IJ≡MS

Original article

Comparison of Prooxidant-antioxidant Balance Method with Crocin Method for Determination of Total Prooxidant-antioxidant Capacity

*[1,3]Daryoush Hamidi Alamdari, [2]Stella A. Ordoudi, [2]Nikolaos Nenadis, [2]Maria Z. Tsimidou, [1]George Koliakos, [3]Seyyed Mohmmad Reza Parizadeh, [3]Mohammad Safarian, [3]Maryam Sabery Karimian, [3]B. Fatemeh Nobakht M. Gh

Abstract

Objective(s)
Comparison of prooxidant-antioxidant balance (PAB) assay with crocin assay.
Materials and Methods
Twenty eight serum samples were chosen, PAB and the total antioxidant capacity were measured by PAB assay and crocin, respectively, and the correlation of both assays, along with their correlation with other clinical and biochemical parameters, were determined.
Results
A significant negative correlation was established between PAB assay and crocin assay. Also a significant negative correlation was established between PAB and uric acid and creatinine.
Conclusion
The results showed that by increasing the total antioxidant capacity, which is showed by crocin, the PAB shifts in favor of antioxidants, which is showed by PAB assay. Now, it could be considered that the PAB, along with other risk factors, might help in the prediction of the risk for cardiovascular events; and further research could clarify whether by application of PAB assay and appropriate interventions for correcting oxidative stress, progression of the cardiovascular disease could be reduced.

Keywords: Crocin, Oxidative stress, Prooxidant-antioxidant balance assay

1- Department of Biological Chemistry, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece
2- Department of Chemistry, Laboratory of Food Chemistry and Technology, Aristotle University of Thessaloniki, Thessaloniki, Greece
3- Department of Nutrition and Biochemistry, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
*Corresponding author: Tel: +98-511-8828573; Fax: +98-511-8828574; email: dhamidialam@yahoo.com

Introduction

In spite of the well documented role of oxidative stress in the atherosclerotic transformation, the determination of prooxidant-antioxidant balance (PAB) is not yet a routine clinical laboratory test, mainly because of the lack of a universally accepted method (1). Many methods have been developed that can measure separately the total prooxidant and antioxidant capacities (2, 3). The estimation of the PAB by determination of both the oxidant and the antioxidant status separately, is indirect, imprecise, time and cost consuming (1). Usually multiple prooxidant and antioxidant markers are used together in order to evaluate the levels of the oxidative stress (1).

We presented recently a simple, rapid and cost-effective method (the PAB assay), which can measure the prooxidant burden and the antioxidant capacity in one assay (1). This method can measure the balance of oxidants and antioxidants simultaneously, by using 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) and two different kinds of reactions; one enzymatic reaction in which the chromogen TMB is oxidized to a color cation by peroxides, and a chemical reaction in which the TMB cation is reduced to a colorless compound by antioxidants; and gives a redox stress index (1).

The "crocin bleaching assay" (CBA) was suggested by Bors *et al* (4) as suitable for screening radical scavenging activity. Originally, inhibition of crocin bleaching by a range of substances was monitored by competition kinetics in the presence of photolytically produced alkoxyl radicals. Obtained relative rate constants were found to correlate well with the known antioxidant activities of selected compounds (phenolic antioxidants, model phenols, polyhydric and heterocyclic radical scavengers, etc). At that time the chemistry behind the method was considered to be the same as that of the 2, 2-diphenyl-1- picrylhydrazyl (DPPH) radical assay, which was regarded as H-atom abstraction. In CBA, abstraction of hydrogen atoms and/ or addition of the radical to the polyene structure of crocin results in a disruption of the conjugated system accounting for crocin bleaching (4). The latter is recorded as reduction of absorbance in the presence or absence of radical scavengers. Later on, Bors and co-workers (5) found that the absolute rate of crocin bleaching depends heavily on the sort of radical attacking the polyene structure. In the latter, peroxyl radical formation was achieved by using azo-initiators (hydrophilic or lipophilic). In this way, Tubaro and co-workers (6) made an effort to average antioxidant and pro-oxidant effects of the constituents of complex natural mixtures. Results were expressed with reference to α-tocopherol (for lipophilic molecules) or Trolox (for hydrophilic ones). In all applications, the analytical protocols are based on modifications or adjustments of that proposed by Bors *et al* (4) as adapted by Tubaro *et al* (6).

The aim of this study was to evaluate the PAB assay by crocin assay. To the best of our knowledge, the correlation of PAB and crocin assay has been hitherto not reported.

