bode AM. Glutathione-dependent ascorbate recycling activity of rat serum albumin. Free Radic Biol Med. 1999; 26:1591–8. [PubMed: 10401626]

30. Viant MR, Lyeth BG, Miller MG, Berman RF. An NMR metabolomic investigation of early metabolic disturbances following traumatic brain injury in a mammalian model. NMR Biomed. 2005; 18:507–16. [PubMed: 16177961]

31. Vislisel JM, Schafer FQ, Buettner GR. A simple and sensitive assay for ascorbate using a plate reader. Anal Biochem. 2007; 365:31–9. [PubMed: 17433246]

32. Washko PW, Welch RW, Dhariwal KR, Wang Y, Levine M. Ascorbic acid and dehydroascorbic acid analyses in biological samples. Anal Biochem. 1992; 204:1–14. [PubMed: 1514674]

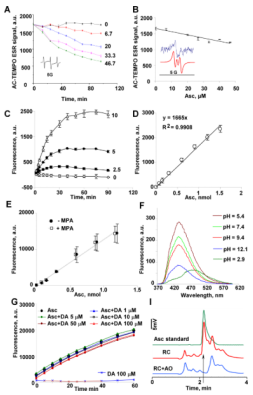

Fig. 1.

Measurement of ascorbate by AC-TEMPO. **A** – Time-course of AC-TEMPO ESR signal decay produced by ascorbate (0–46.7 μM). Insert: initial ESR signal. N=3. **B** – Dose-dependence of AC-TEMPO ESR signal at 25 min after addition of ascorbate. Insert – Blue: superposition of Asc• and TEMPO•. Red – simulated spectra. The reaction mixture contained 100 μM AC-TEMPO. N=3. C – Time-course of accumulation of fluorogenic product (AC-TEMPO-H) after reaction of AC-TEMPO with ascorbate. Conditions: AC-TEMPO – 50 μM, ascorbate concentration (in μM) indicated. Note, that after 40 min the reaction reaches saturation. N=3. **D** – Typical calibration curve utilized for ascorbate measurement in biological samples. AC-TEMPO – 20 μM, $AscH^-$ 3.75–75 μM (0.5–10 μM in the well). N=18. **E** – Calibration curves of freshly prepared ascorbate standards (−MPA) and standards treated the same way as the tissue samples (+MPA). N=6. **F** Fluorescence spectra of AC-TEMPO-H at different pH measured at 30 min. Conditions: AC-TEMPO – 50 μM, ascorbate 10 μM. **G** –. Specificity of AC-TEMPO assay. Time course of accumulation of fluorogenic product (AC-TEMPO-H) after reaction of AC-TEMPO with ascorbate in the presence of dopamine (DA). Conditions: AC-TEMPO – 50 μM, ascorbate – 10 μM, DA (in μM) as indicated. Note that in the absence of ascorbate, DA did not significantly affect AC-TEMPO reduction by ascorbate. **I** –Typical HPLC profile of ascorbate measurement in rat right cortex (RC). Arrow indicates ascorbate peak in standard and RC sample. Note that ascorbate peak completely disappears in AO-treated sample.

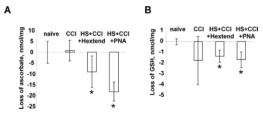

Fig. 2.

A, **B** – Assessments of ascorbate (**A**) and GSH (**B**) loss in murine brain samples after controlled cortical impact (CCI) and CCI + HS followed by fluid resuscitation with Hextend® or polynitroxylated albumin (PNA). Significant difference between naïve and other groups indicated by * (p value < 0.05, N=4–5).

Table 1

Comparative analysis of ascorbate measurements in rat tissues measured with HPLC and AC-TEMPO.

Sample	HPLC	AC-TEMPO	Predicted value (for spiked sample)
	Asc, nmol/mg protein		
Liver 1	2.3 ± 0.1	2.6 ± 0.4	
Liver 2	2.4 ± 0.1	2.9 ± 0.2	
Lung 1	3.9 ± 0.2	4.4 ± 0.1	
Lung 2	3.5 ± 0.2	4.4 ± 0.1	
Left cortex	33.9 ± 0.1	33.3 ± 4.5	
Right cortex 1	31.4 ± 0.4	29.2 ± 3.5	
Right cortex 2		26.2 ± 1.6	26.6
Right cortex 3		28.5 ± 0.6	25.8
Right cortex 4		44.8 ± 1.0	42.5
Mean ± SD, N=3			

Table 2

Rates of AC-TEMPO reduction (expressed as pmols per minute ± SD) by ascorbate alone or by ascorbate in the presence of glutathione (GSH), cysteine,dopamine, urea and bovine serum albumin (BSA) (N = 3–15). Conditions: ascorbate (Asc), 10 μ; AC-Tempo, 50 μ.

Concentrations, μM	GSH	
	−Asc	+Asc
0		25.37±3.46
10	1.00±1.89	27.63±3.52
50	1.37±1.25	28.62±3.80
100	1.95±0.99	26.83±1.86
500	2.87±1.78	26.11±1.94
5000	4.20±2.38	29.25±0.66

Concentrations, μM	Cysteine	
	−Asc	+Asc
0		25.37±3.46
1	1.50±0.42	28.28±1.45
5	0.62±0.79	28.67±0.95
10	0.49±0.88	28.09±1.23
50	−0.71±1.09	27.87±1.85

Concentrations, μM	Dopamine	
	−Asc	+Asc
0		25.37±3.46
1	−0.33±0.61	25.40±0.59
5	−0.18±0.46	27.10±2.16
10	−0.22±0.57	26.34±1.44
50	−0.10±0.69	25.33±1.15
100	−0.03±0.39	24.48±1.48

Concentrations	−Asc	+Asc
Urea 3.3 mM	−5.21±0.03	24.92±0.92
BSA 3.3 μg/ml	4.58±0.11	32.46±0.11

Table 3

Assessment of precision of the proposed assay.

Parameter	Level 1 (30 μM)	Level 2 (40 μM)
Number of runs	25	25
Number of replicates in each run	3	3
Within-Run Imprecision	2.6%	2.0%
Total Imprecision	7.6%	7.1%