ANALYTICAL METHOD COMPARISON OF ADVANCED OXIDATION PROTEIN PRODUCTS (AOPP) WITH MODIFIED AOPP

Sunaiba M[1], Avinash S.S[2,*], Arunkumar K[3], Malathi M[4], Shivashankara A. R[5], Ashwini K[6]

[2,3,4,5] Department of Biochemistry, Father Muller Medical College, Father Muller Road, Kankanady, Mangalore-575002, Karnataka State.
[1, 6] Department of Medical Biochemistry, School of Health Sciences, Kannur University, Thalassery Campus, Palayad-670661, Kerala State.

***Corresponding Author:**
E-mail: dravinash.ss@gmail.com

ABSTRACT

Introduction: *AOPP (Advanced oxidation protein products) are the dityrosine containing protein cross linking products indicating the oxidised tyrosine residues of the plasma protein albumin, fibrinogen and lipoproteins. AOPP are elevated in oxidative stress and inflammation. Unlike AOPP the mAOPP estimation is not interfered by triglyeride levels.*

Objective: *Analytical method comparison of AOPP with mAOPP.*

Materials and Methods: *Measurement of AOPP involves the principle of spectrophotometric determination of oxidation of I- plasma AOPP under acidic condition by Witko Sarsat method. To remove the interference by the triglycerides especially seen in hypertriglyceridemia, the plasma was treated with lipid precipitating agent and the mAOPP were measured. In this study using pooled serum samples we compared the performance characteristics like precision, accuracy, linearity limit and reference range of AOPP assay with the mAOPP assay under our laboratory conditions as per CLIA guidelines of method validation.*

Results and Discussion: *The intrarun, intraday, interday and overall precision of AOPP (4.25%, 5.18%, 3.89%, 5%) was better than that of mAOPP (5.76%, 5.5%, 5.8%, and 5.8%). But on linear regression, mAOPP = 0.5×AOPP + 9.8 chloramine T equivalents (r2-0.62; p < 0.001) and the AOPP was 18% higher than mAOPP. In eutriglyceridemia however, mAOPP = 0.7xAOPP + 4.8 chloramine T equivalents (r2 = 0.75; p < 0.001) and the AOPP was only 14.5% higher. Also the correlation (r = 0.74) of AOPP – mAOPP levels with TG showed that the difference was significantly higher (p < 0.001) in the persons with higher TG levels clearly establishing the overestimation of AOPP.*

Conclusion: *Hence mAOPP may prove to be a better estimate of protein oxidation status and a better diagnostic and prognostic indicator compared to AOPP especially in hypertriglyceridemic patients.*

INTRODUCTION

AOPP (Advanced oxidation protein products) are the dityrosine containing protein cross linking products indicating the oxidised tyrosine residues of the plasma protein albumin, fibrinogen and lipoproteins [1]. AOPP are produced when the plasma albumin is subjected to oxidation by various oxidants like ROS (Reactive oxygen species) and chloramines or hypochlorous acid [2]. AOPP are elevated in persons having a condition in which there is an observed increase in oxidative stress and inflammation [3]. AOPP are considered as a novel marker of oxidative stress in uremia [1]. AOPP are increased in diabetes, atherosclerosis, nephropathies and cancer [4]. AOPP can be considered as a marker for diagnosis and monitoring of the oxidative stress in various stages of progression of the above mentioned diseases conditions. Measurement of AOPP involves the principle of spectrophotometric determination of oxidation of I^- to I^{-3} by plasma AOPP under acidic condition [1]. Several studies have reported the positive interference of triglycerides (TG) on the AOPP measurement [5]. This is of importance in estimation of AOPP in hyper triglyceridemic state frequently associated with diabetes, atherosclerosis and nephropathy [5]. To remove the interference by the triglycerides the plasma was treated with lipid precipitating agent. Further the supernatant was used for measurement of AOPP. The AOPP measured with this modification in assay is termed as mAOPP (Modified AOPP). mAOPP can be used as a better alternative to AOPP [6]. Several studies have reported no interference of triglycerides on mAOPP and also that mAOPP measured is lower than the AOPP level across all groups. In order to corroborate the above hypothesis and various evidences, in this study we compared the performance characteristics like precision, accuracy, linearity limit and

reference range of AOPP assay with the mAOPP assay under our laboratory conditions.

