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Total Antioxidant Capacity Determination with Colorimetric Method and Microplate Reading Format and Comparison with Chemilumin Essence Assay

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Abstract

Total antioxidant capacity (TAC) is related to a variety of molecules that protect biological systems against reactive oxygen species (ROS). The importance of total antioxidants results in the requirement of TAC assay methods. This study is done to design a simple, rapid and inexpensive method for TAC measurement. In this method, ABTS (2, 2′-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid]) was incubated with met myoglobin and H2O2 to produce green radical cation ABTS+. Antioxidants were suppressed the color production, thereby reducing the intensity of the color that was proportional to the concentration of antioxidants, measured at 600nm with a microplate reading format. Trolox was served as a standard or control antioxidant. Sensitivity, precision and accuracy were examined to check the validity of the method. Sensitivity of this method was 0.10 U/ml. The coefficient of variation percent for intra and inter assay was less than 9.6%. According to parallelism and recovery tests, ratio% and recovery% ranged from 90.9-108% and correlation coefficient in comparison with the sensitive chemiluminescence method was 0.9897 (n=60). Data from this study showed that this method has a good sensitivity, precision and accuracy and can be suitable for clinical and research uses.

Keywords: Total antioxidant capacity, Colorimetry, ABTS, Microplate reading format

Introduction

Antioxidants play an important role in the inhibition of reactive oxygen species (ROS) and prevent ROS generation (Stanczyk, Gromadzinska, Wasowicz, 2005). Antioxidant species are divided into three categories: enzyme systems (catalase, glutathione peroxidase, superoxide dismutase, etc.), small molecules (ascorbate, uric acid, glutathione, vitamin E, etc.) and proteins (albumin, transferrin, etc.) (Percival, 1998). Any disruption in the function of antioxidant systems will lead to oxidative stress that depending on the amount and the place of it, cause or accelerate disorders and diseases such as severe damage to DNA (Nisha, Deshwal, 2011), coronary heart disease (Gawron, Chrzczanowicz, Nowak, Nonas, Drygas, Jegier, Kostka, 2005), lung disease (Ziyatdinova, Budnikova, Pogoreltzev, 2006), severe renal disease (De Vega, Fernandez , Mateo, Bustamante, Herrero, Munguira, 2002), diabetes (Taheri, Djalali, Saedisomeoli, Malekshahi, Jazayeri , Qorbani, 2012), epilepsy (Aryaeian, Djalali, Shahram, Jazayeri, Chamari, Nazari, 2011), hepatitis (Venturini, Simao, Barbosa, Lavado, Narciso, Dichi, Dichi, 2010), Parkinson's and

Alzheimer's diseases (Sofic, Sapcanin, Tahirovic, Gavrankapetanovic, Jellinger, Reynolds, 2006) and cancer (Emin, Yordanova, Dimov, Ilieva, Koychev, Prakova, Vlaykova, 2010). Measurement of total antioxidant capacity (TAC) helps in prevention, diagnosis and treatment of many known or unknown human disorders and diseases. Furthermore, TAC determination is necessary in many aspects of cellular and molecular research. Various methods have been used to measure the total antioxidant capacity in clinical studies such as Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) (Gupata, Sharma, Lakshmy, Prabhakaran, Reddy, 2009; Benzie, Strain, 1996).

In TEAC assay metmyoglobin reduces hydrogen peroxide and produces ferryl myoglobin radical which oxidizes the ABTS (2, 2′-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid]) and produces radical cationic and ABTS+ which is green. Antioxidants inhibit the production of ABTS+; thereby reducing the intensity of the color is proportional to the total antioxidant capacity. In this method, Trolox, a water-soluble vitamin E analog serves as a standard or control antioxidant (Resat Apak, Gorinstein, Bohm, Schaich, Özyürek, Kubilay Güçlü, 2013). Another way to measure the TAC, which is highly sensitive is chemiluminescence assay.

In this method, peroxyl radicals produced from 2, 2'-azobis (2-amidinopropane) hydrochloride (AAPH) oxidize luminol which leads to the formation of luminol radicals and photon emitting. Antioxidants inhibit the peroxyl production, thereby reducing the luminescence is proportional to the total antioxidant capacity (Wayner, Burton, Ingold, Barclay, Locke, 1987). Due to a great interest in determination of antioxidant capacity in biological samples, this study aimed to design a method for measuring antioxidant capacity by colorimetric method and microplate reading format. Secondary objectives of the study were the direct use of human serum samples without the need for preparation, shortening the reaction process time and increasing and speeding up the OD reading by using microplate reading format.

