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Oxidative stress tests: overview on reliability and use

Part II

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Abstract. – Although the healthcare field is increasingly aware of the importance of free radicals and oxidative stress, screening and monitoring has yet become a routine test since, dangerously, there are no symptoms of this condition. Therefore, in very few cases is oxidative stress addressed. Paradoxically, patients are often advised supplementation with antioxidants and or diets with increased antioxidant profile, which range from vitamins to minerals which is action against oxidative stress states and even more so no test is advised to assess whether the patient is under attack by free radicals or has a depleted antioxidant capacity.

Hence oxidative stress is an imbalance between free radicals (ROS, Reactive Oxygen Species) production and existing antioxidant capacity (AC), living organisms have a complex anti-oxidant power. A decrease in ROS formation is often due to an increase in antioxidant capacity whilst an increase in the AC may be associated to decreased ROS values. But this is not always apparently so.

Test kits for photometric determinations applicable to small laboratories are increasingly available.

Key Words:

Oxidative stress, Free radicals, Antioxidants, Reactive oxygen species.

Introduction

Oxidation is a process that occurs naturally in the body when oxygen combines with reduced molecules, such as carbohydrates or fats, and provides energy. When there is decreased oxidation or decreased energy production, the cells can no longer function efficiently and disease results. However, this normal process propagates short-lived intermediates known as free radicals, and some free radicals escape and initiate further oxidation setting up a chain reaction. So, potentially harmful reactive oxygen species are produced as a consequence of biological metabolism, and by exposure to environmental factors. Free radicals are then usually removed or inactivated by a team of natural antioxidants which prevent these reactive species from causing excessive cellular damage.

"Oxidative stress" is the general phenomenon of oxidant exposure and antioxidant depletion, or oxidant-antioxidant balance.

Although Reactive Oxygen/Nitrogen Species (ROS/RNS) play an important role in immune-mediated defence against invading microorganisms and serve as cell-signalling molecules, at high concentrations, ROS/RNS are capable of damaging host tissues, i.e., they can modify or damage DNA, lipids, and proteins. As yet mentioned, ROS/RNS levels are controlled through an intricate network of endogenous and exogenous antioxidant molecules that are responsible for scavenging and consumption of specific reactive species. In this regard, intake of dietary antioxidants has received much attention, with the concept being that these molecules can affect disease by modulating the biological reactivity of free radicals.

Over the past four decades, a substantial body of data has accumulated to support the direct or indirect association between free radicals and various human diseases. Given the number of patients world-wide suffering from these disorders, and the association with free radicals, screening of oxidative stress (OS) and consequently lifestyle and dietary changes are fundamental for a preventive approach. The role of OS in ageing, neurodegenerative, vascular and other diseases is more and more widely accepted, the value of an-

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tioxidant strategies may sometimes be controversial although a well-balanced antioxidant diet is undoubtedly important and strongly supported.

Market Available Point of Care Tests

In the last years, several laboratory tests have been investigated and produced to assess the whole antioxidant activity of plasma or serum blood[1-5].

Point-of-care diagnostic testing, or testing performed at the patient bedside, allows physicians to diagnose patients more rapidly than traditional laboratory-based testing. Rapid results can enable better patient management decisions, improved patient outcomes, and a reduction in the overall cost of care. These tests are utilized in hospitals, clinics, commercial laboratories and research institutions for the purpose of diagnosis and monitoring of disease.

Although clinicians may associate point-of-care testing (POCT) with critical care, the reality is that POCT (bedside, decentralized, or near-patient testing) is already being performed in virtually every clinical setting.

POCT began more than 30 years ago, although the phrase came into use within the past 15 years. The driving force behind this type of testing has always been to improve patient care through rapid availability of reliable results. The ability to obtain clinical laboratory test results at the site of care in 2 minutes has immediate medical management benefits as well as resource and time benefits.

The potential benefits of POCT include earlier and more appropriate diagnosis, fewer tests, earlier treatment, and reduction or elimination of unnecessary treatment. An unquantifiable benefit of POCT also offers convenience and decreases the time spent in a department or clinic, which are advantages for providers and patients alike.

By an analytical point of view, the effectiveness of antioxidant plasma barrier can be evaluated by testing its capacity to reduce a specific substrate, i.e. by assessing its capacity to supply oxidized background (e.g. free radicals) with one or more electrons.

For this purpose, different chemical reducing-oxidizing couples are available. For example, transition metals (i.e. iron) exhibit the property of receive one electron thus shifting from the oxidized state (Fe^{3+}) to reduced state (Fe^{2+}). Such compounds are the reference to assess antioxidant power of biological systems. Indeed, the so-called "plasma antioxidant power" is ultimately a measure of the reducing or "electron-giving" activity of blood plasma.

On the other hand, some molecules share the property to change their absorbance just when bound to compounds able to switch from the oxidized to reduced state. For example, some thiocyanates are able to reversibly shift from uncolored to colored derivatives, in the presence of ferric or ferrous salts, respectively[4]. Such "chromogens" can work as excellent "detectors" when coupled with adequate "oxidizing/reducing meters" in test designed to assess antioxidant activity of biological systems.

Indeed, when a ferric salt is dissolved in a uncolored solution containing a particular thiocyanate derivative, the resulting solution becomes red, as a function of the ferric ions concentration. This process is due to the formation of a complex between ferric salt and thiocyanate. Further adding of a small amount of blood plasma will reduce ferric ions to ferrous ions thus making uncolored the initial red solution. Such a chromatic change, may due to the release of ferrous ions by the colored thiocyanate complex, can be read by means of a photometer, previously set on the wavelength of chromogen.

Therefore, the entity of absorbance change will directly correlate with the antioxidant "potential" of blood plasma against the specific substrate which has been used as oxidant/detector (ferric ions). In other words, the capacity of tested plasma to reduce ferric to ferrous ions will provide a direct measure of the capacity of a sample of a such plasma to give reducing equivalents and then neutralize chemical species lacking of electrons, like ROS, obviously in the reduction-oxidation potential range of chosen oxidant-reducing couple (Fe^{3+}/Fe^{2+}).

Generally, researchers in the free radicals field assert that each assay has it's own specific characteristics and therefore advantages and disadvantages. There are differences in the free radical-generating system, molecular target, reaction type, biological matrix, residence in the lipo- or hydrophilic compartments and physiological relevance. It is, therefore, impossible to identify one assay as a gold standard for measuring total antioxidant status in body fluids.

A combination of a biomarkers of OS, i.e. indexes of oxidative damage and the antioxidant profile,provides a global assessment of the oxi-

dant/antioxidant balance of the organism as well as on the nutritional needs of patients and on the possible antioxidant strategies.

In the following paragraphs, the main Professional Point of Care assays actually available on the International market will be discussed. The FORD(patent pending) and FORT as well as BAP and dROMs are assays that brings laboratory testing to the near patient testing fields. Test kits have been developed to provide operators with highly reliable, rapid and user-friendly methods for the global evaluation of the oxidative status (radical-induced damage index and the total antioxidant capacity) in the body from a single drop of a capillary blood. In particular both FORT and FORD test are completely stored at room temperature and work employing lyophilized chromogens to reduce operator handling and contact with chemical compounds.