AIMS AND OBJECTIVES

Aim:

- To perform Analytical method comparison of Advanced Oxidation Protein Products with Modified Advanced Oxidation Protein Products.

Objectives:

1. To calculate and compare precision of AOPP with mAOPP method.
2. To compare performance of AOPP and mAOPP method by regression analysis.
3. To analyse and correlate interference of triglyceride levels on AOPP and mAOPP method.

MATERIALS AND METHODS

Preparation of pooled serum:

The venous blood was collected in a plain vacutainer using aseptic precautions. Serum was separated by centrifugation at 4000 rpm for 10 minutes. The separated serum was collected and put into a separate glass container. Pooled serum was filtered using Whatman filter paper into another container. Pooled serum was put into 1.5 ml eppendorf tubes.

Storage of pooled serum:

All the eppendorf tubes containing pooled serum was stored under -20 degree Celsius.

Standardisation of AOPP and mAOPP method:

Both the methods were standardised using chloramine T. various standards of chloramines T were prepared ranging from 0 – 100 micromoles/L, and a standard graph was prepared by plotting increasing standard chloramines T concentration against their absorbance.

Estimation of serum AOPP by Witko-Sarsat method:

200 microL of serum diluted (1:5) with PBS was added to 10 microL of 1.16M KI. The reaction was stopped by adding 20 microL of acetic acid and the absorbance was read immediately at 340 nm. The measured AOPP was expressed in chloramines T equivalents [1].

Estimation of serum mAOPP by modification of Witko-Sarsat method:

200 microL of serum was precipitated by adding 5 microL of 2M MgCl2 and 20 microL of 4% PTA in 0.19M NaOH. The mixture was centrifuged at 1000 g for 20 min. AOPP was determined in the supernatant. The measured mAOPP was expressed in chloramines T equivalents [6].

Comparison of serum AOPP levels with serum mAOPP levels:

Intra run precision of AOPP and mAOPP method:

AOPP and mAOPP were estimated in 20 replicates of pooled serum in duplicate within a single run. Mean, SD and CV were calculated. The CV was taken as intra run precision [7].

Intraday precision of AOPP and mAOPP method:

AOPP and mAOPP were estimated in 20 replicates of pooled serum in duplicate in two different runs across a single day. Mean, SD and CV were calculated. The CV was taken as intraday precision [7].

Inter day precision of AOPP and mAOPP method:

AOPP and mAOPP were estimated in replicates of pooled serum in duplicate across 20 different days. Mean, SD and CV were calculated. The CV was taken as inter day precision [7].

Overall precision of AOPP and mAOPP method:

Overall precision of AOPP and mAOPP was calculated as an average of intra run, intraday and inter day CV [7].

***Method comparison of mAOPP with AOPP by regression analysis**:*

AOPP and mAOPP were estimated in duplicate in 40 different patient samples across 5 different days covering a wide range of AOPP and mAOPP levels. The comparison between AOPP and mAOPP levels was done by linear regression analysis after removing the outliers by Bland Altman's plot. Triglyceride levels were also estimated in all the samples for the purpose of correlation with AOPP and mAOPP [8].

***Calculation of linearity limit for AOPP and mAOPP method**:*

Linearity of the AOPP and mAOPP assay was calculated using chloramines T standard graph.

***Establishment of normal reference range of AOPP and mAOPP**:*

AOPP and mAOPP were estimated in 20 different normal persons sample covering all age and gender group. The mean and confidence interval were calculated. The reference range was established as mean ± 95% CI.

***Correlate serum triglyceride levels with serum AOPP levels and serum mAOPP levels**:*

The triglyceride levels estimated in 40 different patient samples were correlated with respective AOPP and mAOPP levels.

***Correlate serum triglyceride levels with difference between serum AOPP level and serum mAOPP level**:*

The triglyceride levels estimated in 40 different patient samples were correlated with respective difference in AOPP and mAOPP levels.