Materials and methods

First, the required solution was prepared by using chemical compounds with high purity (Sigma, United States of America) as follows:

Standard solutions

Trolox standard solutions (100, 80, 60, 40, 20, and 10 µM) were prepared by using serial dilution of 1 µM stock solution.

Phosphate buffered saline (PBS, 10mM pH 7.4)

First a liter of 10 µM sodium dihydrogen phosphate solution (1.2 g/L) was prepared. Then a liter of 10 µM dipotassium hydrogen phosphate solution (1.74 g/L) was prepared. PH meter electrode was put in the first solution, and the second solution was added to it until pH reached at 7.4. Then, 150 mmol NaCl (8.775 g) weighed and was added to the above buffer. PBS was used as a diluent buffer solution.

Blank solution

Diluent buffer was used as a blank solution.

ABTS Solution

ABTS solution (250 µM) was prepared by using 2.5 µM stock solution.

Metmyoglobin solution

Metmyoglobin solution (2 µM) was prepared by using 20 µM stock solution. PBS was used as a solvent.

Hydrogen peroxide solution

Hydrogen peroxide solution (50 mM) was prepared by using 5.0 M stock solution.

Procedure

According to Table 1, reagents were added to the wells of test serum sample, blank and standard. The absorption was measured at 600nm with a microplate reader (Sun Rise, TECAN A-5082, TECAN Groups Ltd., Salzburg, Austria) just after 5min. Based on absorption of Trolox solutions, a standard curve was drawn and according to it, antioxidant concentration of the test serum samples was calculated.

Table 1: Method procedure

Test Tube / Reagent	Test Sample	Blank Solution	Standard Solution
Diluent Buffer	90 microliter	90 microliter	90 microliter
Sample	20 microliter	----	----
Diluent Buffer	----	20 microliter	----
Standard	----	----	20 microliter
Metmyoglobin Solution	50 microliter	50 microliter	50 microliter
ABTS Solution	20 microliter	20 microliter	20 microliter
5 min incubation at room temperature			
Hydrogen peroxide Solution	20 microliter	20 microliter	20 microliter
5 min incubation at room temperature then absorbance reading at 600nm			

Evaluation of validity of the method

Sensitivity

Sensitivity of the method was calculated based on the mean zero standard signals plus three standard deviation (3SD) method.

Precision

To examine the precision, three samples were selected in the range of low, medium and high concentration to determine the intra and inter assay. The reproducibility was calculated based on coefficient of variations (CV %) and replication number 12 in intra assay and 10 in inter assay.

Parallelism

To verify the accuracy based on the parallelism test, first the total antioxidant capacity in a sample was measured by the method under study. Then the same sample was diluted by 2, 4, 8, 16 and 32 times. By using the expected and the measured data, ratio% of parallelism test was determined.

Recovery

To verify the accuracy based on the recovery test, 0, 10, 20, 40, 80, 100 µl of the standard solution was added to a sample. By using the expected and the measured data, recovery% was calculated.

Method comparison

The comparison between the method under study and chemiluminescence method was performed by using 60 human serum samples and correlation coefficient of these two methods was calculated. Chemiluminescence assay was carried out by TAC-Peroxyl kit (Applied Bioanalytical Labs, USA) and luminometer (Lumistar, BMG, Austria).

Results

Table 2 and 3 contain the data for intra assay and inter assay precision. These findings show that the considered method has acceptable precision.

Table 2: Evaluation of precision by intra assay test

Concentration	Replication No.	Mean	SD	CV%
Low	12	6.53	0.51	7.8
Medium	12	10.26	0.82	8.0
High	12	14.32	1.08	7.5

Table 3: Evaluation of precision by inter assay test

Concentration	Replication No.	Mean	SD	CV%
Low	10	6.17	0.59	9.6
Medium	10	9.95	0.88	8.8
High	10	14.01	1.018	7.3

Based on the mean zero standard signals plus three standard deviation (3SD), sensitivity of the method was calculated about 0.10 U/ml.

In evaluating the accuracy of the method, parallelism and recovery test results are provided in Table 4 and 5. The results show the acceptable accuracy of the method.

Table 4: Evaluation of accuracy by parallelism test

Step	Dilution	Expected	Measured	Ratio%
1	01:01	50.00	47.24	94.48
2	01:02	25.00	26.34	105
3	01:04	12.50	13.01	104
4	01:08	6.25	6.75	108
5	01:16	3.13	3.0	95
6	01:32	1.56	1.4	92

Table 5: Evaluation of accuracy by recovery test

Step	Standard Addition	Expected	Measured	Recovery %
1	0+x	2.75	2.71	98.3
2	10+x	6.37	6.07	95.3
3	20+x	11.37	10.34	90.09
4	40+x	21.37	22.51	105.3
5	80+x	41.38	43.86	106.0
6	100+x	51.37	47.02	91.5

Figure 1 also contains the comparative results of the considered method with the chemiluminescence method in the same samples.

