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ORIGINAL ARTICLE

Performance Evaluation of the Innovative PAT Test, Comparison with the Common BAP Test and Influence of Interferences on the Evaluation of the Plasma Antioxidant Capacity

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SUMMARY

Background: **Antioxidants (AOs) represent the main barrier of defense against damaging aggression due to reactive species, in particular by reactive oxygen species (ROS). The plasma AO capacity is a measure of physiological, environmental, and nutritional factors (exposure to ROS and antioxidant supplementation) determining the redox status in humans and can underline the oxidative stress (OS) conditions in the progression/development of many diseases. Moreover, changes in AO plasma content after supplementation may provide information on the absorption and bioavailability of nutritional compounds and efficacy of AO therapy.**
Aim: **The aim of the study was a comparison between the common BAP (Biological Antioxidant Potential) test, used for the evaluation of the antioxidant capacity, and the innovative PAT (Plasma Antioxidant Test) and to assess both the *in vitro* interferences of phosphates on the iron reduction and the interference of the plasmatic concentration of phosphates in relation to the plasma antioxidant capacity measured with the two methods.**
Methods: **Thirty-six apparently healthy volunteers were involved in the study for the comparison of the two methods.**
Results: **BAP test and PAT performed on 36 plasma samples demonstrated that plasma antioxidant capacity dosage using the BAP test resulted in overestimated levels in relation to plasma phosphate. Increased BAP values due to phosphates correspond to increased differences between BAP and PAT value (correlation coefficient R = 0.812, p = 0.001).**
Conclusions: **PAT can be considered an innovative and predictable method for the measure of the antioxidant power of plasma.**
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KEY WORDS

oxidation, plasma, antioxidant, phosphates, BAP test, PAT

INTRODUCTION

In aerobic living organisms, the production of reactive species (RS), in particular reactive oxygen species (ROS), is under the control of the antioxidant reserve (RAO). Antioxidants (AOs) can derive from either endogenous or exogenous agents, can be located in both the hydrophilic and lipophilic compartments of plasma, and are actively involved as a defense system against ROS, which are continuously generated in the body due to both normal metabolism and disease [1-4]. Higher levels of ROS and consequent lower levels of AOs lead to a condition of oxidative stress (OS), an emerging risk factor for the development of many diseases (diabetes, cancer, cardiovascular and neurodegenerative diseases, etc.) [1-3,5-8]. OS is also induced by a wide range of chemical, physical, and biological environmental factors (UV stress, pathogen invasion, pollution, smoke,

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drugs).
ROS, hydrogen peroxide (H_2O_2), and superoxide (O_2^-) are physiologically produced in a number of cellular reactions, including the iron-catalyzed Fenton reaction [9, 10] and by various enzymes, such as lipoxygenases, peroxidases, NADPH oxidase, and xanthine oxidase. More than 100 different tests are used for the determination of OS and the prevalent methods are those regarding biomarkers of lipid, DNA, and protein oxidation or the antioxidant capacity of the body [1]. In order to measure the antioxidant capacity of plasma samples, different methods have been developed based on the ability of plasma AOs to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}).
The quantified antioxidant potential is attributable to the major components of plasma barrier to oxidation (vitamin C, vitamin E, uric acid, bilirubin) [11-14].
An unspecific chemical reaction, such as that of iron (III) with thiocyanate, is commonly used. An innovative method, much more precise and specific, has been developed, the Plasma Antioxidant Test (PAT) [15]. The question is: "Can a non-specific reaction become specific?".
Experiments on overexpression of antioxidant production do not always result in the enhancement of the antioxidative defense, and hence increased antioxidative capacity does not always correlate positively with the degree of protection. The use of biological fluids can create a multitude of artifacts because of the presence of interferences. In plasma, for example, one of the most common interferences is phosphate which has the capacity to bind iron and form a stable complex. As a result, the biochemical dosage for assessing the total plasma antioxidant capacity based on iron reduction (e.g., BAP test [11]) is influenced by the serum concentration of phosphates leading to an overestimation of the plasma antioxidant capacity.
This overestimation can be negated by introducing the correct quantity of zirconium salts into the dosage, which have the capacity to bind the phosphates without causing precipitation.
The solution is made stable by adding a stabilizer that avoids the uncontrolled reaction development and makes the compound free from polarity modifications. In this way, the prevalent and stable species formed will be $Fe(SCN)_3$. Characteristic of these species is the red-brown colour of the combined solution that is detectable at 505 nm. The stabilizer also allows a pH-independent reaction and avoids the problem of plasma protein precipitation (in particular albumin).
An adult male has approximately 600 g of phosphates expressed as phosphorous. In plasma, the phosphorus content in normal subjects oscillates between 2.6 and 4.5 mg/dL (0.84 - 1.45 mmol/L) corresponding to values of phosphates ($H_2PO_4^-$, HPO_4^{2-}) equal to 7.8 and 13.5 mg/dL, respectively [16]. This concentration can vary depending on meals and the secretion of hormones such as PTH (parathyroid hormone). Serum phosphate exists in the form of monovalent and bivalent phosphate anions. Approximately 10% is attached to proteins, 35% is a sodium, calcium, and magnesium complex, and the remaining 55% is present in free-form. Although both organic and inorganic phosphates are present in serum, only the inorganic phosphate is measured as the organic part is primarily located in haematic cells.
Based on this assumption, the aim of this research is to assess both the *in vitro* interferences of phosphates on the iron reduction and the interference of the plasmatic concentration of phosphates in relation to the plasma antioxidant capacity measured by the BAP test and PAT.

