Original article:

Determination of reference limit and evaluation of precision to measure Total Antioxidant Capacity (TAC) by Ferric Reducing Antioxidant Power (FRAP) method

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Abstract

Introduction: Total Antioxidant Capacity (TAC) is an important parameter and is a better alternative as all the antioxidants present in plasma need not to be measured separately.

Method: Blood was collected from 150 healthy volunteers to establish reference limit. TAC is measured by Ferric Reducing Antioxidant Power (FRAP) method, which is based on the principle that antioxidants present in the plasma reduce Fe^{+++} TPTZ complex to Fe^{++} TPTZ a blue coloured complex, the change in absorbance directly reflects the TAC of plasma. The absorbance is measured at 630nm, slightly modified from the original method, so that it can be measured in a semi-auto analyzer.

Observation: Mean of the study was found to be 1330.18 µMole/L with SD 253.36. Median of TAC was 1303 µMole/L. 95% reference limit for lower and upper case was 928.55 & 1802.50 µMole/L respectively. This method was found to be linear up to at least 2500 µMole/L. The overall precision of the method was quite satisfactory as intra & inter assay CV were 3.14% and 3.78% respectively. The plasma samples could be stored for at least 14 days in deep freeze without significant alteration. TAC level was estimated in plasma with high glucose concentration and was found to be significantly lower than normal (844.11$\pm$ 269.10 µmole/L).

Conclusion: FRAP is a simple, rapid and precise assay to measure TAC in plasma.

Keywords: Total Antioxidant capacity (TAC), Ferric Reducing Antioxidant Power (FRAP)

Introduction

Different exogenous factors and endogenous metabolic processes are known to give rise to Reactive Oxygen Species (ROS), which exerts detrimental effects on the biomolecules. To combat these effects, antioxidant defence system is present in human which comprises of antioxidant enzymes (like Superoxide dismutase SOD, Glutathione peroxidase GPx, Glutathione reductase GR etc), nutrients (like β carotene, Vitamin C and Vitamin E etc), and metabolites (like Uric acid, Albumin, Bilirubin etc)[(1)]. The concentration of these antioxidants can be measured individually but this approach is time consuming and expensive. Nowadays measurement of Total Antioxidant Capacity (TAC) is considered as a better and practicable alternative[(2)]. Though several

methods are available to measure TAC, a method which is simple, inexpensive yet sensitive is always preferred for better patient care service. Ferric Reducing Antioxidant Power (FRAP) method seems to fulfil all these criteria. Many study groups have used this method and established the reference values for the parameter [3] but most of the available literature is from the Western countries. The same may not be applied in a Bengali population due to gross difference in genetic makeup, life style and diet. Moreover the International Federation of Clinical Chemistry (IFCC) recommends that each laboratory should establish its own reference value to aid clinicians in interpreting the observed values[4].

In this background, the present study is undertaken with the following objectives:

1) To establish 95% reference limit of TAC in plasma by FRAP method along with 90% confidence interval.
2) To find out the linearity and precision of the test.
3) To determine whether the samples can be stored up to 14 days or not in deep freeze.
4) To find out whether the method can be applied in clinical samples, so that it can be used for diagnostic purposes.

Material and Methods

This cross sectional, descriptive and observational study was conducted in the Department of Biochemistry of College of Medicine and Sagore Dutta Hospital. Healthy individuals, inclusive of both male and female were recruited in the study after having their written informed consent. All the students and the staff of the Institution along with the accompanying persons of the patients coming for OPD treatment were approached randomly for the purpose. The wellbeing was assessed by the guidelines of IFCC[5] and accordingly individuals with following conditions were excluded:

1) Pathophysiological states: Renal failure, Congestive heart disease, Chronic respiratory disease, Liver diseases, Malabsorption syndromes and Nutritional anaemia.
2) Systemic diseases: Hypertension, Diabetes.
3) Intake of Pharmacologically active agents: alcohol, tobacco, oral contraceptives, replacement or supplementation therapy e.g. Insulin.
4) Modified Physiological states: pregnancy, psychological and mental disorders such as severe stress and depression.

The study was approved by Institutional Ethics Committee.

Clinical and Laboratory Standard Institute (CLSI)[6] has recommended a minimum of 120 observations to be made to establish a reference limit. In this study blood was collected from 150 healthy individuals using EDTA as anticoagulant ensuring a minimum of 10 hour fasting condition and adequate rest prior to venepuncture.

Plasma was separated and divided into 2 aliquots with proper labelling. One was analysed on the day of blood collection and the other one was stored in the deep freeze for future analysis. Randomly 5 samples were chosen for 8 days where plasma was not stored but analysed in duplicate on the same day.

TAC was measured by FRAP assay by Benzie & Strain[7] slightly modified. It is based on the principle that at low pH, Ferric Tripyridyl Triazine (Fe III TPTZ) complex gets reduced to ferrous form developing an intense blue colour. Ascorbic acid standards (Merck) prepared in concentration of 500, 1000, 1500, 2000 & 2500 µmole/L were used for comparison. The working reagent was prepared by mixing (a) 300 mM acetate

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buffer (pH- 3.6), (b) 10 mM TPTZ (Loba) in 40 mM HCl and (c) 20 mM $FeCl_3$ (Merck) in ratio of 10:1:1 at the time of use. 100 µl sample or standard was mixed with 3 ml of working FRAP reagent, vortexed and incubated at room temperature for 4 minutes. The colour developed was measured at 630nm in a semiautoanalyser. The absorbance of the standards was used to establish the linearity of the test.