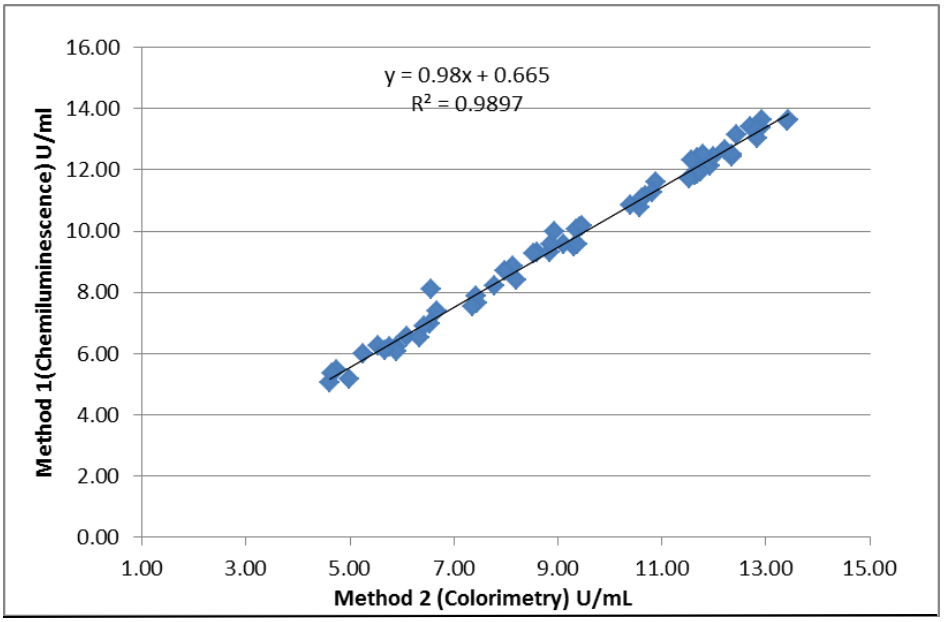

Figure1: Evaluation of accuracy by method comparison (n=60)

Discussion

In this study, given the importance of measuring the total antioxidant capacity, a simple, rapid and inexpensive method was submitted to measure the TAC in human serum samples and its results were compared with the chemiluminescence method.

Living organisms and blood have a large number of antioxidants which entrap the free radicals. Free radicals are produced as a natural byproduct of the normal cell metabolism and as a response to environmental factors. Excessive free radicals accumulation will lead to many diseases and quantitative measurement of the total antioxidant capacity in plasma may provide important biological information about the prevention, diagnosis and treatment of some of them.

Various methods have been presented for antioxidant capacity determination but none of them is a reference method, therefore, more studies are still required. So far, many methods have been reported based on spectrophotometry (Apak, Ozyurt, Demirata, 2011), colorimetry (Schnabel, Lackner, Rupprecht, Espinola-Klein, Torzewski, Schnabel, 2005), fluorometry (Ghiselli, Serafini, Maiani, Azzini, Ferro-Luzzi, 1995) and chemiluminescence assays for TAC determination from which some of them are used as commercial kits.

TAC assay kits produced by BioVision (www.biovision.com), Abcam (www.abcam.com), BioAssay Systems (www.bioassaysys.com) companies are based on the reduction of Cu+2 to Cu+ and reading absorption at 570 nm. TAC assay kits produced by RANDOX (www.randox.com) and SIGMA-ALDRICH (www.sigmaaldrich.com) companies are according to the TEAC method.

ABTS radical cation which is produced in the second reaction of TEAC method, can be solubilized in both aqueous and in organic media. Also, TEAC assay permits to study over a wide pH range. The procedure of TEAC is simple and is used in many research laboratories. Regarding the advantages of TEAC assay, the basic approach of this research is using its principle with microplate reading format which resulted in reducing the time of reading and the volume of samples. Also, in this method there is no need for complex preparation of sample before measurement and the sample is used directly.

This study has developed a simple, rapid, and inexpensive method for measurement of TAC. In addition, the foundation of the validity of a method is ensured from precision, accuracy and sensitivity of that method, and the proposed method has acceptable precision, accuracy and sensitivity. The common commercial kits assay TAC with a sensitive and efficient method is expensive. Therefore, this method can be suitable for clinical and research laboratory tests that provide reliable data.

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