MATERIALS AND METHODS

In vitro evaluation: interference of phosphates on iron reduction

A solution of sodium phosphate ($Na_2HPO_4.2H_2O$) in distilled water was used with five different concentrations in terms of phosphorus (Pi) (2.5 mg/dL; 5 mg/dL; 10 mg/dL; 20 mg/dL; 40 mg/dL). This solution was used as sample for the determination of PAT. Each determination was made in triplicate and the results were plotted in a dose/effect curve expressed on the basis of the concentration value of Pi or as logarithm (Log). The same relations were also evaluated in the same conditions and with the same Pi concentrations in absence of zirconium salt.

BAP test and PAT comparison: evaluation on human plasma samples

- BAP test dosage method

The BAP test was carried out using the H&D srl (Parma, Italy) diagnostic kit which is commercially available as mono-dosed and pre-dosed equipment.
40 µL of R2 reagent (iron solution) were added to the cuvette containing R1 reagent (thiocyanate derivate pre-dosed solution), followed by 10 µL of the sample. The reading was taken at 505 nm following a 5-minute incubation period at 37°C using the dedicated FRAS 4 Evolvo system (H&D srl, Parma, Italy).

- PAT dosage method

PAT was carried out using the H&D srl (Parma, Italy) diagnostic kit which is commercially available as mono-dosed and pre-dosed equipment.
This test is designed to measure the plasma antioxidant capacity in the presence of zirconium salts added to the reaction mixture. 40 µL of R2 reagent (iron solution) were added to the cuvette containing R1 reagent (thiocyanate derivate pre-dosed solution), followed by 10 µl of the sample. The reading was taken at 505 nm following a 1-minute incubation period at 37°C using the dedicated FRAS 4 Evolvo system (H&D srl, Parma, Italy).

Table 1. PAT and BAP values in presence and absence of zirconium salts (mean values of 3 determinations).

Pi (mg/dL)	PAT (µmol/L Vit C)	BAP (µmol/L Vit C)
40	2818	6144
20	1156	3794
10	608	2290
5	350	1346
2.5	204	962

Table 2. BAP and PAT values (mean ± SD) on human plasma samples (n = 36).

BAP (µmol/L)	PAT (µmol/L)	ΔBAP-PAT (µmol/L)
2374 ± 188	1710 ± 169	653 ± 185

Table 3. Mean ± SD of plasma phosphorus (Pi) levels (mg/dL), BAP (µmol/L Vit C), and PAT (µmol/L Vit C) in capillary human plasma samples (n = 16).

