Analytical Biochemistry 441 (2013) 109–114

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# Measuring the antioxidant capacity of blood plasma using potentiometry

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ARTICLE INFO

*Article history:*
Received 4 April 2013
Received in revised form 26 June 2013
Accepted 8 July 2013
Available online 16 July 2013

*Keywords:*
Potentiometry
Total antioxidant capacity
Plasma
Exhaustive exercise

ABSTRACT

The use of potentiometry to measure plasma antioxidant capacity to contribute to oxidative stress evaluation is presented. In this assay, plasma ($n = 60$) diluted (0.3 to 1 ml) in phosphate buffer, pH 7.4, NaCl 9%, was submitted to potentiometry. A platinum wire was the working electrode and saturated calomel the reference. The results are presented as the difference between sample and buffer potential ($\Delta E$). $\Delta E$ presented a good inverse correlation with added increasing concentrations of ascorbate (2.5–75 μmol/L; $R = -0.99$), urate (9.0–150 μmol/L; $R = -0.99$), and bilirubin (0.78–13 μmol/L; $R = -0.99$). Increase in the antioxidant capacity decreased $\Delta E$. Depletion of the antioxidant capacity by *tert*-butylhydroperoxide (6.5–50 μmol/L) presented a direct correlation (0.97) with $\Delta E$. Furthermore, $\Delta E$ presented an inverse correlation ($R = -0.99$) with increased antioxidant capacity of plasma (FRAP) induced by the addition of ascorbate (2.5–75 μmol/L). The response of the potentiometric method proved be adequate for measuring the plasma antioxidant depletion induced by acute exhaustive exercise in rats (control, $n = 15$; exercised, $n = 15$). This exercise decreased the concentration of urate ($p < 0.05$), decreased FRAP ($p < 0.5$), increased TBARS ($p < 0.5$), and decreased the potentiometer sensor response ($p = 6.5 \times 10^{-3}$). These results demonstrate the adequacy of potentiometry for evaluating the antioxidant capacity of blood plasma samples.



The aerobic metabolism produces, as side products, reactive oxygen species (ROS)[1] that alter the redox status of cells and tissues. To maintain suitable concentrations of these species, living organisms developed antioxidant systems. The first line of defense is the enzymatic antioxidant system, which neutralizes the action of $O_2^-$ with superoxide dismutase and of $H_2O_2$ with catalase and the glutathione peroxidase/glutathione reductase system. The second line of defense is constituted mainly by protein reduced thiols and low-molecular-weight antioxidants (LMWAs). The LMWAs comprise a wide variety of molecules, including dietary products (tocopherols, ascorbate, retinols, polyphenols, etc.) and metabolic products (urate, ascorbate, and reduced glutathione), and they are the compounds that contribute a significant part to the antioxidant capacity of tissues and biological fluids. Some of these compounds can penetrate cells and achieve specific locations where the oxidative attack may be occurring [1–4].

When, in a biological fluid, the attack from ROS or other oxidants overcomes the antioxidant capacity, oxidative stress is established. This condition has been related to the onset and progression of various pathologies such as cancers, Alzheimer disease, Parkinson disease, psoriasis, heart disease, hypertension, and diabetes [5,6]. Oxidative stress is also related to physiological processes such as aging, physical training, and fatigue [7–10].

Several methods have been proposed to monitor the redox status. These methodologies include the direct detection of radicals (electron spin resonance) or the detection of the lesion products of membranes, proteins, and DNA (HPLC). Other methods quantify the activity of the antioxidant enzymes and/or the LMWAs individually or as a whole [2–4,11]. The evaluation of total antioxidant defense capacity has advantages over the measurement of each antioxidant separately, because it takes account of all the antioxidants, known or unknown, and their synergism [12,13].

The reducing capacity of biological samples depends on all redox couples present in the biological fluids, cells, organelles, and tissues [14]. Thus, the reducing capacity that represents the redox status is a zone of variation, just as the physiological pH is [2,15].

Because the LMWAs have electronegativity, they can be measured using electrochemical methods [16], very attractive because of their experimental simplicity, low cost, and high sensitivity [17]. In this sense, both amperometric and voltammetric methods have

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[1] *Abbreviations used:* LMWA, low-molecular-weight antioxidant; ROS, reactive oxygen species; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; TBOOH, *tert*-butylhydroperoxide; FRAP, ferric reducing ability of plasma; TBARS, thiobarbituric acid-reactive substances; TEAC, Trolox equivalent antioxidant capacity; Co, control group; Ex, exercised group.

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http://dx.doi.org/10.1016/j.ab.2013.07.012

been used to measure the antioxidant capacity of biological samples. Amperometric methods are suitable and have been used to determine the activity of antioxidant enzymes and some LMWAs [18–23]. Voltammetry is able to quantify hydrophilic and lipophilic LMWAs within a concentration range of 1–10 μmol/L, suitable for measuring antioxidant concentrations in biological samples [24–32]. However, the high concentrations of proteins that adhere to the electrode can interfere with the result. To resolve this problem, before each measure, the electrode must be polished to maintain the sensitivity, and this procedure increases the time to take the measurements [25,26].

Another electrochemical method that can be used is potentiometry. This method is based on the relationship between the electrochemical cell potential and the activity of all chemical species that are part of it [33]. The lower the potential of a sample, the greater its ability to donate electrons and, therefore, its antioxidant capacity. Potentiometry presents advantages compared with voltammetry, because it does not need application of current or potential modulation, allowing a simpler apparatus to be used in the research field. In this method the sample is the single independent variable, leading to more accurate results.