Oxidative stress testing is of fundamental importance for preventive medicine and health care, disease managment as well as the control of relevant therapies during pathologies, in a wide range of fields and applications. Some examples are:

- Monitoring antioxidant therapies;
- Testing pharmacological treatments;
- Monitoring lifestyle changes;
- Sports sector (e.g., during various phases of training or before and after a performance);
- Preventive medicine;
- Anti-ageing fields;
- Disease management in several fields;
- Clinical research.

The FORT Test

Principle and Standardization of Method

FORT (Free Oxygen Radicals Testing) is a colorimetric test based on the properties of an amine derivative employed as chromogen, $ChNH_2$ (4-Amino-N-ethyl-N-isopropylaniline hydrochloride) to produce a fairly long-lived radical cation[6]. When sample is added to a $ChNH_2$ solution, the coloured radical cation of the chromogen is formed and the absorbance at 505 nm, which is proportional to the concentration of hydroperoxyl molecules, is associated to the oxidative status of the sample.

The visible spectrum of the $ChNH_2$ radical cation, reported in Figure 1, shows two peaks of absorbance at the 505 and 550 nm. The overall spectral intensity increased with time.

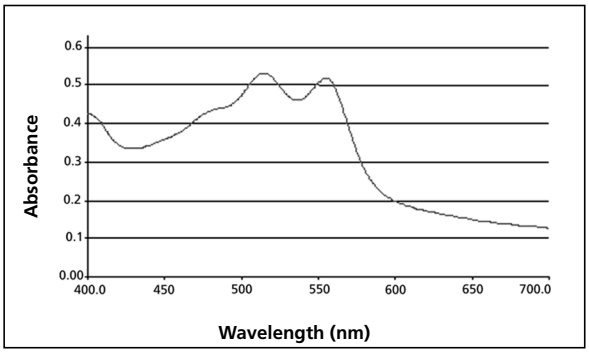

Figure 1. Visible spectrum of the FORT chromogen radical cation

As shown in Figure 2, the increase in absorbance in the first 7-10 minutes is fairly linear, then reaches a plateau after a time interval which depends on temperature (the reaction is completed in approximately 60 minutes at 37°C).

Hence, a kinetic analysis of the colourimetric reaction at 37°C was selected.

The $ChNH_2$ solution is also EPR active, and under high magnetic field modulation (m.a. = 1 mT) it exhibited a single broad line (Figures 3A and 3B).

Hydroperoxides (ROOH) are fairly stable molecules under physiological conditions, but their decomposition is catalyzed by transition metals. Both Fe^{2+} and Fe^{3+} are effective catalysts in the reaction of degradation of these compounds resulting in several secondary reactive radical species formation[7]. In biological samples, hydroperoxides concentration represents a good index of free radical attack because it is indicative of intermediate oxidative products of lipids, peptides and amino acids.

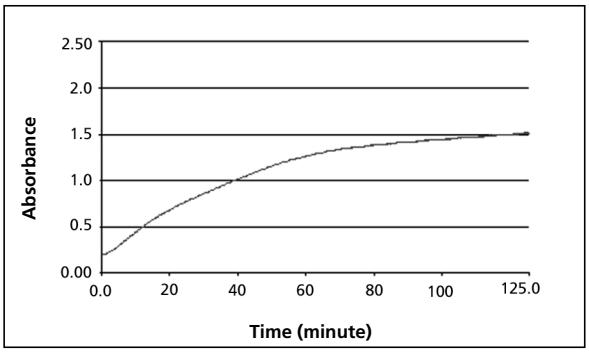

Figure 2. Time course of the FORT chromogen radical cation formation at 37°C.

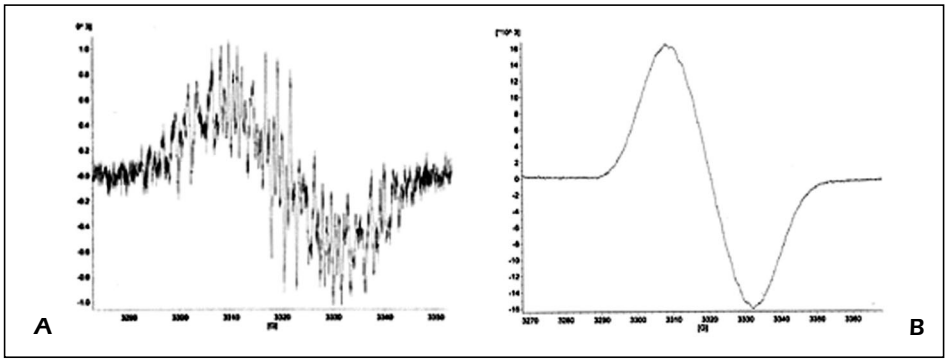

Figure 3. The EPR spectrum of the FORT chromogen radical cation formed in a buffer solution in presence of ferrous ions and TBH at 0.05 mT of modulation amplitude ***(A)*** or human plasma ***(B)*** at 1 mT of modulation amplitude.

The reactions occurring in the FORT test conditions are based on the capacity of transition metals to catalyse the breakdown of ROOH into derivative radicals, according to Fenton's reaction. Once they are formed, ROOH mantain their chemical reactivity and oxidative capacity to produce proportional amounts of alkoxy ($RO^{\cdot}$) and peroxy ($ROO^{\cdot}$) radicals .

These derivative radicals are then preferentially trapped by a suitably buffered FORT chromogen and develop, in a linear kinetic based reaction at 37°C, a coloured fairly long-lived radical cation photometrically detectable. The intensity of the colour correlates directly with the quantity of radical compounds, according to the Lambert-Beer's law and it can be related to the oxidative status of the sample.

$$R\text{-}OOH + Fe^{2+} \rightarrow R\text{-}O^{\bullet} + OH^{-} + Fe^{3+}$$
$$R\text{-}OOH + Fe^{3+} \rightarrow R\text{-}OO^{\bullet} + H^{+} + Fe^{2+}$$
$$RO^{\bullet} + ROO^{\bullet} + 2CrNH_2 \rightarrow RO^{-} + ROO^{-} + [Cr\text{-}NH_2^{+\bullet}]$$

Definition of Unit of Measure for the FORT test

Considering the chemical heterogeneity of the secondary radical species deriving from the iron-dependent breakdown of ROOH during the FORT test, it has been decided to relate the absorbance readings to hydrogen peroxide (H_2O_2) concentration. A reference curve was created and stored into the dedicated instrument (FORMPlus, FORmox and photometers CR3000, Callegari Spa, Catellani Group, Parma, Italy) which performs automatically the calculation of equivalent concentrations of H_2O_2.

In order to define a dedicated unit of measure for the FORT test, conventional units called FORT units have been defined. One FORT unit corresponds to approximately 7.6 mmol/l of H_2O_2 (equivalent to 0.26 mg/l). Transformations are automatically performed by the dedicated instruments so that the results are expressed both as concentration of H_2O_2 equivalent and as FORT units. So doing the value interpretation results easier for any operators including lay users.

