

Determination of Total Antioxidant Capacity of Human Plasma from Patients with Lung Diseases Using Constant-Current Coulometry

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Abstract

Original coulometric method for total antioxidant capacity (TAC) determination based on the reaction of plasma antioxidants with electrogenerated bromine is developed. TAC is expressed in units of quantity of electricity (Coulombs) spent for titration per a liter of plasma. Coulomb is the universal unit of the TAC measurements because one can express TAC in units of different antioxidants using Faraday constant and the number of electrons involved in the reaction between antioxidant and electrogenerated bromine.

The TAC in the plasma being determined for patients with different types of lung diseases is significantly lower than the one for control group. The data obtained by coulometry correlate with results of voltammetric determination based on oxidation of plasma on the glassy carbon electrode ($Y=a + bX$, where $a = -6,7 \pm 0,5$, $b = 1,07 \pm 0,04$, $R = 0,9934$).

So, the proposed method could be recommended for clinical laboratories for estimation of antioxidant status and its subsequent correction using additional antioxidant therapy.

Keywords: Total antioxidant capacity; Constant-current coulometry; Voltammetry; Blood; Lung diseases

1. Introduction

The current scientific literature is replete with data concerning oxidative stress in biological systems and the role of various antioxidants both in physiological states such as growth and aging, or in pathological conditions as atherosclerosis, neurogenerative and inflammatory diseases and cancer.

Oxidative stress may be defined as a violation in the balance between oxidants and reductants in living organism. The living cells are exposed to oxidants originating from a large variety of exogenous and endogenous sources. Exogenous sources include air pollutants, natural deleterious gases, ozone and high concentrations of oxygen or hyperbaric oxygen for example, effects of ionizing and non-ionizing irradiation, chemicals and toxins, and pathogenic bacteria and viruses [1-3].

Oxidants can be classified in various ways; according to their reactivity towards biological targets, according to their production site, according to their chemical nature or according to their belonging to a radical or non-radical subgroup. The radical group includes species such as superoxide anion-radical ($O_2^{\bullet-}$), hydroxyl radicals (HO^{\bullet}) and nitric oxide radicals (NO^{\bullet}). The non-radical oxidant group includes metabolites as hydrogen peroxide, hypochlorous acid and aldehydes. These species can cause biological damage on their own or may serve as a sources for more reactive and damaging species [4].

Endogenous sources include enzymes which can indirectly produce reactive oxygen species (ROS). The enzyme xanthine oxidase converting xanthine to uric acid also converts oxygen to superoxide anion-radicals during this process and nitric oxide synthase can produce nitric oxide radicals which can interact with superoxide anion-radicals resulting in the production of the deleterious oxygen species peroxynitrite [5]. Neutrophils may also serve as major contributors of ROS. Following activation these cells undergo a respiratory burst resulting in the release of an efflux of ROS as well as proteinase, cationic proteins and other compounds which may act synergistically to cause oxidative damage in tissues [6].

The living organism has adapted itself to an existence under a continuous efflux of ROS. Among the different adaptive mechanisms the antioxidant defense system is of major importance [7, 8].

This system can be classified into two major groups: the proteins and the low-molecular weight antioxidants (LMWA). The antioxidant proteins contain enzymes (superoxide dismutase, catalase, peroxidase and some supporting enzymes such as glucose-6 phosphate dehydrogenase and glutathione reductase) and the properly proteins (albumin, transferrin, caeruloplasmin and ferritin). The LMWA group contains a large number of compounds capable of preventing oxidative damage by direct and indirect interaction with ROS [9].

The efficiency of antioxidants is expressed as total antioxidant capacity (TAC) parameter summarizing overall activity of all types of antioxidants in living systems.