STATISTICAL ANALYSIS

The significance of difference between means of serum AOPP and serum mAOPP levels were calculated by independent t test.

Correlation of serum triglyceride levels with serum AOPP, serum mAOPP levels and difference between serum AOPP and serum mAOPP levels were done by Karl Pearson's product moment correlation test.

Comparison of the relationship between AOPP with mAOPP was done by simple linear regression analysis.

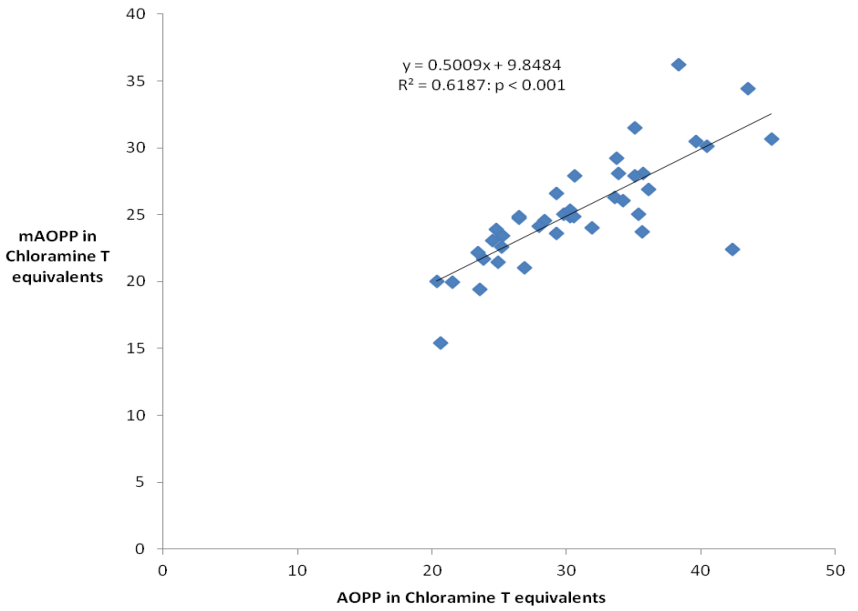
RESULTS

Table 1: **Precision tests in pooled serum assays for AOPP and mAOPP.**		
Precision	AOPP	mAOPP
Intrarun	4.26	5.76
Intraday	5.18	5.5
Interday	3.89	5.8
Overall	5	5.8
Legend Table 1: Precision of AOPP is better than mAOPP.		

The independent one tailed t test with unequal variance for the significance of difference between AOPP (mean: 30.85; SD: 6.4 chloramine T equivalents) and mAOPP (mean: 25.3; SD: 4.1chloramine T equivalents) in serum samples gave value of $p < 0.001$. The % mean difference between AOPP and mAOPP was 18%. The % mean difference between AOPP and mAOPP in persons with normal triglyceride levels was 14.5%.

The **analytical method comparison** between mAOPP (y) and AOPP (x) assay in the serum samples of all persons under study by simple linear regression analysis yielded the equation of $y = 0.5*x + 9.8$ chloramine T equivalents. The r^2 was 0.62 and $p < 0.001$. (Figure 1)