Method Performances

Linearity: The linearity of the FORT test system was tested using two different methods (LOF test and Mandel test). With both of them the linearity resulted statistically verified. Range of linearity: 148-608 FORT units.

Precision: intra-assay coefficient of variation, CV < 5%.

Repeatability: intra-assay coefficient of variation, CV < 5%.

Sensitivity: it is determined by the linearity range of the FORT reaction, that is 160-600 FORT units. S = 4.0528, the sensitivity (S) is defined as: S = ΔAbs/ΔC; Abs = absorbance, C = concentration.

Accuracy: BIAS of the FORT test was determined analyzing a series of H_2O_2 solution in water. Ten replicates were performed for each level of concentration (C).

Predicted BIAS was calculated as: [(C expected-C obtained)/C obtained] × 100.

BIAS < 4% for 1.43 mM $H_2O_2 \leq C \leq 4.23$ mM H_2O_2;

BIAS < 7% for C = 1.214 mM H_2O_2;

BIAS < 12% for C ≥ 4.74 mM H_2O_2.

Sample: 20 µl of whole blood; 10 µl for serum or plasma.

Normal values: up to 310 FORT units (corresponding to approximately 2.36 mmol/l of H_2O_2)[8].

The higher the FORT result obtained, the higher is the oxidative status of the sample. Although the assay is very reproducible for the same subject during the day and the CVs, both inter- and intra-assay, are very low, the value of the FORT measured on healthy subjects may be variable. Since the oxidative stress state of an individual depends on the hereditary, dietary and environmental factors, there is a large heterogeneity in the population that may be related to disease incidence and longevity. For this reason, it is advisable to establish reference value for a patient.

Interference Factors

The FORT test is based on Fenton's reaction. Fenton chemistry was discovered about 100 years ago and it has proven to be a cornerstone of free radical biochemistry[9,10]. Fenton's reagent is a mixture of H_2O_2 and ferrous iron, which produces secondary radicals[11,12], according to the reactions:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$
$$RH + OH^{\bullet} \rightarrow H_2O + R^{\bullet} \rightarrow \text{further oxidation}$$
$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

The ferrous iron (Fe^{2+}) initiates and catalyses the decomposition of H_2O_2, resulting in the production of hydroxyl radicals ($OH^{\cdot}$). Hydroxyl radicals can oxidise organics (RH) by abstraction of protons producing organic radicals ($R^{\cdot}$), which are highly reactive and can be further oxidised, initiating a radical chain oxidation. During the FORT reaction, overall organic radicals present in the sample are trapped by the FORT chromogen and photometrically measured.

The reactions above suggest that the presence of iron is required in the test reaction, and thereby use of any kind of iron-chelating agents (e.g., EDTA, citrate, desferal), external hydroperoxide and/or antioxidant sources (e.g., H_2O_2, benzoyl peroxide, BHT, ascorbic acid) affect the FORT assay blocking the Fenton's chemistry. In fact, solutions of the FORT chromogen and organics lacking the iron showed no specific EPR signal. Therefore, the FORT test cannot be applied when iron chelating substances are present.

The hydrogen peroxide reacts in the Fenton's reaction, so external sources of H_2O_2, such as some disinfectants, can potentially interfere with the test resulting in not reliable FORT values. Analogously, the presence in the sample of molecules such as BHT (3,5-di-tert-butyl-4-hydroxytoluene) having antioxidant action interfere with the correct scheme for the test reaction in accordance with the Fenton's chemistry.

When the test is performed on whole blood, abnormal haematocrit values and haemolysed samples may affect the results. Nevertheless, there is no significant interference when hematocrit is between 38% and 48%.

The FORD test

Principle and Standardization of Method

FORD (Free Oxygen Radicals Defence) is a colorimetric test based on the ability of antioxidants present in plasma to reduce a preformed radical cation. The principle of the assay is that at an acidic pH (5.2) and in the presence of a suitable oxidant solution ($FeCl_3$), 4-Amino-N,N-diethylaniline, the FORD chromogen, can form a stable and colored radical cation.

Antioxidant molecules (AOH) present in the sample which are able to transfer a hydrogen atom to the FORD chromogen radical cation, reduce it quenching the color and producing a decoloration of the solution which is proportional to their concentration in the sample.

Preliminary experiments showed that the choice of oxidant solution and the ratio between the concentration of the chromogen substance and the concentration of the oxidative compound are essential for the effectiveness of the method.

$$\text{Chromogen}_{(\text{uncolored})} + \text{oxidant } (Fe^{3+})\ H^{+} \rightarrow \text{Chromogen}^{\cdot+}_{(\text{purple})}$$

$$\text{Chromogen}^{\cdot+}_{(\text{purple})} + AOH \rightarrow \text{Chromogen}^{+}_{(\text{uncolored})} + AO$$

The UV-visible spectrum of the FORD chromogen radical cation (Figure 4) shows maximum of absorbance at approximately 330 nm, 510 nm and 550 nm. Hence, an end-point analysis of the colorimetric reaction at 505 nm and at 37°C was selected.

The time course of the FORD chromogen radical formation obtained with an oxidant solution of $FeCl_3$ which gives a stable colored solution is reported in the Figure 5. It is outlined that to

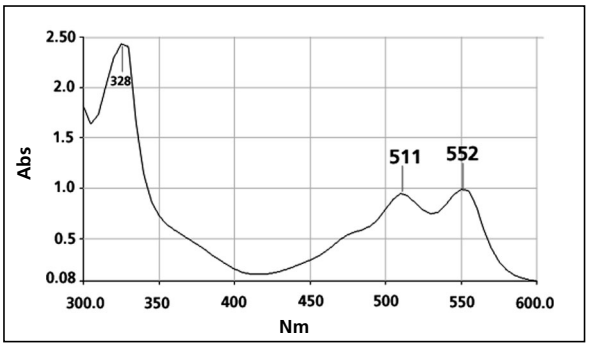

Figure 4. UV-visible spectrum of the FORD chromogen radical cation.

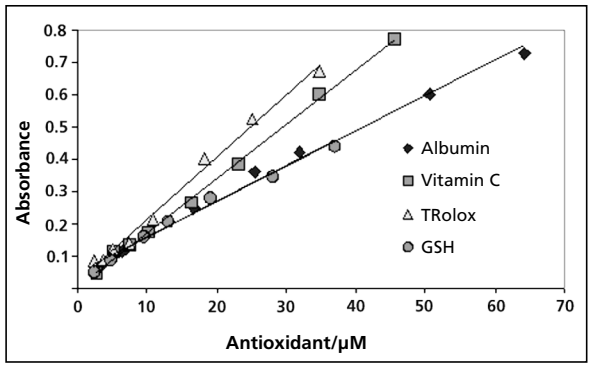

Figure 6. Degree of inhibition of the FORD chromogen absorbance as a function of antioxidant concentration.

have both high sensitivity of the measurements and a sufficient inhibition range, a starting point between 0.80 and 1.00 of absorbance at 505 nm is necessary. This absorbance readings are typically reached after 3-4 minute, after that the optical density remains stable. Hence, a lag-time of 4 minutes was adopted between starting the reaction and measure of the chromogen radical cation absorbance reading.