There are three major approaches for the evaluation of antioxidant status: determination of antioxidants, measurement of the products of macromolecules oxidation and direct detection of free radicals. For assessing endogenous antioxidant capacity, most studies have examined the concentrations of antioxidants (vitamin E, vitamin C, carotenoids, folate, sulfur containing amino acids) in plasma and cells and the cellular activities of antioxidant enzymes (glutathione reductase, superoxide dismutase, catalase and glutathione peroxidase). Because glutathione is rapidly oxidized to disulfide form by radicals and other reactive species and it is exported from cells, intracellular glutathione:disulfide ratio can provide a valid index of oxidative stress [10]. Assessments of lipid peroxidation have included the analysis of lipid peroxides, isoprostanes, diene conjugates, and breakdown products of lipids (malonaldehyde, ethane, pentane and 4-hydroxynonenal). Among these products, malonaldehyde is often used as a reliable marker of lipid peroxidation. For assessing ROS-induced protein oxidation, most investigators have determined the production of protein carbonyls, the loss of free thiol groups in proteins, and nitration of protein-bound tyrosine residues [11]. Indeed, protein nitrotyrosine has been widely used as a convenient stable marker for the production of reactive nitrogen-centered oxidants (nitrite oxide and peroxyxynitrite). Direct detection of free radicals has been performed using electron spin resonance and spin trapping techniques [12]. Although the electron spin resonance technique is suitable for detecting free radicals in solution chemistry, it has limited application to biological tissues owing to their usually high content of water. However, this problem can be overcome by the use of the spin trapping technique, which involves the conversion of highly reactive free radicals to relatively inert radicals, followed by electron spin resonance analysis [13].

One of the perspective ways for TAC evaluation is electrochemical methods [14-18].

The aim of work presented is development of coulometric titration for the TAC analysis in biological objects.

2. Experimental

2.1. Subjects

Thirty healthy blood donors, aged 21-54 years, were used as control group for the determination of the reference range of the TAC. They were on a normal diet.

A group of 70 patients with lung diseases was examined. Patients were on the ordered diet and regimen. The etiologies of the patients' lung disease were chronic obstructive pulmonary disease (30 persons), tuberculosis (20 persons) and cancer of lung (20 persons).

2.2. Blood sampling

Venous blood was collected in glass tubes containing a small amount of heparin as an anticoagulant. Plasma was obtained by centrifugation at 3000 rpm for 5 min and immediately analyzed for TAC.

2.3. Determination of TAC

2.3.1. Constant-current coulometry

Determination of TAC is based on the coulometric titration of plasma by electrogenerated bromine [19].

TAC is expressed in units of quantity of electricity (Coulombs) which is spent for titration on one liter of plasma.

2.3.2. Voltammetry

The plasma TAC was measured using voltammetric analyzer "Ecotest -VA". A three-electrode system consisted of glassy carbon electrode, a silver-silver chloride reference electrode with KCl saturated (Ag/AgCl) and a counter electrode (platinum wire). Measurements were carried out in phosphate buffer solution, pH 7.4 (15 mL) at a potential scan rate of $200 \text{ mV}\cdot\text{s}^{-1}$. Voltammograms were recorded in the potential range from 0 to +0.8 V. Each sample was analyzed five times.

2.4. Statistical analysis

Statistical analysis of the results was performed using SPSS for Windows. All data are expressed as the mean value \pm SE. The differences of parameters were tested by Student's t-test. A $p < 0.05$ was considered as statistically significant.

3. Results and discussion

Method for estimation of plasma TAC using constant-current coulometry is developed. TAC determination is based on the reaction of plasma antioxidants with the electrogenerated

bromine as active species. Electrochemical oxidation of bromide on platinum electrode in acidic medium leads to formation of Br_2 , Br_3^- , and radicals Br^\bullet . They take part in radical, redox reactions and reactions of electrophilic substitution and addition to double bonds. Therefore, one can investigate wide range of biologically active compounds of various structure including antioxidants containing in human plasma. TAC is expressed in units of quantity of electricity (Coulombs) spent for titration per a liter of plasma. Coulomb is the universal unit of the TAC measurements because one can express TAC in units of different antioxidants using Faraday constant and the number of electrons involved in the reaction between antioxidant and electrogenerated bromine as mentioned above.

The TAC in the plasma of patients with lung diseases is significantly lower than the one for control group (Table 1).

