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The application of coulometry for total antioxidant capacity determination of human blood

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Abstract

New coulometric method for estimation of blood and plasma total antioxidant capacity (TAC) based on using electrogenerated bromine was proposed. TAC of blood from patients with chronic renal disease undergoing long-term hemodialysis was investigated. Statistical significant changes in TAC level of venous and arterial blood were found. Catalase activity and low density lipoproteins (LDL) concentrations were determined. Linear correlation between TAC and parameters mentioned was found. Contribution from some individual antioxidants was investigated. The developed method for TAC assay is expressive, simple, stable and reliable, and successfully could be used for TAC determination of some biological fluids. This method could be applied in clinic for estimation of blood TAC from patients.

Keywords: Total antioxidant capacity; Coulometric titration; Chronic renal failure; Blood; Catalase; Low density lipoproteins

1. Introduction

Living cells are continuously exposed to a variety of challenges that exert oxidative stress. These could stem from endogenous sources through normal physiological processes such as mitochondrial respiration and haemoglobin oxidation. Alternatively, they could result from exogenous sources such as exposure to pollutants, ionizing irradiation and other extreme factors [1].

Oxidative stress is often associated with or leads to the generation of reactive oxygen species (ROS) including free radicals. Their amount depends not only from generation rate but also from antioxidant defense system of human body particularly of blood. Violation in its antioxidant properties leads to the development of pathological state [2,3] because ROS are strongly implicated in the pathophysiology of diseases such as cancer, heart diseases and atherosclerosis, aging, diabetes mellitus, renal, inflammatory, infectious and neurological diseases [4].

There are different mechanisms of antioxidant action in biological fluids. The first is decreasing the level of active products from oxygen reduction and another way is removing the transition metals group (Fe, Cu) by their bounding with proteins. This leads to inhibition of free radical reactions [5,6]. Free radicals are also eliminated from the body by their interaction with antioxidants.

Two classes of antioxidants are known: the low-molecular weight (LMW) compounds (tocopherols, ascorbate, β-carotene, glutathione, uric acid, bilirubin, etc.) and the proteins (albumin, transferrin, caeruloplasmin, ferritin, superoxide dismutase, catalase, glutathione peroxidase, etc.) [7]. TAC parameter summarizes overall activity of antioxidants and antioxidant enzymes. The depletion of TAC induced by oxidative stress is eliminated by release of stock organ antioxidants, mainly from liver and adipose tissue and the induction or activation of antioxidant enzymes. At a later phase of oxidative stress, the TAC falls due to depletion of

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antioxidants. LMW antioxidants penetrate specific location in the cell where oxidative stress may occur and protect against ROS. The clinical importance of determining TAC consists in identifying patients with increased risk of diseases mentioned above and deficient nutrition [8,9]. A significant increase of TAC really occurs after supplementation with vitamins C, E, and β-carotene [10] and phenolics of green and black tea [11]. TAC can be also used for monitoring and optimization antioxidant therapy.

At present, estimation of the body antioxidant status as clinical index of oxidative stress has three approaches: determination of the concentration of total or individual LMW antioxidants in serum or plasma, determination of enzymes activity and monitoring markers of oxidative stress, products of LDL peroxidation for example.

The TAC of biological fluids has been assayed by a number of different methods including oxygen consumption during lipid peroxidation [12], luminol-enhanced chemiluminescence [13], measurement of R-phycoerythrin bleaching (oxygen radical absorbance capacity) [14–16], sensitivity of erythrocytes to hemolysis [17], ferric reducing activity [18], lipids generation [19,20], and crocin bleaching assay [21,22].

Original method was proposed for estimation of TAC in Trolox-equivalent units. Method based on the formation of blue–green cation radical of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] cation in the presence of methmyoglobin and hydrogen peroxide [23].

Electrochemical methods are developed for the investigation of antioxidant properties of biological fluids particularly of blood, estimation of its TAC and clarifying the role in antioxidant defense system [24,25]. Cyclic voltammetry has been validated for quantitation of the LMW antioxidant capacity of blood, plasma, tissue homogenates [26] and for the measurement of the plasma TAC from patients with chronic renal disease during dialysis therapy [27].

Patients undergoing long-term hemodialysis exhibit the increased levels of oxidative stress [28,29]. So, investigation of TAC for patients with chronic renal disease undergoing long-term hemodialysis is very actually.

The aim of the study is to propose new simple coulometric method for TAC determination and its application in clinic.

2. Experimental

2.1. Subjects

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

A group of 100 patients (44 males and 56 females) with chronic renal failure receiving hemodialysis at the Center of an-of Kidney Organism Clearing (Department of Hemodialysis, Kazan) was examined. All patients were dialyzed three times/week for 3 h each session. Two groups of patients were chosen. The first group was received regular hemodialysis less than 1 year and the second — more than 1 year. Patients with higher proteinuria (over 300 mg/l) were excluded from the study. Patients were examined before and after standard session of hemodialysis using a dialyzer with polysulfon membrane (Fresenius) with an area of 1.3 m^2. Patients were on the ordered diet and regimen.