The system was tested by using different concentrations of several antioxidant compounds, namely ascorbic acid, albumin, glutathione (GSH), uric acid and Trolox, the α-tocopherol analogue with enhanced water solubility. The dose-response curves obtained (Figure 6) showed that inhibition of the starting absorbance is linear. Moreover, results revealed a relevant participation of FORD from ascorbic acid, Trolox, albumin and GSH, and no response from uric acid.

Antioxidants tested have comparable kinetics in FORD chromogen radical scavenging, and the absorbance inhibition induced is immediate. Hence, a lag-time of 2 minutes was selected between addition of sample containing antioxidants and measure of the color inhibition.

This provides an assay based on the extent of radical cation reduction at a fixed time point and not on the rate of reduction. This feature rules out complications due to the monitoring of the time course of colour inhibitions.

FORD color quenching is determined especially by contribution of antioxidants like proteins, reduced glutathione, vitamins etc. These antioxidants (together with uric acid which is not detected by the FORD test but is measured by a different test) are among the most important contributors to antioxidant plasmatic barrier.

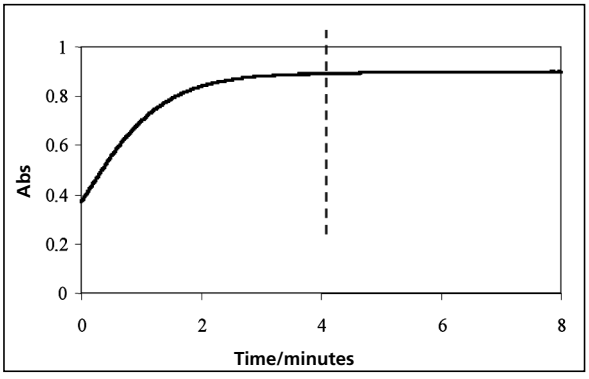

Figure 5. Time-course of the FORD chromogen radical formation at 37°C and 505 nm.

Unit of Measure

Like many other methods[4,13], FORD results are express like Trolox equivalents (mmol/l) using a calibration curve plotted with different amounts of standard Trolox that is stored on the dedicated instrument (FORM Plus, FORM ox and CR3000 series diagnostic analyzers, Callegari SpA, Catellani Group, Parma, Italy). An example of dose-response curve obtained by using Trolox is shown in Figure 7. Each data is the mean of four determinations performed in four different days. The standard deviation is very low and the curve is highly reproducible (Coefficient of Variation, CV < 5%).

Reference Values

Based on a preliminary number of 70 human blood donors (male/female ratio, 37/33; aged 20-70 years, mean age 36 years) and the values cited in scientific literature[14], at present the reference

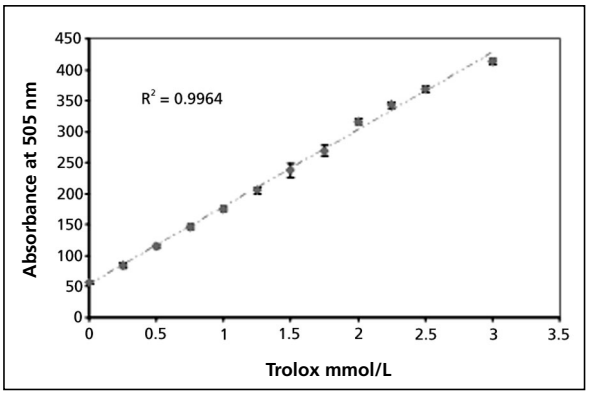

Figure 7. Degree of inhibition of the FORD chromogen absorbance as a function of the Trolox concentration.

values of FORD were estimated to be within the 1.07-1.5 mmol/l Trolox range (mean value = 1.23 mmol/l Trolox) which includes approximately 85% of data.

Method Performances

Linearity

The linearity of the FORD test system was tested using solutions of Trolox as a chemical antioxidant standard. Tests were performed over a wide range of concentrations by subsequent dilutions of a stock solution and measuring the correspondent increment in FORD. The linearity has been statistically verified applying the Mendel's test.

Range of linearity: 0.25-3.0 mmol/l Trolox.

Repeatability and Precision

Three different concentrations of Trolox (2.5, 1.25 and 0.25 mM) were assayed 10 times in the same run for the determination of intra-assay coefficient variation (CV). An intra-assay CV< 5% was demonstrated. Additionally, repeatability and precision were established testing whole human capillary blood. The FORD test was carried out using two different instruments and one level of concentration that is 1.25 mM Trolox equivalent. 20 replicates were performed for each instruments during the same day.

Repeatability (N = 20): CV < 5%;
Precision (N = 40): CV< 5%.

Interferences

Use of any kind of iron-chelating agents (e.g., EDTA, citrate, desferal); external hydroperoxide and/or antioxidant sources (e.g., H_2O_2, benzoyl peroxide, BHT, BHA, ascorbic acid); abnormal haematocrit values and haemolysed samples (when the test is performed on whole blood).

Sample: 50 µl of whole blood.

Clinical Applications

Cancer-related anorexia/cachexia syndrome and oxidative stress play a key role in the progression and outcome of neoplastic disease. Mantovani et al[15] have developed an innovative approach consisting of diet with high polyphenol content (400 mg), p.o. pharmaconutritional support enriched with n-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) 2 cans (237 mL each) per day, medroxyprogesterone acetate 500 mg/d, antioxidant treatment with alpha-lipoic acid 300 mg/d plus carbocysteine lysine salt 2.7 g/d plus vitamin E 400 mg/d plus vitamin A 30,000 IU/d plus vitamin C 500 mg/d, and selective cyclooxygenase-2 inhibitor Celecoxib 200 mg/d. The treatment is administered for 16 weeks. The following variables are evaluated: (a) clinical variables (stage and Eastern Cooperative Oncology Group performance status); (b) nutritional variables (lean body mass, appetite, and resting energy expenditure); (c) laboratory variables (serum levels of proinflammatory cytokines, C-reactive protein, and leptin and blood levels of reactive oxygen species and antioxidant enzymes); and (d) quality of life variables (European Organization for Research and Treatment of Cancer QLQ-C30, EQ-5Dindex, and EQ-5DVAS). A phase II nonrandomized study has been designed to enroll 40 patients with advanced cancer at different sites with symptoms of cancer-related anorexia/cachexia syndrome and oxidative stress. As of January 2004, 28 patients have been enrolled: 25 patients were evaluable and 14 of them have completed the treatment (20 patients have completed 2 months of treatment). As for clinical response, five patients improved, three patients remained unchanged, and six patients worsened. The Eastern Cooperative Oncology Group performance status (grade) 1 remained unchanged. As for nutritional/functional variables, the lean body mass increased significantly at 2 and 4 months. As for laboratory variables, reactive oxygen species decreased significantly and proinflammatory cytokines interleukin-6 and tu-

mor necrosis factor-alpha decreased significantly. As for quality of life, it comprehensively improved after treatment. The treatment has been shown to be effective for clinical response, increase of lean body mass, decrease of reactive oxygen species and proinflammatory cytokines, and improvement of quality of life. The treatment has been shown to be safe with good compliance of patients.