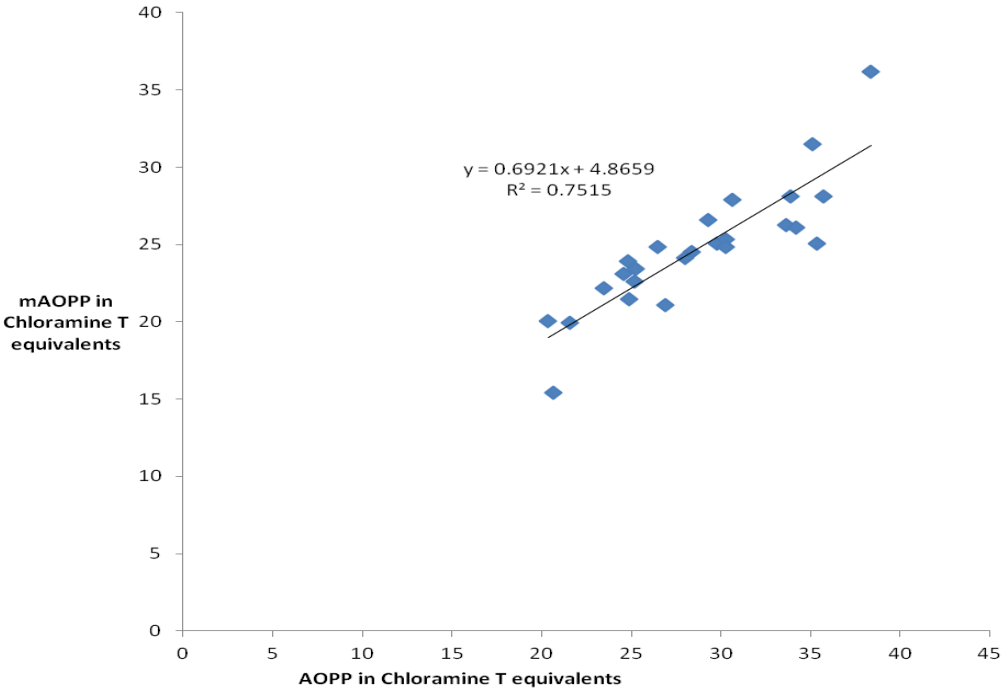
Figure 1: Analytical method comparison of mAOPP vs AOPP



Legend to figure 1: This simple linear regression analysis compares the relationship between mAOPP and AOPP assay.

The **analytical method comparison** between mAOPP (y) and AOPP (x) assay in the serum samples of persons with normal triglyceride levels (triglyceride less than 150 mg/dl) by simple linear regression analysis yielded the equation of y = 0.7*x + 4.8 chloramine T equivalents. The r^2 was 0.75 and $p < 0.001$. (Figure 2)

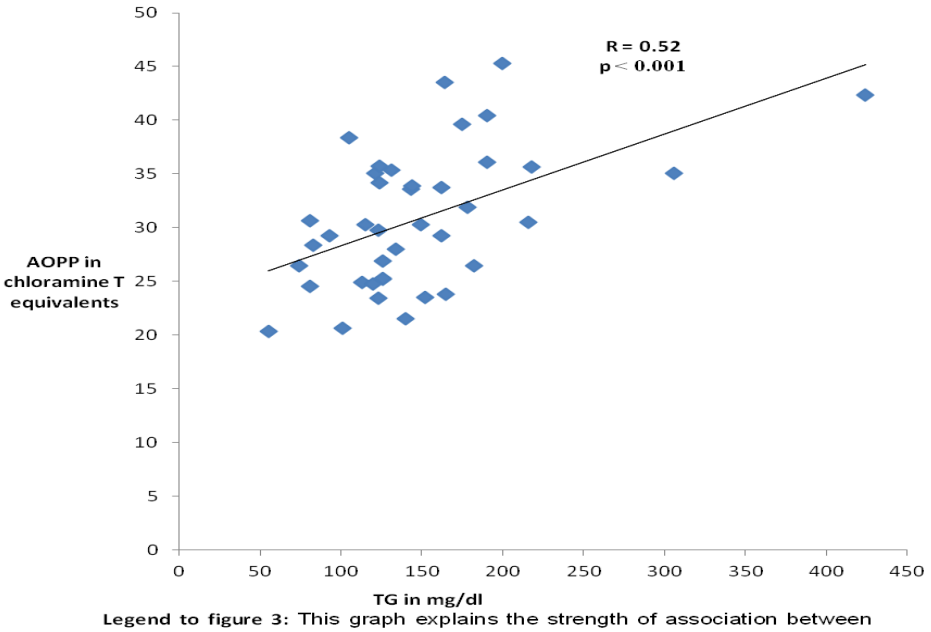
Figure 2: Analytical method comparison of mAOPP vs AOPP in persons with normal TG levels



Legend to figure 2: This simple linear regression analysis compares the relationship between mAOPP and AOPP assay in persons with normal TG levels (TG < 150 mg/dl).

The Karl Pearson product moment correlation between AOPP levels and TG levels yielded r value of 0.52 and $p < 0.001$ (Figure 3).