2.2. Blood sampling

Blood was collected before and after dialysis and also in 24 and 48 h after session of hemodialysis. Venous and arterial 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.

2.2.1. Serum preparation

Blood was placed in pure drawn tubes. Before centrifugation, the clot was rimmed with a wooden applicator stick. Then samples were centrifuged at 2000 prm for 20 min. The serum was removed and immediately analysed for LDL and TAC.

2.3. Determination of TAC

Determination of TAC is based on the coulometric titration of blood or plasma by electrogenerated bromine [30].

Bromine was electrogenerated from aqueous 0.2 M KBr solutions in 0.1 M H_2SO_4 using a P-5827 M potentiostat at a current density 5 mA/cm^2. The end-points in amperometric titrations were measured with two polarized platinum electrodes (ΔE = 300 mV). A smooth platinum plate with a surface area of 1 cm^2 served as the working electrode, and a platinum coil separated from the anodic compartment with a semipermeable diaphragm served as the auxiliary electrode.

Coulometric titration was carried out in a 50.0 mL cell that contained 20.0 mL of the supporting electrolyte. The generating circuit was switched on; when a certain value of the indicator current was attained, an aliquot portion (20 μL) of blood or plasma was added to the cell, and a timer was simultaneously started. The titration end-point was detected by the attainment of the initial value of the indicator current. The timer was stopped, and the generating circuit was turned off [31–33].

All experiments were carried out at temperature 25 ± 2 °C.

TAC is expressed in units of quantity of electricity (Coulombs (Cl)) which is spent for titration on 1 L of plasma or blood.

2.4. Determination of catalase activity

Catalase activity was measured by an assay of hydrogen peroxide based on formation of its stable complex with ammonium molybdate [34]. Blood (0.2 mL) was incubated in 1 mL reaction mixter containing 65 mM hydrogen peroxide in 60 mM sodium–potassium phosphate buffer, pH 7.4 at 25 °C

for 4 min. The enzymatic reaction was stopped with 1 mL of 32.4 mM ammonium molybdate and the concentration of the yellow complex of molybdate and hydrogen peroxide was measured at 450 nm. Enzyme activity was expressed in mkat (mkat/mL of blood).

2.5. Determination of low density lipoproteins (LDL)

Photometric determination of LDL was performed according to [35]. Colloid stability of blood proteins in heparin and calcium chlorous presence is broken, so pure LDL is precipitated. Heparin is able to form complex with LDL which is precipitated with calcium chlorous addition. Quantity LDL is determined by dimness intensity of solution.

2.6. Investigation of individual antioxidants contribution

Liophilic albumin from human serum (REANAL, Hungary) and 10% human serum albumin solution were used as standards.

Coulometric titration of standard albumin solution was performed by electrogenerated bromine and iodine. Electrogeneration of iodine and bromine were carried out from 0.1 M KI in tartrate buffer solution with pH 3.56 and 0.2 M KBr in 0.5 M H_2SO_4, respectively.

Ascorbate, glutathione, urea, uric acid, cysteine, vitamins B_1, B_2, B_6, PP (chemical grade) standard solutions were prepared by exact weighting and dissolving in double-distilled water.

2.7. Statistical analysis

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

The correlation analysis of data was performed using parametric methods with the aid of the Origin v 6.1 (OriginLab Corporation).

3. Results and discussion

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 blood. So, S-containing amino acids, ascorbic and uric acids can be oxidized by electrogenerated bromine. Polyphenols are capable to react by radical mechanism, electrophilic substitution and redox processes. Porphyrins are inclined for addition to double bonds.

Table 1
Total antioxidant capacity of venous blood from patients with chronic renal failure during hemodialysis

Duration of hemodialysis	Total antioxidant capacity (kCl/L)			
	Before hemodailysis	R.S.D.	After hemodailysis	R.S.D.
Less than 1 year	27 ± 2	0.05	39 ± 2	0.05
More than 1 year	27 ± 1	0.04	35 ± 2	0.04

The electrogenerated bromine was used as reagent for estimation of the integrated antioxidant properties of biological fluids particularly of blood and plasma.

3.1. TAC of blood and plasma

TAC of venous blood for patients is authentically lower than for control group on 63% (26 kCl/L versus 42 kCl/L respectively). Patients undergoing long-term hemodialysis exhibit the increased levels of oxidative stress and show the deficit of compounds with antioxidant properties. These experimental data are confirmed by [28]. So, patients with chronic renal failure have the weaken antioxidant defense.