Anemia occurs in more than 30% of patients with epithelial ovarian cancer before any surgery. High levels of proinflammatory cytokines and increased oxidative stress may contribute to the development of cancer-related anemia. Macciò et al[16] assessed a population of previously untreated patients with advanced epithelial ovarian cancer to evaluate whether there was a correlation between hemoglobin (Hb) and parameters of inflammation and oxidative stress, stage of disease, and performance status (PS). In 91 patients with epithelial ovarian cancer and 95 healthy women matched for age, weight, and height, levels of Hb, C-reactive protein (CRP), fibrinogen (Fbg), proinflammatory cytokines, leptin, reactive oxygen species (ROS), and antioxidant enzymes were assessed at diagnosis before treatment. The correlations between Hb, parameters of inflammation and oxidative stress, stage, and PS were evaluated. Hb levels were lower in patients with advanced epithelial ovarian cancer than in control subjects and inversely related to stage and PS. Hb negatively correlated with CRP, Fbg, interleukin 1beta (IL-1beta), IL-6, tumor necrosis factor alpha (TNF-alpha), and ROS, and positively correlated with leptin and glutathione peroxidase (GPx). Multivariate regression analysis showed that stage and IL-6 were independent factors determining Hb values. This evidence suggests that anemia in epithelial ovarian cancer is common and its presence is related to stage of disease and markers of inflammation.

Increases in the inflammatory marker C-reactive protein (CRP) have been associated with a higher risk of incident coronary heart disease (CHD). These epidemiologic data suggest that identifying the determinants of elevated CRP levels before CHD has become clinically manifest could provide insights into the earliest stages of CHD development. The causes of increased CRP, however, are not completely understood. Reports have indicated that increased body mass index (BMI) is strongly related to elevated CRP. Data also indicated that hypertension and smoking may increase CRP. These factors do not appear to explain all of the variability in CRP though, so other factors may contribute. Basic studies have indicated that oxidative stress may have pro-inflammatory effects. Particularly, reports indicate that oxidative stress is critical for activation of nuclear factor kappaB (NF-κB), a transcription factor that increases expression of pro-inflammatory cytokines, chemokines, and cell adhesion molecules. Consistent with these reports, both laboratory and animal investigations have shown that various markers of oxidative stress are indeed related to increased expression of pro-inflammatory cytokines (interleukin 6 and interleukin 8), chemokines (monocyte chemotactic protein-1) and cell-adhesion molecules (vascular cell adhesion molecule-1, intracellular adhesion molecule-1). Additionally, researchers recently observed *in vitro* evidence of an association between an oxidative stress marker (oxidized low-density lipoprotein) and CRP. From all these data, it would be reasonable to hypothesize that oxidative stress might be associated with elevated levels of CRP in humans. Yet studies of oxidative stress and CRP in healthy humans have been somewhat sparse. A few studies have indicated that oxidative stress markers such as urinary F_2 isoprostanes, hydrogen peroxide production from mononuclear cells, and oxidized-LDL show positive associations with CRP in humans without CHD, but the precise association between oxidative stress markers and CRP in healthy humans is not firmly established. Studies suggest that oxidative stress may have pro-inflammatory effects, but data on the relationship between oxidative stress and CRP in healthy individuals is insufficient. Abramson et al (17) conducted a cross-sectional study of oxidative stress markers and high sensitivity CRP (hsCRP) among 126 adults without CHD. These researchers investigated whether markers of oxidative stress were associated with CRP in persons without clinical CHD, and whether these oxidative stress markers predicted CRP independently of BMI and other CRP determinants. They focused on two markers of oxidative stress which had not previously been investigated in relation to CRP in healthy humans. The first measure was the free oxygen radical test assay. The FORT provides an indirect measure of hydroperoxides, which are an useful measure of oxidative stress because they indicate the intermediate oxidative products of lipids, amino acids and peptides. The second measure was the plasma ratio of reduced to oxidized glutathione, known as the GSH/GSSG couple. The steady

state balance between the GSH/GSSG couple can be expressed as a redox potential (E_h) GSH/GSSG, and this redox potential is considered a dynamic and quantitative measure of oxidative stress[18]. In this study of adults without clinical CHD, they found a positive association between oxidative stress, as measured by the FORT and CRP. In unadjusted analyses, these researchers found that the FORT explained a statistically significant 29% of the variance in ln(hsCRP). In a linear regression model that adjusted for age, sex, body mass index, and other potential hsCRP determinants, the FORT was positively related to log-transformed hsCRP and explained 14% of free of CHD, oxidative stress, as measured by the FORT, is significantly associated with higher hsCRP levels, independent of BMI and other CRP determinants. They also found a positive association between (E_h) GSH/GSSG and ln(hsCRP) which was not statistically significant. This results suggest that oxidative stress may be a determinant of CRP levels and promote pro-atherosclerotic inflammatory processes at the earliest stages of CHD development. These finding of an association between the FORT and CRP extends the basic findings noted above and lends credibility to the notion that oxidative stress may be related to inflammation in healthy humans. This suggests that oxidative stress could potentially be a key factor in the early stages of CHD. Of particular note in this study was the finding that the FORT rivalled BMI as a predictor of CRP, and that the FORT explained a substantial amount of CRP variability independent of BMI. Prior investigations have reported that increasing BMI level are strongly predictive of elevated CRP. This study suggests that oxidative stress may also be a relevant determinant of CRP levels in healthy persons, independent of BMI, and that therefore, the link between oxidative stress and CRP may involve mechanisms that are not necessarily related to BMI. It is of interest that even if they found a correlation between hsCRP and the FORT, (E_h) GSH/GSSG correlated poorly with hsCRP. At first glance the differences between these two markers of oxidative stress seem puzzling. However, it should be stressed that they may reflect very different oxidative processes. Increases in FORT values most likely indicate increases in oxidation of lipids, which occurs in lipid bilayers and in lipid particles. In contrast, (E_h) GSH/GSSG may represent oxidation in thiols that are most likely to occurr in hydrophilic compartments such as the cell cytoplasm. As such, these findings suggest the importance of recognizing that different oxidative markers may be representative of different cellular oxidation events.