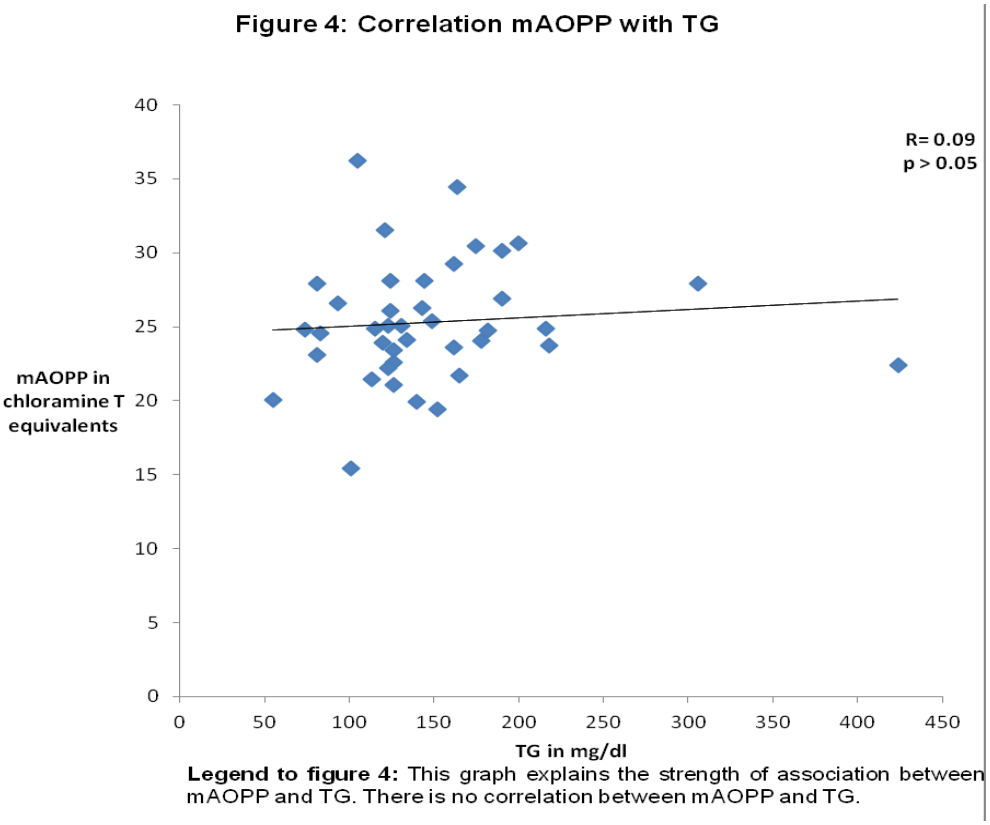
Figure 3: Correlation AOPP with TG



Legend to figure 3: This graph explains the strength of association between AOPP and TG. As the TG levels increases the AOPP levels also increase significantly.

The Karl Pearson product moment correlation between mAOPP levels and Triglyceride levels yielded r value of 0.09 and $p > 0.05$ (Figure 4).