Potentiometry has been used to measure redox status to detect sediment levels in estuaries of rivers [34], to detect fermentation-induced current [35], and to quantify very low concentrations of metallic ions [36], antioxidant activity in wines [37], and plant extracts [16].

Thus, the aim of this paper was to standardize and apply a potentiometric method to the determination of the redox status of the blood plasma.

## Materials and methods

### *Apparatus*

Potentiometric measurements were performed using a platinum wire (length 2.5 cm, diameter 0.5 mm) welded to a nickel/chrome wire (coated with glass) as the working electrode. The tip of the electrode in contact with the sample was 1 cm in length (Fig. 1). Saturated calomel with double junction (R-683; Analion, Ribeirão Preto, Brazil) was used as the reference electrode. The two electrodes were connected to the potentiometer/pH meter HI-9321 (Hanna Instruments, Woonsocket, RI, USA). Both electrodes were immersed in a 3-ml cell with controlled temperature (20 ± 1 °C) to which the sample was added.

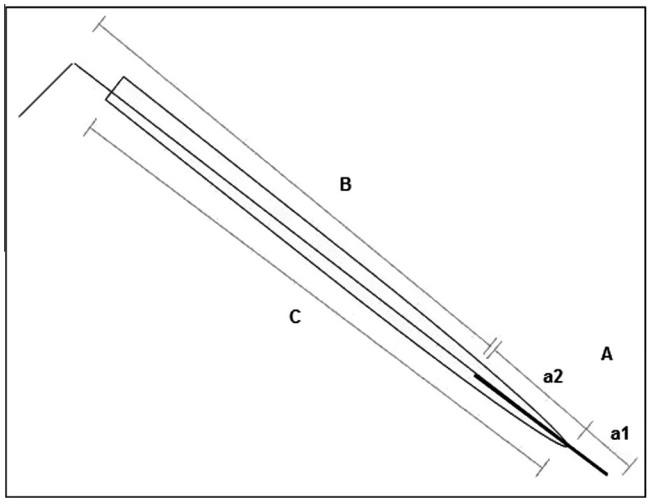


**Fig.1.** Diagram of the working electrode. (A) Platinum wire 0.5 mm in diameter and 2.5 cm in length, (a1) 1 cm exposed to sample contact and (a2) 1.5 cm covered with a glass protection (C). (B) The platinum wire was welded to nickel/chrome wire that connects to the potentiometer.

The working electrode was cleaned with double-filtered water and polished, whenever necessary, with aluminum hydroxide (0.5 μm). The electrode was considered suitable to use if it presented $E = 195 \pm 5$ mV when immersed in the standard solution (5 mmol/L $Na_4Fe(CN)_6$ plus 5 mmol/L $Na_2Fe(CN)_6$ dissolved in 5 mmol/L phosphate buffer, pH 7.0). The reference electrode was cleaned with water only.

For the measurements the blood plasma (300 μl) was dissolved in 1 ml of total volume using PBS (phosphate buffer, 5 mmol/L, pH 7.4, plus NaCl 0.9%). Before each measure the potential of the PBS solution was recorded. The redox potential ($\Delta E$), as presented here, is the difference between the sample potential ($E_s$) and the buffer potential ($E_b$): $\Delta E = E_s - E_b$. All measurements were made 120 s after sample addition, the time required to stabilize the electrodes.

### *Reagents*

All reagents used in this work were purchased from Sigma or Merck. The solutions were prepared using double-filtered water (Milli-Q; Millipore, Billerica, MA, USA), used also to clean glassware and equipment.

### *Experiments using human plasma*

Plasma (heparin as anticoagulant) of 60 volunteers (19 ± 1 years of age) was used to perform the experiments. The blood collection was done by an accredited pharmacist using appropriate procedures. After collection, the blood was centrifuged and the plasma of all volunteers was mixed and stored frozen (−70 °C) until use. The project was previously submitted and approved by the Institutional Ethics Committee on Human Research (CEP/FOP/UNICAMP—Protocol 19/2004).

To improve the antioxidant capacity, increasing concentrations of ascorbate (2.5–75 μmol/L), urate (9.0–150 μmol/L), and bilirubin (0.78–13.0 μmol/L) were added to plasma samples, separately. The added antioxidant concentrations were close to physiological variations, as described by Burtis et al. [38]. The measurements were performed immediately after the antioxidant additions.

To decrease the antioxidant capacity, the samples were incubated with increasing concentrations (6.5–50 μmol/L) of *tert*-butylhydroperoxide (TBOOH), for 60 min, at room temperature, under vigorous stirring, to provide enough oxygen. The TBOOH compound is an organic peroxide that oxidizes biological membranes [39], DNA [40], and proteins [41].

The antioxidant capacity of the samples with and without ascorbate (2.5–75 μmol/L) was assessed using the FRAP method described by Benzie and Strain [42].

### *Exhaustive exercise protocol*

Thirty male Wistar rats, 2 months of age, body mass 381.6 ± 20.8 g, were housed in collective (5 animals each) cages in a temperature-controlled environment (20 ± 1.0 °C) with a reversed light cycle (12:12 h). To acclimate, they were subjected to shallow water (34 °C), for 10 min, on 2 consecutive days [43,44]. The animals were randomly divided into two groups: control (Co) and exercised (Ex). The Ex group animals were fasted 24 h and subjected to an acute exhaustive swimming protocol. They swam with 10% overweight in controlled-temperature water (34 ± 1 °C) until exhaustion. Exhaustion was determined as the time at which the animal remained submerged for more than 10 s [45]. After the exercise the animals were fasted and sacrificed 6 h after. The Co group was subjected to the same fasting and sacrificed at the same time.