Fatigue is a multidimensional symptom that is described in terms of perceived energy, mental capacity, and psychological status: it can impair daily functioning and lead to negative effects on quality of life. It is one of the most common side effects of chemotherapy and radiotherapy. L-carnitine (LC) supplementation has been demonstrated to be able to improve fatigue symptoms in patients with cancer. Gramignano et al[19] tested the efficacy and safety of LC supplementation in a population of patients who had advanced cancer and developed fatigue, high blood levels of reactive oxygen species, or both. These researchers evaluated fatigue and quality of life in relation to oxidative stress, nutritional status, and laboratory variables, mainly levels of reactive oxygen species, glutathione peroxidase, and proinflammatory cytokines. From March to July 2004, 12 patients who had advanced tumors (50% at stage IV) at different sites were enrolled (male-to-female ratio 2:10, mean age 60 y, range 42-73). Patients were only slightly anemic (hemoglobin 10.9 g/dL) and hemoglobin levels did not change after treatment. LC was administered orally at 6 g/d for 4 wk. All patients underwent antineoplastic treatment during LC supplementation. Fatigue, as measured by the Multidimensional Fatigue Symptom Inventory-Short Form, decreased significantly, particularly for the General and Physical scales, and for quality of life in each subscale of quality of life in relation to oxidative stress. Nutritional variables (lean body mass and appetite) increased significantly after LC supplementation. Levels of reactive oxygen species decreased and glutathione peroxidase increased but not significantly. Proinflammatory cytokines did not change significantly. Improvement of symptoms with respect to fatigue and quality of life in relation to oxidative stress may be explained mainly by an increase in lean body mass, which may be considered the most important nutritional or functional parameter in assessing the cachectic state of patients. In this view, fatigue with related symptoms can well be considered an important constituent of cancer-related anorexia cachexia syndrome.

Mantovani et al[20] conducted an open early-phase II study, according to the Simon two-stage design, to evaluate the efficacy and safety

of an integrated treatment based on a pharmaconutritional support, antioxidants, and drugs, all given orally, in a population of advanced cancer patients with cancer-related anorexia/cachexia and oxidative stress. The integrated treatment consisted of diet with high polyphenols content (400 mg), antioxidant treatment (300 mg/d alpha-lipoic acid + 2.7 g/d carbocysteine lysine salt + 400 mg/d vitamin E + 30,000 IU/d vitamin A + 500 mg/d vitamin C), and pharmaconutritional support enriched with 2 cans per day (n-3)-PUFA (eicosapentaenoic acid and docosahexaenoic acid), 500 mg/d medroxyprogesterone acetate, and 200 mg/d selective cyclooxygenase-2 inhibitor celecoxib. The treatment duration was 4 months. The following variables were evaluated: (a) clinical (Eastern Cooperative Oncology Group performance status); (b) nutritional [lean body mass (LBM), appetite, and resting energy expenditure]; (c) laboratory [proinflammatory cytokines and leptin, reactive oxygen species (ROS) and antioxidant enzymes]; (d) quality of life (European Organization for Research and Treatment of Cancer QLQ-C30, Euro QL-5D, and MFSI-SF). From July 2002 to January 2005, 44 patients were enrolled. Of these, 39 completed the treatment and were assessable. Body weight increased significantly from baseline as did LBM and appetite. There was an important decrease of proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha, and a negative relationship worthy of note was only found between LBM and IL-6 changes. As for quality of life evaluation, there was a marked improvement in the European Organization for Research and Treatment of Cancer QLQ-C30, Euro QL-5D(VAS), and multidimensional fatigue symptom inventory-short form scores. At the end of the study, 22 of the 39 patients were "responders" or "high responders". The minimum required was 21; therefore, the treatment was effective and more importantly was shown to be safe.

d-ROMs test

Principle and Standardization

In the d-ROMs test, ROMs (Reactive Oxygen Metabolites, mainly hydroperoxides, ROOH) of a blood sample, in presence of iron (that is released from plasma proteins by an acidic buffer) are able to produce alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals, according to the Fenton's reaction. Such radicals, in turn, are able to oxidize an alkyl-substituted aromatic ammine ($A\text{-}NH_2$, that is dissolved in chromogenic mixture) thus transforming them in a pink-colored derivative ($[A\text{-}NH_2^{\cdot}]^+$), accordingly to the reactions (the first two for alkoxyl radicals and the others two for peroxyl radicals):

$$ROOH + Fe^{2+} \rightarrow RO^{\cdot} + Fe^{3+} + OH^-$$
$$RO^{\cdot} + A\text{-}NH_2 \rightarrow RO^- + [A\text{-}NH_2^{\cdot}]^+$$
$$ROOH + Fe^{3+} \rightarrow ROO^{\cdot} + Fe^{2+} + H^+$$
$$ROO^{\cdot} + A\text{-}NH_2 \rightarrow ROO^{\cdot} + [A\text{-}NH_2^{\cdot}]^+$$

Finally, this colored-derivative is photometrically quantified. Indeed, the intensity of developed color is directly proportional to the level of ROMs, according to the Lambert-Beer's law.

The d-ROMs test is based on spectrophotometer studies on increases in red colour intensity after the addition of a small quantity of human blood to a solution of N,N-diethylparaphenylendiamine (chromogen), buffered to pH 4.8. Such colouring is attributed to the formation, via oxidation, of the cation radical of the amine which formationis due to alkoxyl and peroxyl radicals. These latter derive from the reaction of the Fe^{2+} and Fe^{3+} ions released by proteins in acidic conditions as created *in vitro*.

After the experimental validation by means of the ESR, analytical performances of d-ROMs test either manually or automatically was evalauted by the biochemical and clinical viewpoints by means of spectrophotometry.

The effect of temperature on velocity of reaction (assessed as mAbs/min) in the most frequently utilized range (1-4 min) was evident so that a thermostating system (with optimum at 37°C) is required during kinetic measurements.

Definition of Unit of Measure for the d-ROMs Test

The results of d-ROMs test will be expressed as Carratelli Units (CARR U), according to the following formula:

$$CARR\ U = F \times (\delta\ Abs/min)$$

where:

- F is a correction factor with an assigned value-approximately 9000 at 37°C (according to the results obtained with the standard).
- (δ Abs/min) are the mean differences of the absorbances recorded at 1, 2 and 3 min.

The results of d-ROMs test are expressed as CARR U because of the heterogeneity of hydroperoxides which are specifically detected with this technique. Such unities can be obtained by multiply absorbance changes, as photometrically detected, for a known correction factor, i.e. the F factor which values are between 9.000 and 10.000 (according to the prescriptions of manufacturer, as assessed by means of a specific control serum). In any case, in order to have an absolute reference, it has been experimentally established that 1 CARR U corresponds to 0.08 mg of H_2O_2/dL.

Method Performances

Analytical parameters which was considered on human blood samples were the kinetic of reaction, the effect of temperature on reaction velocity, the linearity of reaction, the sensitivity of technique and its analytical imprecision, the stability of sample over the time at various temperatures and the analytical interferences.

The kinetic of reaction, the effect of temperature on velocity of reaction and the linearity between signal and concentration were manually assessed by carrying out d-ROMs on two different spectrophotometers[21]. In such conditions, by monitoring over the time the increase of absorbance at 505 nm, the reaction of d-ROMs test was shown to be linear at 37°C in the most frequently utilized measurement range (1-4 min).

The linearity between signal and concentration (assessed as mAbs/min) in the most frequently utilized measurement range (1-4 min) as assessed by increasing volume of sample or by its dilution was shown to be excellent.