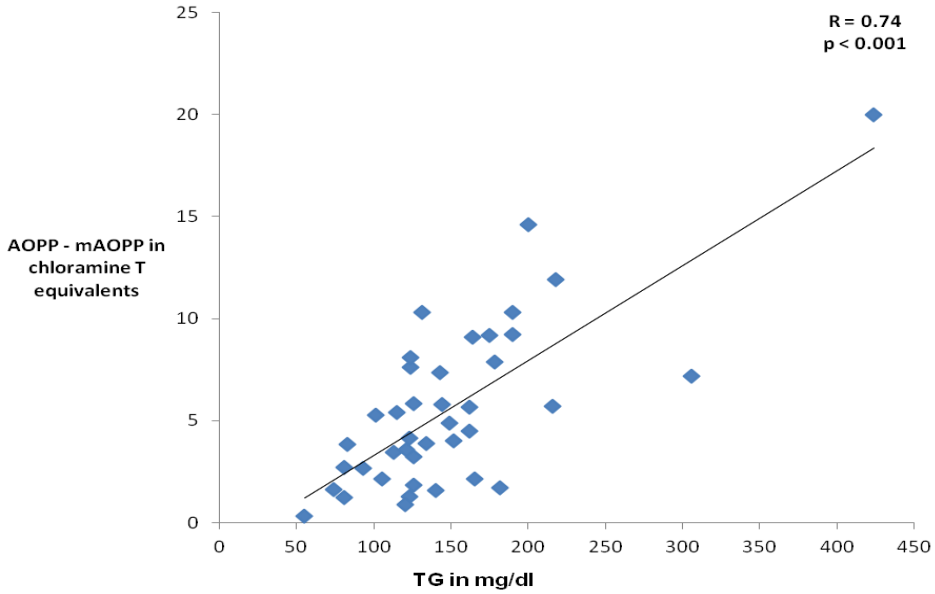
Figure 4: Correlation mAOPP with TG



Legend to figure 4: This graph explains the strength of association between mAOPP and TG. There is no correlation between mAOPP and TG.

The Karl Pearson product moment correlation between AOPP-mAOPP levels and Triglyceride levels yielded r value of 0.74 and $p < 0.001$ (Figure 5).

Figure 5: Correlation of AOPP – mAOPP with TG



Legend to figure 5: This graph explains the strength of association between AOPP - mAOPP and TG. The difference between AOPP and mAOPP increases with TG. This indicates that mAOPP does not vary w

Linearity limit:

The chloramines T standard absorbance was found be linear up to 100 micromole/L.

Reference range:

The normal reference range of AOPP at 95% confidence interval was 28.93 ± 1.98 chloramine T equivalents. The normal reference range of mAOPP at 95% confidence interval was 24.71 ± 1.58 chloramine T equivalents.

DISCUSSION

In our laboratory condition the AOPP assay was compared with mAOPP assay.

Precision:

In our set up we found that precision of AOPP was better than mAOPP. The intrarun (4.25 %), intraday (5.18 %), interday (3.89 %) and overall precision (5 %) were all better for AOPP when compared to the intrarun (5.76 %), intraday (5.5 %), interday (5.8 %) and overall precision (5.8 %) mAOPP assay (Table 1). This was due to an extra step of precipitation that was necessary for mAOPP assay. Precipitation of lipids by lipid precipitating reagents, centrifugation and removal of supernatant yielded in more random errors during the measurement of mAOPP causing more imprecision.

Accuracy by analytical method comparison:

In our lab set up we also found that there is a significant ($p < 0.001$) difference between AOPP levels (mean-30.85; SD-6.4 chloramine T equivalents) and mAOPP levels (mean-25.3; SD-4.1chloramine T equivalents). Since there was a significant difference in the AOPP and mAOPP levels, we compared the relationship between two by regression analysis. It yielded the equation mAOPP = 0.5* AOPP + 9.8 chloramine T equivalents (r^2-0.62; $p < 0.001$) (Figure 1). It showed that mAOPP levels were only little higher than half the level of AOPP, clearly stating that mAOPP levels were significantly lower (18 %) when compared to AOPP levels. The r^2 was only 0.62 indicating less goodness to fit maybe due to less number of samples analysed (n=40). This is of importance in estimation of AOPP levels as novel marker of oxidative stress in uremic state, diabetes and atherosclerosis. In the all the above

conditions the AOPP would be overestimated. Hence mAOPP may prove to be a better estimate of protein oxidation status and a better diagnostic and prognostic indicator compared to AOPP. This needs to be further substantiated by predictability (ROC) studies.

Interference studies:

Several studies have shown a positive interference of TG on AOPP assay. When we correlated the AOPP levels with TG levels it suggested a significant ($p<0.01$) positive correlation ($r = 052$). This shows that increase in TG levels causes falsely overestimated AOPP values (Figure 3). But when we correlated mAOPP levels with TG levels we found no correlation between the two ($r =0.09$) (Figure 4).

Also the correlation ($r = 0.74$) of AOPP – mAOPP levels with TG showed that the difference was significantly higher ($p < 0.001$) in the persons with higher TG levels (Figure 5). This again proves that AOPP if falsely overestimated in persons with high TG levels and mAOPP is unaffected by rising TG levels. This was also proved when we again compared the relationship between AOPP and mAOPP in persons with normal TG (triglyceride < 150 mg/dl). It yielded the equation of mAOPP = 0.7*AOPP + 4.8 chloramine T equivalents ($r2 = 0.75$; $p < 0.001$) (Figure 2).