Table 1. Total antioxidant capacity of plasma from patients with lung diseases.

Group	Total antioxidant capacity, $\text{kCl}\cdot\text{L}^{-1}$
Control	26 ± 2
Chronic obstructive pulmonary disease	10.5 ± 0.5
Cancer of lung	8 ± 1
Tuberculosis	6 ± 3

Simultaneously, determination of plasma's TAC using voltammetry was performed. Plasma components oxidize on the glassy carbon electrode in phosphate buffer at 0.38 V vs. Ag/AgCl with formation of wave or peak (Fig.1).

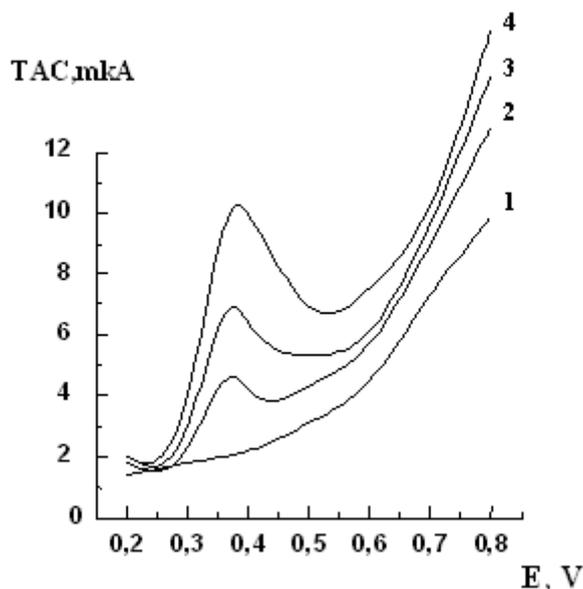


Fig.1. Voltammograms of different volume (mL) plasma oxidation on glassy carbon electrode in phosphate buffer (pH 7.4): 1 - 0; 2 – 0.15; 3 – 0.20; 4 – 0.30. Potential scan rate is $200 \text{ mV}\cdot\text{s}^{-1}$.

The peak potential or inflection point of current wave characterizes the reducing power of antioxidants and their ability to donate electrons. The anodic current corresponds to the concentration of antioxidants in plasma. It should be noted, that anodic current of the peak linearly grows with increase of plasma volume entered in electrochemical cell.

The values of plasmas TAC are presented in Table 2.

Table 2. Result of voltammetric determination of plasma TAC ($P=0.95$).

Pathology	Total antioxidant capacity, mA	RSD
Chronic obstructive pulmonary disease	2.0 ± 0.2	0.01
Cancer of lung	3.6 ± 0.2	0.04
Tuberculosis	0.46 ± 0.09	0.09

Although TAC values were determined in different conditions, correlation between TAC received by voltammetry and coulometry has shown ($Y=a + bX$, where $a= -6.7\pm 0.5$, $b=1.07\pm 0.04$, $R =0.9934$).

It should be noted, that human respiratory tract stretches from the nasal and oral cavity to the alveoli of the lung, making the total surface area that is directly exposed to the external environment [20]. Thus, the large endothelial surface makes the lung a major target site for

circulating oxidants and xenobiotics. Since the lung is constantly exposed to many atmospheric pollutants including tobacco smoke, fuel emissions, ozone and nitrogen dioxide and given the natural oxidizing nature of the atmosphere (21 % O₂) the lung is always at risk of oxidative injury [20, 21].

Respiratory tract infections or exposure to airborne pollutants that overwhelm local antimicrobial and antioxidant defenses result in an initiation of the inflammatory/immune system and importantly the recruitment and activation of phagocytic cells. The airway epithelium functions as an effector of such responses, which include an increase in mucus secretion and the production of inflammatory mediators and cytokines that further induce the recruitment of inflammatory and immune cells [20, 22]. The response in the lung is characterized by activation of endothelial cells, epithelial cells and resident macrophages, and the recruitment and activation of phagocytic cells such as neutrophils, eosinophils, monocytes and lymphocytes.