Overlapping results were done by performing d-ROMs test with other and different equipments, either diluting samples, according to the manual procedure or increasing the volume of samples, according to the automatic procedure. The linearity range of d-ROMs test as assessed by automatic technique was between 50 and 500 CARR U. Therefore, for values up to 500 CARR U the dilution of sample is required[22].

The first study, in which d-ROMs test was performed according to the kinetic procedure provided encouraging results with a within run imprecision, as CV, of 2.1% (n = 20, fresh sera) and a between run imprecision of 3.1% (n = 20, frozen sera). Substantially overlapping results were found, in the same analytical conditions, by performing d-ROMs test with a different analytical instrument. Taken together these findings clearly indicate that d-ROMs test can be performed either manually or automatically, with acceptably low analytical imprecision. Heparin, was shown to be not able to influence results of d-ROMs test.

The results of d-ROMs test, when analysis is carried out repeatedly in the same subjects and in the same day they do not differ substantially, unless a factor able to induce a sudden increase of ROMs generation (e.g. a muscular effort) intervenes[23].

Normal values. In a population of healthy subjects was shown that blood ROMs levels (mainly hydroperoxides) as measured by d-ROMs test have an unimodal distribution that peaks between 250 and 300 CARR U (i.e. between 20 and 24 mg/dL H_2O_2).

Clinical Applications

Newborns, independently of the gender, was found to have significantly lower levels of ROMs than these of adults[24-26] whereas pregnancy was shown to be related to higher values of d-ROMs test compared to those of non-pregnant women.

The d-ROMs test was proven to be useful also in patients suffering from oxidative stress-related diseases. In the field of neuropsychiatry, a placebo-controlled trial demonstrated that chelant therapy with D-penicillamine is able to reduce serum levels of d-ROMs in Alzheimer's disease[27]. Patients with amyotrophic lateral sclerosis was shown to have higher d-ROMs test values compared to healthy controls[28].

The d-ROMs test has been performed also to assess oxidative stress which can be related to kidney diseases, especially in chronic renal failure and in its treatment, i.e. dialysis and kidney transplantation[29-32] and it has been proven that kidney-transplanted individuals are at high risk for oxidative stress.

d-ROMs test was proven useful in the assessing of oxidative stress in Down's syndrome[33].

	d-ROMs test	FORT test
Principle	Both are colorimetric tests based on the Fenton reaction. The principle of photometry and chemistry of transition metals is well known to the scientific community and well documented in scientific literature.	
Chromogenic substrate	Both are derivatives of phenilendiamine	
Chromogen state	Liquid	Lyophilized
Steps involved	5	5
Classification of toxicity: Harmful	Yes	No
Chromogen storage	4-8°C	At room temperature (15-30°C)
Modalities of execution	Laboratory-Not IVD certification	Point of care- Full IVD certification and CE compliance
Precision in manual use	Poor (Iamele et al., Clin Chem Lab Med 2002; 40: 673-676, 34) plus clinical report from Freiburg Hospital for Tumorbiology (Dossett A, Arends J, Hydroperoxide im venösen Blut: Vergleich von zwei photometrischen Ansätzen., 35)	Good (Dal Negro et al., It J Chest Diseases 2003; 57: 199-209) plus clinical report from Freiburg Hospital for Tumorbiology. (Dossett A, Arends J, Hydroperoxide im venösen Blut: Vergleich von zwei photometrischen Ansätzen., 35)
Validation by ESR	Documented	Documented
Certification IVD	No	Yes, CE0344
Patents	Callegari S.p.A. has taken court action against manufacturers to cancel the d-ROMs patent worldwide	Patent pending
Results expression	Arbitrary unit, referred to as Carr. U.	mmol/l of H_2O_2, official unit FORT units, conventional unit, fully documented
Analytical performances	Coefficient of variation (CV) not documented	Variability = 0,79% ± 3,47sd, well documented (Normal values a reproducibility of the major oxidative stress obtained thanks to FORM system: Dal Negro et al., It J Chest Diseases 2003; 57: 199-209)
Assessment of the normal range	Not documented/unavailable	Well documented (Normal values and reproducibility of the major oxidative stress obtained thanks to FORM system. Dal Negro et al., It J Chest Diseases 2003; 57: 199-209)
Values in newborns	Documented	Well documented (Parmigiani S et al., J Perinat Med 2003; 31 (Suppl 1): 264)
Clinical usefulness	Documented	Well documented
Comparison with other test	Documented	Well documented by the University of Modena and Padua (Giovannini et al., Biochimica Clinica 2004; 28: 200, 36)
Manufacturer of the complete system	No	Yes
Guidelines	Available worldwide	Available worldwide
Years of experience in clinical routine	More than 10	Since 1930 (therefore 76 years)
At least 20 citations on PUBMED	No	No
Appreciations/State Reimbursement	International Union of Angiology/NO	Yes. e.g. reference test for cancer wards authorized by governamental body in Italy (Cagliari Hospital) and in Germany the test is reimbursed by insurance companies

BAP Test

BAP (Biological Antioxidant Power) test is based on the capacity of a colored solution, containing a source of ferric (Fe^{3+}) ions adequately bound to a special chromogenic substrate, to a decolour when Fe^{3+} ions are reduced to ferrous ions (Fe^{2+}), as it occurs by adding a reducing/antioxidant system, i.e. a blood plasma sample.

Therefore, in the BAP test, a small quantity of blood plasma (10 µl) to be tested is dissolved in a coloured solution, which has been previously obtained by mixing a source of ferric ions (i.e. ferric chloride, $FeCl_3$) with a special chromogenic substrate (i.e. a thiocyanate derivative).

After a short incubation (5 min), at 37°C, such solution will decolor and the intensity of this chromatic change will be directly proportional to the capacity of plasma to reduce, during the incubation, ferric ions (initially responsible for the color of solution) to ferrous ions, according to these reactions:

$$FeCl_3 + AT_{(uncolored)} \rightarrow FeCl_3 - AT_{(colored)}$$
$$FeCl_3 - AT_{(colored)} + BP_{(e-)} \rightarrow FeCl_2 + AT_{(uncolored)}) + BP$$

where :

- $FeCl_3$ is ferric chloride;
- $AT_{(uncolored)}$ is a thiocyanate derivative (uncolored);
- $FeCl_3$-$AT_{(colored)}$ is the colored complex of ferric chloride with the thiocyanatederivative;
- $BP_{(e-)}$ is a molecule of blood plasma barrier with reducing/electron giving/antioxidant activity against ferric ions;
- BP is the oxidized form of $BP_{(e-)}$;
- $FeCl_2$ is the ferrous chloride obtained by the reducing activityof $BP_{(e-)}$.

By photometrically assessing the intensity of decoloration, the concentrations of reduced ferric ions can be adequately determined thus allowing a measurement of reducing capacity or antioxidant potential of tested blood plasma. Such "potential" is obviously not absolute but relative to the tested substrate, i.e. ferric ions. Considering that such ions are naturally occurring components of our body, BAP test provides a measure of antioxidant power of the fraction of plasma barrier to oxidation which is directly involved, due to the implicated reducing-oxidant potentials, against the attack of reactive chemical species in "physiological" or "biological" conditions.