	Phosphorus (Pi) (mg/dL)	BAP (µmol/L)	PAT (µmol/L)	Δ BAP-PAT (µmol/L)
Mean ± SD	3.59 ± 0.50	2381 ± 212	1636 ± 150	743 ± 206

Phosphate interference in BAP test determination

- Plasma phosphate dosage method

The Gesan Production (Italy) LR phosphorus kit was used for the quantitative determination of the inorganic phosphate level in human plasma. The photometric analysis is based on the reaction of the inorganic phosphate with ammonium molybdate which forms a phosphomolybdate compound in an acid environment with a characteristic absorption at 340 nm. The following were added to 1000 µL of R1 reagent: 10 µL of distilled water in the blank sample cuvette, 10 µL of standard in the cuvette for the standard and 10 µL of serum/plasma in the sample cuvette. The reading was carried out at 340 nm against the blank reagent after a 5-minute incubation period at 37°C. The following formula was used for the calculation: phosphorus [mg/dL] = Abs sample/Abs standard x standard concentration. The reference adult plasma values are 2.5 - 5.5 mg/dL, without any difference relating to gender.

Volunteer details

Thirty-six (15 male and 21 female) seemingly healthy subjects, ages between 21 and 72, were involved in the study for the comparison between BAP test and PAT. The influence of phosphates in the evaluation of the antioxidant capacity measured with BAP test was also determined in a subgroup of sixteen subjects (3 male and 13 female).

A fasting capillary blood sample was taken using a heparinized microvette from each volunteer after obtaining written and informed consent.

The plasma was extracted by centrifugation of the entire blood sample (6000 rpm for 90 seconds).

Statistics

The graphs and data analysis were produced using the software application Excel and Origin 6.0. A paired sample *t*-test was used for the statistical analysis, any p values < 0.05 were considered significant.

RESULTS

***In vitro* evaluation: interference of phosphates on iron reduction**

The innovative PAT test is an improvement of the common BAP test.

Table 1 gives the BAP and PAT values obtained at the different concentrations of phosphorous (Pi) in the presence (PAT test) and in the absence (BAP test) of zirconium salts. Both the BAP value and the PAT value are expressed in µmol/L of vitamin C.

As represented in Figure 1, the data from both tests

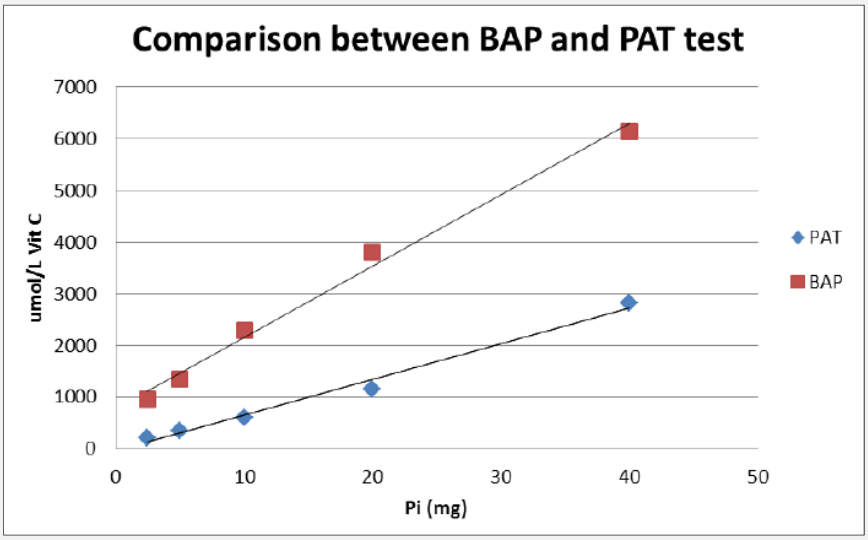

Figure 1. Values (µmol/L Vit C) at different concentrations of phosphorus (Pi) in presence (PAT test) or absence of zirconium salts (BAP test).