Neutrophils and other phagocytes attack pathogens using a mixture of oxidants, for example, O₂^{•-}, NO[•], H₂O₂, and HOCl. The process of phagocytosis begins when the neutrophil travels to a site of infection as directed by certain chemotactic signals that are generated at the infection site. When foreign microbes, which may be bound by serum-derived glycoproteins (opsonized, for example), perturb the plasma membrane of a neutrophil, a dormant pyridine-nucleotide-dependent oxidase is activated. This pyridine nucleotide oxidase is believed to be a reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) or a reduced nicotinamide adenine dinucleotide oxidase (NADH oxidase), whose action might involve a b type cytochrome [23, 24]. This process initiates a “respiratory burst”, which lasts for 15 to 20 min and reduces O₂ to O₂^{•-} (a one-electron reduced product) and H₂O₂ (a two-electron reduced product). It also serves as the first source of oxidant production in phagocytes.

Simultaneously, the plasma membrane of the phagocyte invaginates around the foreign particle, surrounding it and subsequently pinching off to become a phagosome. Also during this time period, degranulation occurs: granular lysosomes migrate toward the phagosome, fuse with it, and empty their granular contents into it; after fusion with lysosomes, the phagosome becomes a phagolysosome. The contents of the lysosomes primarily include digestive enzymes like acid hydrolases, neutral proteases, and alkaline phosphatases. These lysosomes also contain cationic proteins, lipopolysaccharides, lactoferrin, myeloperoxidase, and biopolymers that might be involved in bactericidal reactions. This entire process occurs extremely rapidly taking no longer than a few minutes [23, 24].

Inside the phagolysosome, the heme containing enzyme myeloperoxidase forms an enzyme-substrate complex with H_2O_2 that can then catalyze the two-electron oxidation of halides like Cl^- , Br^- , and I^- by H_2O_2 . The oxidation of the halides, particularly Cl^- , forms a toxic agent with potent antimicrobial properties. In the case of chloride, the oxidation product is HOCl, which subsequently kills the ingested microorganism. The H_2O_2 needed for this process is generated in the respiratory burst and can be detected in the phagosome [25]. Furthermore, a recent study on the myeloperoxidase-hydrogen-peroxide-chloride system showed that myeloperoxidase also generates Cl_2 gas and that human neutrophils employ chlorine gas as an oxidant during phagocytosis [26].

TAC value for patients with chronic obstructive pulmonary disease is lower than the one healthy subjects and this difference is statistically significant.

The pathogenesis of chronic obstructive pulmonary disease is complex [27]. It involves both airway inflammation [28] and an oxidant/antioxidant imbalance [29]. The inflammatory response is usually accompanied by an influx of neutrophils and release of proteases (neutrophil elastase, tryptase) and ROS. Excessive transmigration is responsible for the enhanced number of neutrophils that is found in the airways and in a bronchoalveolar lavage fluid of patients with chronic obstructive pulmonary disease [30]. During this transmigration process various mediators are released. These involve cytokines that regulate the expression of adhesion molecules, inflammatory cell degranulation and migration [31]. This inflammatory process is also accompanied by oxidative stress. Proinflammatory cytokines and growth factors stimulate release of ROS which act as signaling mediators for a variety of signal transduction pathways and gene expression [32-34]. These observations suggest that inflammation and oxidative stress are co-dependent and strongly interrelated processes [35].

Moreover, oxidative stress may be also involved in the systemic consequences of chronic obstructive pulmonary disease. Oxidative stress occurs when the balance between oxidants, for instance ROS, and antioxidants shifts in favor of the ROS. A few studies indicate that the antioxidant status is impaired at the systemic level. Increased levels of lipid peroxidation products have been demonstrated in serum of patients with stable chronic obstructive pulmonary disease [36].

TAC level for tuberculosis and cancer is significantly differ from one for other groups as one can assume. It is caused by active destructive processes in the lung.

So, the significantly lower TAC level for patients could be explained by approaches mentioned above.

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