TAC of blood for patients in process of hemodialysis increases irrespectively at duration of hemodialysis (Table 1). TAC increases during hemodialysis on contrary to that has been reported by Gerardi et al. [29]. Increased TAC level of blood after hemodialysis session is probably caused by decreasing of oxidant and pro-oxidant contents during extracorporal blood clearing process.

TAC of blood during the session of hemodialysis increases. It's alteration with duration shows authentically increasing in the course of first 24 h, average increase being 13.4% (Fig. 1). In 48 h after hemodialysis session, blood antioxidant capacity significantly decreases (till the 25 kCl/L). But this value is more than TAC primary level (23 kCl/L).

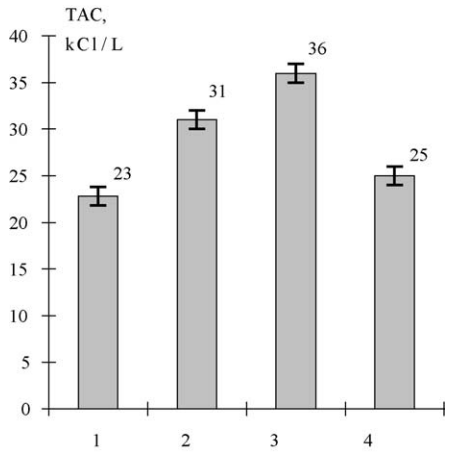

Fig. 1. Variation in total antioxidant capacity of arterial blood from hemodialyzed partients with the time: (1) before session of hemodialysis; (2) after session of hemodialysis; (3) over 24 h after session; (4) over 48 h after session.

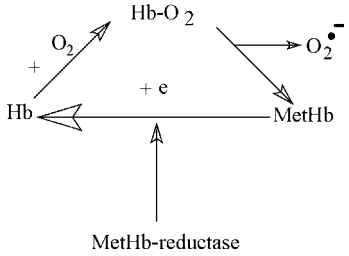

Fig. 2. Formation of superoxide anion-radical during haemoglobin oxidation.

TAC increases during the first 24 h after end of procedure. It is possibly associated with hemodialysis properties. Hemodialysis possesses the clearing function. But also, it actuates trigger mechanisms which promote the appearance and shift of antioxidants in peripheral blood. The following decreasing of TAC to primary level is coincided with periodicity of hemodialysis in Russia (48 h). It is consequent of metabolism in living cells.

The levels of antioxidant capacity for venous and arterial blood are slightly different (26 and 23 kCl/L, respectively). As known, arterial blood is characterized with the smaller antioxidant capacity as it is saturated by oxygen. The first product of oxygen molecular activation is superoxide anion-radical ($O_2^{\bullet -}$). It is source of all active forms of oxygen in vivo which intensively decrease the antioxidant capacity. Haemoglobin transporting oxygen has ability to form $O_2^{\bullet -}$ in oxidation (Fig. 2) [36].

Antioxidant capacity of plasma is less than for whole blood (for arterial blood in 1.9 and for venous in 1.7 time) (Table 2). It is apparently connected with the content of porphyrins in whole blood which reacts with electrogenerated bromine.

3.2. TAC correlation with LDL concentration and catalase activity

LDL is the main object of peroxidation. Therefore, concentration of serum LDL has important prognostic function. One can assume that lipid peroxidation intensity and following effect from antioxidant therapy for alteration of antioxidant status.

ROS may be involved in a broad pattern of tissue injury in patients on regular hemodialysis therapy and, in fact, increasing evidence suggests that the antioxidative system is compromised in these patients.

Table 2
Total antioxidant capacity of plasma and blood

Analyte	Total antioxidant capacity (kCl/L)			
	Before hemodialysis	R.S.D.	After hemodialysis	R.S.D.
Arterial plasma ($n = 41$)	12.2 ± 0.6	0.03	17 ± 1	0.03
Arterial blood ($n = 41$)	23 ± 1	0.04	31 ± 1	0.04
Venous plasma ($n = 34$)	15 ± 1	0.04	21.6 ± 0.8	0.05
Venous blood ($n = 34$)	26 ± 1	0.05	34.3 ± 0.9	0.03

LDL level in serum for hemodialyzed patients is significantly higher than for control group. This data agree with earlier data [37,38] that patients with chronic renal failure have significantly increased lipoprotein level as compared with the reference group.

Relationship between TAC and LDL concentration was found. So, TAC is inverse proportional to LDL concentration ($Y = a + bX$; $a = 20.0 \pm 0.9$; $b = -(1.0 \pm 0.2)$, $R = -0.785$, $p < 0.001$). Reverse correlation between TAC level and LDL concentration is well conformed with theory. So, TAC parameter found by coulometric titration method can use to prediction antioxidant defense system state.