Materials and Methods

Chemicals

TMB powder (3,3′,5, 5′-Tetramethylbenzidine. 2 HCl, Sigma), peroxidase enzyme (Applichem: 230 U/mg, A3791, 0005, Darmstadt, Germany), chloramine T trihydrate (Applichem: A4331, Darmstadt, Germany), hydrogen peroxide (30%) (Merck). Saffron red stigmas were donated by Saffron Cooperative of Kozani (Greece) and Verdu-Canto Saffron S.L. (Alicante, Spain). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was obtained from Sigma Chemical Co. (St Louis, USA). All the other reagents used were reagent grade and were prepared in double distilled water.

Sample collection and preparation

This research project was confirmed by Ethics Committee of Aristotle University of Thessaloniki. All individuals gave both oral and written informed consent. Twenty eight serum samples were chosen. Ten subjects were obese patients with type 2 diabetes, 2 subjects had asthma, and 16 subjects were obese. For serum preparation, the blood samples were centrifuged at 2,000×g for 15 min, and the serum aliquots were separated and stored at

-80 ºC. For all persons, clinical parameters including weight, height, waist circumference and routine biochemical parameters were measured using the standard protocols.

Prooxidants-antioxidants balance (PAB) method

A modified PAB was applied based on a previously described method (1). The standard solutions were prepared by mixing varying proportions (0-100%) of 250 μM hydrogen peroxide with 3 mM uric acid (in 10 mM NaOH).

Sixty mg TMB powder was dissolved in 10 ml DMSO; for preparation of TMB cation, 400 μl of TMB/DMSO was added in 20 ml of acetate buffer (0.05 M buffer, pH 4.5), and then 70 μl of fresh chloramine T (100 mM) solution in distilled water was added into this 20 ml, mixed well, incubated for 2 hr at room temperature in a dark place; 25 units of peroxidase enzyme solution were added into 20 ml TMB cation, dispensed in 1 ml and put at -20 °C; in order to prepare the TMB solution, 200 μl of TMB/DMSO was added into 10 ml of acetate buffer (0.05 M buffer, pH 5.8); the working solution was prepared by mixing 1 ml TMB cation with 10 ml of TMB solution, incubated for 2 min at room temperature in a dark place and was immediately used. Ten microliters of each sample, standard or blank (distilled water) were mixed with 200 μl of working solution, in each well of a 96 well plate, which was then incubated in a dark place at 37 °C for 12 min; at the end of the incubation time, 100 μl of 2N HCl was added to each well; and measured in an ELISA reader at 450 nm with a reference wavelength of 620 or 570 nm. A standard curve was provided from the values relative to the standard samples. The values of the PAB are expressed in arbitrary HK units, which are the percentage of hydrogen peroxide in the standard solution. The values of the unknown samples were then calculated based on the values obtained from the above standard curve.

Crocin method

A) Crocin stock and working solution preparation: Raw saffron (0.5 g) was washed three times with diethyl ether (3×15 ml×

5 min), and the residual ether drops were evaporated under a nitrogen stream. Purified saffron was suspended in 25 ml of methanol, stirred manually for 5 min, and filtered through RC 55 (0.45 μm, 0.25 mm) filters. The filtrate (20 mg of saffron/ml) was stored at -18 ºC for a maximum of 1 month and used as the crocin stock solution. Crocin working solutions were daily prepared in methanol so that after adjustment the A_{433} value was 3.0 (7). All treatments were carried out away from direct exposure to light.

B) crocin Kinetic Study: The peroxyl radical scavenging activity of plasma was evaluated according to the protocol of Tubaro (7) with the modifications reported by Ordoudi and Tsimidou (8).

Briefly, crocin concentration was adjusted to 10 μM, on the basis of the extinction coefficient reported in the literature (433 MeOH) 133000 M^{-1} cm^{-1}. A certain volume of crocin working solution was diluted with methanol to 2 ml (total volume) so that the A_{433} value was 0.67. The same volume of crocin working solution was then transferred into a 2 ml volumetric flask, along with different volumes of plasma (10-70 μl). Stock AAPH solution (0.125 M) was daily prepared in 0.01 M PBS (0.08% w/v NaCl) and stored at 4 °C during the different sets of experiments. The reaction started with the addition of AAPH (200 μl) (t= 0 min). After dilution to 2 ml (total volume) with PBS and stirring for 30 s, the test solution was transferred into a quartz cell of 0.5 cm-path length and absorbance monitoring (A_{440}) was started at exactly 1 min after the addition of initiator.