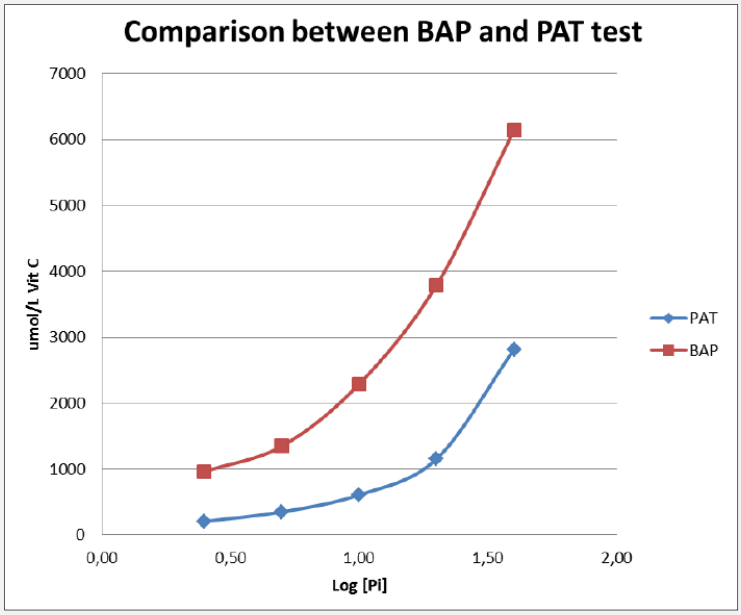

Figure 2. Values (µmol/L Vit C) at different concentrations of phosphorus (Pi) expressed as Log [Pi] in presence (PAT) or absence of zirconium salts (BAP test).

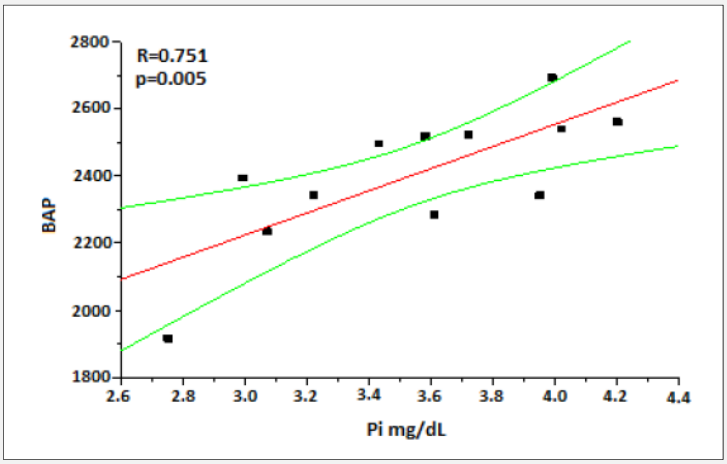

Figure 3. Correlation between BAP values and plasma phosphorus (Pi) levels in 16 capillary human plasma samples (green lines indicate the confidence limits).

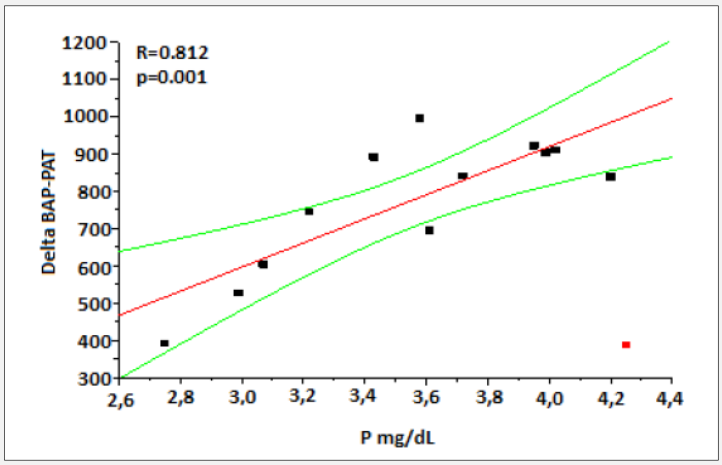

Figure 4. Correlation between ΔBAP-PAT and phosphorus (Pi) levels (mg/dL) in 16 capillary plasma samples (green lines indicate the confidence limits).

have a linear distribution. The addition of zirconium salts to the PAT test solution significantly influences the concentration curve.