To evaluate precision of the study, repeatability was tested both in sample and standard. A total of randomly chosen 40 samples over 5 days were run in duplicate to find out the intra assay coefficient of variation in samples as described by Schultheiss O C et al[8].

All the 5 standards were run in 4 replicates for 5 days to find out the Total coefficient of variation (Both, Within run and Between run). This is according to the protocol of CLSI document EP 15 – A2.

A total of 31 samples were analysed after 14 days and compared with its previous value. The comparison of values of initial and stored samples was done by t-test.

A total of 48 plasma samples were collected from Central Laboratory of College of Medicine & Sagore Dutta Hospital, where blood glucose level was found to be high (Fasting value more than 120 mg%). It was considered that these patients are suffering from Diabetes Mellitus. As diabetic patients are well known to have decreased TAC level[9], the level of TAC was estimated in all these 48 samples and the mean value was compared with that in normal subjects by t test.

All the data were tabulated and analysed using the software "Analyse – it" for Microsoft Excel version 2.30 (http://analyse-it.com/;2012).

Observations & Result

Reference limit of TAC obtained by FRAP method is presented in Table 1. Mean and SD of the method was found to be 1330.18 ± 253.36 µmole/L. 95% Reference limit was set as lower limit: 928.55 µmole/L and upper limit: 1802.50 µmole/L. Coefficient of variation was found as 3.14% and 3.78% in sample and standards respectively. The method was found to be linear upto 2500 µmole/L. The samples was found to be stored at least up to 14 days as t test shows no significant difference of mean values of TAC estimated on 0 (zero) day and 14th day (Table 2). The method can also demonstrate significant ($p < 0.0001$) low level of TAC (Mean ± SD = 844.11± 269.10 µmole/L, Table 3) in cases with high plasma glucose concentration, which are probably cases with diabetes mellitus (DM).

Table 1: Reference interval and precision of TAC by FRAP assay

Parameters (n = 150)	Values
Mean (µmole/L)	1330.18
SD	253.36
Median	1303.00
95% Reference limit	
lower	928.55
upper	1802.50
90% Confidence interval	
lower	802.00 to 978.00

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upper	1703.00 to 2498.00
Intra assay CV in sample (n = 40, replicate 2)	3.14%
Total CV in standard (n = 20 per standard, total 5 standards, replicate per run= 4)	3.78%
a) Within run	3.04%
b) Between run	2.1%

Table 2: Effect of Refrigeration on TAC

TAC	Mean (µmole/L)	SE	SD
On 0 (zero) day (n = 31)	1352.9	36.71	204.4
After 14 days (n = 31)	1357.7	37.14	206.8
t statistic	- 0.96		
Degree of freedom	30		
2 tailed p	0.3462 (Non significant)		

Table 3: Comparison of mean value of TAC in blood samples of normal & diabetic subjects

TAC	Mean (µmole/L)	SE	SD
Control (n =150)	1330.18	20.69	253.36
Case (Diabetes) (n = 48)	844.11	38.84	269.10
t statistic	- 11.40		
Degree of freedom	196		
2 tailed p	<0.0001 (significant)		

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Discussion

In this study TAC in plasma was measured by FRAP method. Age of the study population was 20 years to 55 years. No healthy volunteer was obtained beyond 55 years. Whether TAC varies among gender and with increase in age had not been evaluated in this study. It is almost similar to the finding of Eugene HJM et al who reported a mean value of 1392 µmole/L with an SD of 158[(10)].

In this study, absorbance was measured in a semiautoanalyser at 630nm, whereas Benzie and Strain[(7)] measured the absorbance at 593nm in a spectrophotometer. As most of the semiautoanalysers do not have filters to measure the absorbance at 593nm, the nearest wavelength of 630nm was chosen in this study. Thus the method becomes more widely applicable.

The linearity beyond 2500 µmole/L was not done as none of the data from the healthy volunteers were found to be beyond this value. Literature also suggests that lower level of TAC is important in disease conditions. Thus higher value seems to be insignificant.

The method appears to be quite precise with inter assay and intra assay coefficient of variation well below10% (Table 1), which is the acceptable.

Samples were stored for 14 days to perform a repeat test. This time span is chosen as per convenience. Whether the samples can be stored beyond this time has not been evaluated.

The method was also found to be reliable for application in disease condition. In DM, lower level of TAC was found to be inversely associated with fasting blood glucose level by other study groups due to oxidative stress. [(9)] Significant lower value of TAC was found in patients with high plasma glucose in this study also.

Conclusion

FRAP assay can be done without any sophisticated instrument like spectrophotometer. The method is found to be linear up to at least 2500 µmole/L, which is well beyond its reference limit (1330.18 µmole/L $\pm$ 253.36). The samples can be stored in the deep freeze for 14 days at least. The method can differentiate conditions with lower level of TAC from normal in a significant way as found in samples with high glucose concentration.

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