C) Expression of Results: Loss in absorbance values within 10 min of reaction, in the absence (ΔA_0) or in the presence of plasma (ΔA), was calculated, and relative bleaching rates ($\Delta A_0/\Delta A$) were plotted against the different volumes of plasma. Given the complexity of plasma composition as well as the competition kinetic model of CBA (8), the linear regression slopes of each plot represent relative rate constants ($k_{rel} = \sum k_{AHi} /k_C$), multiplied by total concentration of antioxidants in plasma. Regarding the latter as 1 mM of AH, the aforementioned slopes correspond to k_{rel} values which divided by the respective value of Trolox, result in the index

"Trolox Equivalent Value, TEV$_{krel}$". For each of the plasma samples, krel calculation was based on at least two series of experiments.

Within-day repeatability (as %CV) of bleaching rate values in the absence of plasma (ΔA_0) ranged from 4.5-7.2 at three random days of analysis (n=5). For one plasma dilution (20 µl), within-day repeatability of ΔA values was 2.6% (n=5). Repeatability of krel measurements has been thoroughly examined for Trolox in Ordoudi study (8).

The clinical and biological parameters
All clinical and biochemical parameters were measured by the routine clinical tests.

Statistical analysis
For the statistical analysis, the GraphPad Instant statistical package was used (GraphPad Software, Inc). All parameters were given as mean±SD. The group comparisons were assessed by the unpaired t-test. Parametric and non-parametric correlations were assessed using the Pearson correlation coefficients and the Spearman correlation coefficients, respectively. The level of statistical significance was set to $P<0.05$.

Results
Correlation between PAB assay and crocin assay
A significant negative correlation was established between PAB assay and crocin assay (r=-0.31, P <0.04), Figure 1.
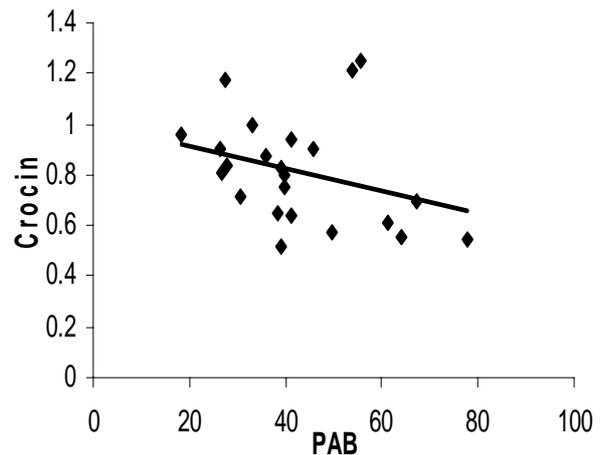

Figure 1. Correlation of PAB results with crocin results (r=-0.31, P <0.04).

Correlation of clinical and biochemical parameters with PAB results and crocin results
The clinical and biochemical parameters of subjects, along with their correlation with PAB results and crocin results, are presented in Table 1. It should be noted that PAB correlates with the levels of uric acid, creatinine and total protein. However, no correlation was established between anthropological parameters, blood pressure, sugar, HbA1c, triglycerides, total cholesterol, HDL as well as LDL concentrations and PAB. The crocin results have the same correlations with other parameters like PAB results except uric acid concentration.

Table 1. The clinical and biochemical parameters and their correlation with PAB values and crocin values.

Parameters	Mean±SD	Correlation with PAB (r, *p*)	Correlation with crocin (r, *p*)
Age (yrs)	56± 9.3	no, 0.08, 0.75	no, 0.19, 0.4
Waist /Hip	0.90+0.1	no, 0.2, 0.2	no, 0.37, 0.07
BMI (Kg/m^2)	33.26± 6.6	no, 0.24, 0.2	no, 0.03, 0.9
Systolic BP (mm Hg)	134.2± 19.4	no,-0.17, 0.42	no, 0.24, 0.29
Diastolic BP (mm Hg)	84.1± 9.0	no, 0.04, 0.8	no,-0.07, 0.7
Fasting Blood Sugar (mg/dl)	109.6± 26.1	no,- 0.31, 0.11	no, 0.08, 0.7
HbA1c (%)	5.8± 0.9	no, -0.34, 0.11	no,- 0.03, 0.9
Triglycerides (mg/dl)	136.8± 72.3	no, 0.18, 0.3	no, 0.15, 0.47
Total cholesterol (mg/dl)	206.8± 36.7	no, -0.07, 0.7	no, -0.23 0.28
LDL cholesterol (mg/dl)	124.5± 32.1	no, -0.01, 0.92	no, 0.002, 0.9
HDL cholesterol (mg/dl)	49.4± 9.1	no, 0.19, 0.33	no, 0.03, 0.8
Uric acid (mg/dl)	4.8± 1.2	yes, -0.4, 0.04	no, 0.14, 0.5
Serum creatinine (mg/dl)	0.86± 0.13	yes, 0.45, 0.01	yes, 0.3, 0.08
Total protein	5.9± 2.9	yes, -0.68, 0.04	yes, 0.45, 0.01
PAB (HK: arbitrary unit)	44.1± 16.4	-	yes, -0.68, 0.04
Crocin	0.81± 0.21	yes, -0.31, 0.04	-