Also when the interfering factor, high TG were removed the goodness to fit improved from $r2 = 0.62$ to $r2 = 0.75$. (Figure 1, Figure 2). Under this setting percentage difference between AOPP and mAOPP reduced from 18 % to 14.5 %. This clearly shows that in persons with normal TG levels there is not much difference in AOPP and mAOPP levels; nevertheless AOPP is always overestimated compared to mAOPP. This is of importance in persons with uremia, diabetes and atherosclerosis in whom hypertriglyceridemia incidence is high. In such persons mAOPP may serve to be a better indicator of oxidative stress.

Further studies of interference by other chemicals needs to be studied on AOPP as well as mAOPP.

Linearity limit:

The chloramines T standard absorbance was found be linear up to 100 micromole/L. Since chloramines T is the standard used for both AOPP as well as mAOPP assay, this shows that AOPP and mAOPP assay are linear throughout the medical decision limit range**.**

Reference Range:

The normal reference range of AOPP at 95% confidence interval was 28.93 ± 1.98 chloramine T equivalents.

The normal reference range of mAOPP at 95% confidence interval was 24.71 ± 1.58 chloramine T equivalents.

CONCLUSIONS

AOPP estimation is more precise compared to mAOPP due to absence of lipid precipitation step. AOPP is over estimated compared to mAOPP in normal as well as high TG patients. AOPP and AOPP - mAOPP levels correlate significantly with TG levels unlike mAOPP which has no significant correlation with increasing levels of TG. So mAOPP levels are the better estimators of oxidative stress seen especially in patients with uremia, diabetes and atherosclerosis, since such patients have high incidence of hypertriglyceridemia.

REFERENCE:

1. Witko-Sarsat V, Friedlander M, Capeillere-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, Jungers P, Descamps-Latscha B. AOPP as a novel marker of oxidative stress in uremia. Kidney Int. 1996; 49(5): 1304-1313.
2. Capeillere-Blandin C, Gausson V, Descamps-Latscha B, Witko-Sarsat V. Biochemical and spectrophotometric significance of advanced oxidized protein products. Biochim Biophys Acta. 2004; 1689: 91-102.
3. Tilman Drueke and Beatrice Descamps-Latscha Sandrine Canteloup, Jean-Michel Dayer, Paul Jungers, Khoa, Chantal Capeillere-Blandin, Anh Thu Nguyen, Veronique Witko-Sarsat, Miriam Friedlander, Thao Nguyen. Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure. J Immunol. 1998; 161: 2524-2532.
4. Descamps-Latscha B, Witko-Sarsat V, Nguyen-Khoa T, Nguyen AT, Gausson V, Mothu N, London GM, Jungers P. Advanced oxidation protein products as risk factors for atherosclerotic cardiovascular events in nondiabetic predialysis patients. Am J Kidney Dis. 2005; 45: 39-47.
5. Valli A, Suliman ME, Meert N, Vanholder R, Lindholm B, Stenvinkel P, Watanabe M, Barany P, Alvestrand A, Anderstam B. Overestimation of advanced oxidation protein products in uremic plasma due to presence of triglycerides and other endogenous factors. Clin Chim Acta. 2007; 379(1-2): 87-94.
6. Anderstam B, Ann-Christin BH, Valli A, Stenvinkel P, Lindholm B, Suliman ME. Modification of the oxidative stress biomarker AOPP assay: application in uremic samples. Clin Chim Acta. 2008; 393(2): 114-118.
7. CLSI. Evaluation of precision performance of quantitative measurement methods: Approved guideline, 2 ed. Clinical and Laboratory Standard Institute: Wayne P A; 2004.
8. CLSI. Method comparison and bias estimation using patient samples. Approved guideline, (Interim Revision) CLSI Document EP9-A2-IR. 2 ed. Clinical and Laboratory Standard Institute: Wayne P A; 2010.