Free oxygen radicals produced by normal aerobic metabolism have been implicated in several pathophysiological mammalian processes. Mammalian erythrocytes have large amounts of catalase which is a heme-containing enzyme that catalyses the conversion of hydrogen peroxide to water and oxygen.Catalase has a dual functional role; a true catalytic role in the decomposition of hydrogen peroxide and a peroxidic role in which the peroxide is utilized to oxidize a range of H-donors. It is widely distributed in the body compartments, tissues, and cells. Erythrocytes appear to have high catalase activity compared to other cells because of greatly exposure of erythrocytes to molecular oxygen [39–41].

Catalase activity was determined. For hemodialyzed patients, significantly lower catalase activity (on 13%) caused by higher hydrogen peroxide levels, was found. It seems reasonable to assume that the imbalance in the activity of extracellular antioxidant enzymes in chronic renal disease can result in accumulation of free radical species, and in unscheduled oxidation of susceptible molecules. Similar results were obtained by authors [38,42].

Correlation analysis demonstrates the linear dependence between TAC level and catalase activity ($Y = a + bX$; $a = 2 \pm 1$; $b = 0.98 \pm 0.04$, $R = 0.9887$, $p < 0.0001$). Positive correlation TAC with catalase activity demonstrates that coulometric TAC determination method reflects correctly antioxidant properties of biological fluids.

So, obtained correlations between TAC and LDL concentration and catalase activity confirm interrelation of processes with participation of free radicals, antioxidants (both low-molecular and proteins, in particular enzymes) and LDL as substrate of peroxidation. Moreover, it is shows efficiency of common parameters use, TAC for example, for estimation of efficiency of antioxidant defense system in living organism, in particular its antioxidant status.

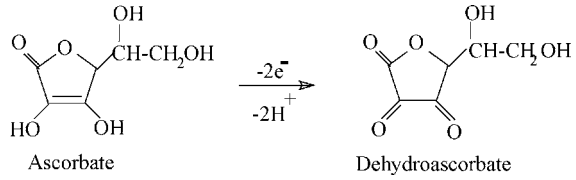

Fig. 3. Scheme of ascorbate electrochemical oxidation.

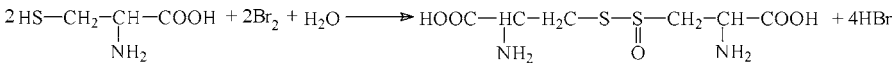

Fig. 4. Cysteine reaction with electrogenerated bromine.

3.3. Individual antioxidants giving contribution in TAC

So far as TAC determined is integral index, investigation of contribution from individual antioxidants is very important. It should be noted that only water-soluble antioxidants have been investigated because TAC measurements were carried out in aquatic media.

From chemical structure of various water-soluble antioxidants, one can assume that TAC of blood is caused by presence of S-containing amino acids, vitamins C, B_1, B_2, B_6, PP, Fe-containing porphyrins, glucose, creatin, creatinin, urea, heparin (as anticoagulant), albumin and uric acid.

So, their reactions with electrogenerated bromine were studied for estimation of their contribution in TAC.

Vitamins B_1, B_2, glucose, creatin, creatinin, urea and heparin don't react with bromine and don't put in any contribution in TAC of blood at condition under investigation. On the other side, as experimental data shown, S-containing amino acids, vitamin C, Fe-containing porphyrins, albumin and uric acid react with the electrogenerated bromine.

Ascorbic acid is quantitatively oxidized by electrogenerated bromine to dehydroascorbic acid with consuming of 2e (Fig. 3).

Uric acid reacts with electrogenerated bromine forming urea and alloxane [43] and gives contribution to TAC.

S-containing amino acids react with the titrant with formation disulfide, sulfonic and sulfoxide compounds.

Glutathione interacts with the electrogenerated bromine being consumed six electrons per molecular during electrolysis [44] with oxidation of HS-group to sulfonic group.

Cysteine also reacts with bromine according to following scheme (Fig. 4).

Taking into account, there are S-containing amino acids contents in blood, one can assume that their contribution in TAC would be significant.

Human serum albumin is by far the most abundant, important and powerful antioxidant. It overwhelms all other antioxidants and has to be removed during attempts to measure antioxidant capacity. It has ability to consume and release electrons. Albumin also binds with metals like copper, zinc, iron and mercury, lowing activity of metals as catalysts [45].

Human serum albumin reacts with the electrogenerated bromine and iodine. Stoichiometric coefficients being found by coulometric titration for model solutions are equal to 1:73 and 1:64, respectively [46].

Experimental data obtained confirms that human serum albumin gives the main contribution in TAC.

It should be noted that TAC of blood is parameter characterizing the balance between antioxidant and pro-oxidant systems. Therefore, fluctuations in concentration of individual antioxidants are controlled by homeostase.

The proposed method for TAC assay is expressed, simple, stable and reliable and could be successfully used for TAC determination of human blood and plasma. This method could be applied in clinic for estimation of blood TAC from patients.

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