Discussion

Many methods have been developed that can measure separately the total prooxidants and antioxidant capacities, but no one is for POX-AO balance (PAB). Therefore, for estimation of prooxidant/antioxidant balance, the only way (if not by PAB assay) is to perform two separate tests. This procedure is more laborious, needs more time and cost since two separate methods should be applied to determine the balance. Balance is then calculated indirectly by the data of two assays. By doing these calculations the imprecision increases significantly (1).

Sugherini et al (9) proposed a novel integrated parameter, called "redox compensation index", obtained by combining the results of the Fox-2 assay for plasma lipid hydroperoxides and the ferric reducing/antioxidant power (FRAP) assay for total antioxidant potential of plasma. Prior and Cao (10) mentioned that no single measurement of antioxidant status is going to be sufficient, but a "buttery" of measurements, will be necessary to adequately assess oxidative stress in biological systems. Trotti et al (11) used the chromogen N, N,-diethylparaphenylen-diamine in two different assays one for estimating prooxidant burden and a second to estimate antioxidant capacity (oxy-absorbent test). Although the same chromogen was used in both tests the two tests cannot be combined in one because of the nature of the second test that necessitates the addition of prooxidants into the assay admixture.

The PAB assay is a simple, rapid and cost-effective method, which can measure the prooxidant burden and the antioxidant capacity in one assay, giving a redox stress index (1). The assay uses TMB-TMB cation a known and well studied redox index (12, 13). The assay showed a linear response against a series of oxidants and antioxidants. The oxidant moiety of the test consists of the well known peroxidase total oxidants assay that measures the total peroxides. Changes in hydroperoxides have been widely used in clinical laboratory settings as an indicator of total oxidant status (TOS) (14). Hydrogen peroxide, although negligible in healthy people plasma, has been widely used as a representative of hydroperoxides in most TOS determination methods that express TOS as μM H_2O_2 equivalent (2). On the other hand, the antioxidant capacity is usually expressed in μM uric acid. It should be noted that hydrogen peroxide and uric acid do not directly interact with each other. Accordingly, PAB assay has been calibrated using a series of mixtures of hydrogen peroxide and uric acid. The results are expressed as the percentage of hydrogen peroxide in the calibration mixture and PAB value shows the oxidative stress index and defined as an arbitrary unit (HK unit).

The modified PAB assay, as applied in the present study, performed equally to the PAB assay that can measure the total pro-oxidants and the total antioxidants in one experiment. PAB assay was previously compared to widely utilized and documented methods that aim to estimate permanent oxidative damage such as carbonyl assay (by immunoassay), advanced glycation end products assay (AGEs) (by spectrofluorometry), advanced oxidative protein products (AOPP) assay (by spectrophotometry) (1).

In this study, we compared the PAB assay with crocin assay which is a well known and documented method for determination of the total antioxidant assay. A negative significant correlation is established between PAB assay and crocin. The results showed that by increasing the total antioxidant capacity, which is showed by crocin, the PAB shifts in favor of antioxidants, which is showed by PAB assay.

The major and independent risk factors for cardiovascular disease (CVD) are cigarette smoking, elevated blood pressure, elevated serum total cholesterol and low-density lipoprotein cholesterol (LDL-C), low high-density lipoprotein cholesterol (HDL-C), diabetes mellitus, and advanced age. These risk factors are usually used for the evaluation of an individual's predisposition to cardiovascular disease (15). However, these typical risk factors can be accounted for no more than 25% to 30% of excess cardiovascular risk factors in patients (16). This suggests that other factors might play a key role in the progression of atherosclerosis.

It is now well documented that oxidative stress has a main role in the development of atherosclerosis (17, 18), which leads to many proatherogenic events (such as LDL oxidation, endothelial dysfunction, vascular smooth muscle proliferation and migration) (19) and arterial thrombosis events (such as plaque disruption, platelets–leukocyte aggregation and thrombus formation) (20). Recently, oxidative stress (OS) has been considered as a significant risk factor for CVD and the unifying mechanism for many cardiovascular risk factors. However, there is a lack of a universally accepted method for determination of OS in the routine clinical laboratory.