Considering the Pi concentration expressed as Log [Pi], it is possible to observe that in the presence of zirconium, the curve shifts to the right as a result of a competitive antagonism (Figure 2).

BAP test and PAT test comparison: evaluation on human plasma samples

Table 2 reports the BAP and PAT values in the analyzed human plasma (n = 36). The mean difference between BAP and PAT value is 650 µmol/L of vitamin C, explained by the significant and massive presence of plasmatic phosphate and other interference. This difference is statistically significant by the paired sample *t*-test ($p < 0.05$).

Phosphate interference in BAP test determination

Table 3 gives the mean values of phosphates levels, BAP and PAT values, in the 16 capillary plasma samples analyzed. The mean phosphate value is 3.59 mg/dL, the mean BAP value is 2318 µmol/L, and the mean PAT value 1636 µmol/L; the mean difference between BAP and PAT is 743 µmol/L. This difference is statistically significant for the paired sample *t*-test ($p = 9.9 \times 10^{-10}$).

The data analysis shows that the BAP test correlates significantly with plasma phosphorus levels ($R = 0.751$; $p = 0.005$) (Figure 3).

The difference ΔBAP-PAT is in fact directly proportional to the phosphorus concentration (Figure 4).

DISCUSSION

The plasma AO capacity is a measure of physiological, environmental, and nutritional factors (exposure to ROS and antioxidant supplementation) determining the redox status in humans and can underline the oxidative stress conditions (OS) in the progression/development of many diseases. AOs represent the main barrier of defense against damaging aggression due to ROS. Moreover, changes in AO plasma content after supplementation may provide information on the absorption and bioavailability of nutritional compounds and on the efficacy of AO therapy. In order to control the antioxidant status, different tests are commercially available. In the present study, a comparison has been done between the common test for the evaluation of the antioxidant capacity, BAP test, and the innovative PAT. The aim of the study was to assess both the *in vitro* interferences of phosphates on the iron reduction and the interference of the plasmatic concentration of phosphates in relation to the plasma antioxidant capacity measured with the two methods.

The presence of phosphates in the sample, in fact, causes an interference during the evaluation of the antioxidant capacity based on iron (Fe^{3+}) reduction. The use of zirconium salts (PAT) avoids this interference and zirconium participates as competitive inhibitor allowing phosphate clearance within plasma physiological concentration. The observed difference between BAP test (absence of zirconium salts) and PAT comes from the presence of interferences, in particular by phosphates. The plasma antioxidant capacity dosage with the BAP test results in an overestimation in relation to the plasma phosphate levels. Increased BAP values due to phosphates correspond to increased differences between BAP and PAT values (correlation coefficient $R = 0.812$, $p = 0.001$).

In order to standardize the BAP and PAT values and to allow a comparison with the data so far reported in literature on the BAP test, an appropriate algorithm has been applied that allows the transformation of the PAT data. A new unit has been introduced, U Cor, where 1 U Cor has been determined by considering the mean phosphate plasma levels (range between 2.6 and 4.5 mg/dL) which lead to an overestimation equal to ~700 µmol/L of Vit C. One U Cor corresponds to µmol/L of Vit C obtained with the algorithm: PAT value x 1.40. The value obtained from the algorithm is equivalent to the BAP value. The multiplication factor of 40% was determined by simultaneously taking into account the phosphate evaluation and BAP test values, which demonstrated that BAP values were overestimated by an average of 40%, with oscillation between 25 and 55%. However, it is possible that in subjects with higher phosphate levels, the percentage might be higher than 40%.

CONCLUSION

The tests carried out in this study show that a significant difference between BAP and PAT values and a positive significant correlation between phosphate concentration and BAP value in capillary human plasma samples exist. The plasma antioxidant capacity dosage using the BAP test is overestimated by around 40%. PAT tests can be considered an innovative and predictable method to measure the antioxidant power of plasma. The new test has been proven to be simple, precise, and fast. In fact, PAT determination takes 1 minute in comparison to the BAP test (5 minutes for the evaluation).

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No portion of this manuscript, other than the abstract, has been published or posted on the internet.

Declaration of Interest:

The authors have nothing to disclose.

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