In other studies (21), we demonstrated that the PAB assay could shows the increased OS in patients with coronary artery disease and we proposed that the PAB assay has this potency to be considered as a routine method for clinical laboratory for evaluation of OS. In this study, we also showed this potency.

Now, it could be considered that the PAB, along with other risk factors, might help in the prediction of the risk for cardiovascular events; and an effective primary prevention to correct OS could reduce progression of the cardiovascular disease by appropriate interventions.

Conclusion

The results showed that by increasing the total antioxidant capacity, which is showed by crocin, the PAB shifts in favor of antioxidants, which is showed by PAB assay. Now, it could be considered that the PAB, along with other risk factors, might help in the prediction of the risk for cardiovascular events; and further research could clarify whether by application of PAB assay and appropriate interventions for correcting OS, progression of the cardiovascular disease could be reduced.

Acknowledgment

References

1. Alamdari DH, Paletas K, Pegiou T, Sarigianni M, Befani C, Koliakos G. A novel assay for the evaluation of the prooxidant-antioxidant balance, before and after antioxidant vitamin administration in type II diabetes patients. Clin Biochem 2007; 40:248-254.

2. Erel O.A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005; 38:1103-11011.

3. Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions. Clin Biochem 2004;37:112-119.

4. Bors W, Michel C, Saran M. Inhibition of the bleaching of the carotenoid crocin. A rapid test for quantifying antioxidant activity. Biochim Biophys Acta 1984; 796:312-319.

5. Bors W, Michel C, Saran M. Determination of rate constants for antioxidant activity and use of the crocin assay. In: Ong ASH, Packer L, editors. In Lipid Soluble Antioxidants: Biochemistry and Clinical Applications. Birkhauser Verlag: Basel, Switzerland; 1992. p. 53-63.

6. Tubaro F, Micossi E, Ursini F. The antioxidant capacity of complex mixtures by kinetic analysis of crocin bleaching inhibition. J Am Oil Chem Soc 1996; 73:173-179.

7. Tubaro F, Ghiselli A, Rapuzzi P, Maiorino M, Ursini F. Analysis of plasma antioxidant capacity by competition kinetics. Free Radic Biol Med 1998; 24:1228-1234.

8. Ordoudi SA, Tsimidou MZ. Crocin bleaching assay step by step: observations and suggestions for an alternative validated protocol. J Agric Food Chem 2006 ;54:1663-1671.

9. Sugherini L, Valentini M, Cambiaggi C, Tanganelli I, Gragnoli G, Borgogni P, *et al*. Determination of a redox compensation index and its relationships to glycaemic control in type 2 diabetes mellitus. Clin Chem Lab Med 2000 ;38:983-987.

10. Prior RL, Cao G. *In vivo* total antioxidant capacity: comparison of different analytical methods. Free Radic Biol Med 1999; 27:1173-1181.

11. Trotti R, Carratelli M, Barbieri M, Micieli G, Bosone D, Rondanelli M, *et al*. Oxidative stress and a thrombophilic condition in alcoholics without severe liver disease. Haematologica 2001; 86:85-91.

12. Josephy PD, Mason RP, Eling T. Cooxidation of the clinical reagent 3, 5 3'5'-tetramethylbenzidine by prostaglandin synthase. Cancer Res 1982;42:2567-2570.

13. MisonoY, Ohkata Y, Morikawa T, Itoh K. Resonance Raman and absorption spectroscopic studies on the electrochemical oxidation processes of 3, 3', 5, 5'-tetramethylbenzidine. J Electroanal Chem 1997; 436:203-212.

14. Gutteridge JM, Halliwell B. The measurement and mechanism of lipid peroxidation in biological systems. Trends Biochem Sci 1990 ;15:129-135.

15. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation 1998 12; 97:1837-1847.

16. Bierman EL. George Lyman Duff Memorial Lecture. J AHA, Atherogenesis in diabetes. Arterioscler Thromb 1992; 12:647-656.

17. Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell 2001;104:503-516.

18. Jay D, Hitomi H, Griendling KK. Oxidative stress and diabetic cardiovascular complications. Free Radic Biol Med 2006; 40:183-192.

19. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004; 84:1381-478.

20. Madamanchi NR, Hakim ZS, Runge MS. Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes. J Thromb Haemost 2005;3:254-267.

21. Alamdari DH, Ghayour-Mobarhan M, Tavallaie S, Parizadeh MR, Moohebati M, Ghafoori F. *et al.* Prooxidant–antioxidant balance as a new risk factor in patients with angiographically defined coronary artery disease. Clin Biochem 2008; 41: 375-380.