Comparative Studies

BAP test versus FRAP test: According to Vassalle's study[37], BAP test provided results greatly comparable to those of FRAP test, against which it appears more quick and simple to perform. Indeeed, the preparation and management of the FRAP reagent is a step somewhat complex, compared to that of BAP test, so that manual procedure of FRAP test can increase analytical variability. In fact, acceptable levels of analytical accuracy with FRAP test were observed with automatic procedures.

Comparison FORD/BAP

Both tests are based on principles widely employed by the scientific community and for commercial applications.

FORD → TEAC principle (Trolox Equivalent Antioxidant Capacity).

BAP → FRAP principle (Ferric Reducing Ability of Plasma).

FORD	BAP
$Chromogen_{(uncolored)}$ + oxidant (Fe^{3+}) $H^+ \rightarrow Chromogen^{\bullet+}{}_{(purple)}$	$FeCl_3 + AT_{(uncoloured)} \rightarrow FeCl_3$-$AT_{(colored)}$
$Chromogen^{\bullet+}{}_{(purple)} + AOH \rightarrow Chromogen \pm_{(uncolored)} + AO$	$FeCl_3$-$AT_{(colored)} + BP_{(e-)} \rightarrow FeCl_2 + AT_{(uncoloured)} + BP$
The FORD test uses preformed stable and colored radicals and measures the decrease in absorbance that is proportional to the blood antioxidant levels of the sample according to the Lambert Beer's law.	The BAP test is based on the capacity of a coloured solution, containing a source of ferric ions adequately bound to a special chromogenic substrate, to decolour when ferric ions are reduced to ferrous ions
In the presence of an acidic buffer and a suitable oxidant the chromogen forms a stable and colored radical cation photometrically detectable	The sample is dissolved in a coloured solution, which has been previously obtained by mixing a ferric ions solution with the chromogen
Antioxidant molecules in the sample reduce the radical cation of the chromogen quenching the colour and producing decoloration of the solution which is proportional to their levels	After incubation the solution decolours and the intensity of decoloration is assessed photometrically. The cromatic change is directly proportional to the capacity of plasma to reduce ferric ions to ferrous ions

Further Information on the BAP Assay

The principle is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. Major negative aspects of this test principle are:

- Some antioxidants are able to reduce ferric ions to ferrous ions. However not all are able to do so. This means that some antioxidants will not be determined by this assay. Furthermore other substances other than antioxidants, are able to reduce the ferric ions into ferrous ions. So in summary:
- The primary known contributors to this assay are uric acid and ascorbic acid, antioxidants that reduce the ferric to ferrous ions.
- Other antioxidants that cannot reduce the ferric to ferrous ions (such as carotenoids and SH-group antioxidants) are not being measured by this assay.
- Some reductors, which are not antioxidants, reduce ferric to ferrous ions. These will cause falsely high results being obtained as these are not antioxidants (e.g. glucose, hydroperoxides, ethanol).
- Since the effects of proteins are weak, the assay practically measures non-protein total antioxidant capacity.
- The major antioxidants contributors of this assay are uric acid and ascorbic acid. However, while uric acid is a powerful antioxidant, gender and metabolic differences, as well as some pathological conditions (kidney diseases, metabolic disorders, diet and strenuous exercise) may be related to an increase of uric acid in plasma, thus introducing a possible confounding factor in the measurement of plasma antioxidant capacity. For example, the toxic effect of chronic ethanol consumption results in a paradoxical increase in plasma uric acid concentrations. The same increase in uric acid amounts can be seen in patients with a renal dysfunction. Furthermore high uric acid levels, left undetected, are dangerous to health.

Some More Specific Information on the FORD Test

The FORD test is based on the capacity of antioxidants to reduce a radical cation of the chromogen. Uric acid is not purposely measured in this assay but is measured separately to avoid misinterpretation of results. Furthermore being determined alone, operators are able to investigate high levels further and diagnose related diseases. The principal antioxidants present in plasma are measured via the FORD test (SH-group antioxidants, vitamin C, albumin and other plasmatic proteins, reduced glutathione (GSH) and bilirubin. These antioxidants (together with uric acid which is measured separately by the FORM-Plus instrument) are amongst the most relevant contributors to the antioxidant plasmatic barrier.

BAP performances (precision, repeatibility, linearity, etc) are not provided. The accuracy only is stated. The accuracy of an analytical method describes the degree of closeness of mean test results obtained by the method to the nominal (standard) or known true value. By definition, accuracy is the degree of conformity with the true values or a standard and it relates to the quality of a result. Consequently, it is insufficient to provide a range of concentrations to express the accuracy: a percentage value or BIAS (that is, the error which arises when estimating quantity) is required.

The expression of the BAP results is µmol/l of antioxidants such as vitamin C. The manufacturers do not specify which antioxidant is used for the expression but make a general statement on the expression. The reference range of plasma TAC (Total Antioxidant Capacity) changes from method to method because there is specified tar-

	FORD	BAP
Procedure type	Both automatic and manual	Only manual
Whole blood volume	50 µl	At least 100 µl
Centrifugation time	60 sec	90 sec
Reading time	4 + 2 min	5 sec + 5 min
Repeatability	CV < 5%	N/A
Precision	CV < 8.5%	N/A
Linearity range	0.25-3 mmol/l Trolox	N/A
Accuracy	BIAS < 15% in the overall linearity range	1500-3000 µM
Reference values	1.07-1.53 mmol/l Trolox	> 2200 µM (corresponding to 2.2 mmol/l)

get molecule or assay standard in literature. In some studies, Trolox, uric acid, vitamin C and ferrous ion solutions have been used for the calibration of the assay. For this reason it is important to specify the type of standard employed. However, Trolox is the most widely used traditional standard and hence Trolox was employed as the assay standard in the FORD method.

Furthermore the BAP normal values are significantly higher than values commonly reported for the most of TAC methods including the FRAP (plasma FRAP values of healthy adults reported in scientific literature are approximately 1000 μM). The FORD reference ranges are similar to the values cited in specific literature.

CE Comparison

The Directive 98/79 EC relates to medical appliances for in vitro diagnostics and their accessories. The Directive refers to the accessories as fully-fledged medical appliances for in vitro diagnostics.

The Directive describes a medical appliance for in vitro diagnostics as follows: "each medical appliance that is a reagent, a reactive product, a calibration material, a control material, a kit, an instrument, a device, an appliance or a system that is used separately or in combination and which the manufacturer intends to be used for the in vitro investigation of specimens originating from the human body, including donor blood and tissue, exclusively or principally with the objective of providing information on the physiological condition, the health, the illness or a congenital defect or for determining the safety thereof and the level of compatibility with potential receptors".

The In Vitro Diagnostics Medical Devices Directive 98/79/EC came into force on 7th December 1998 and has a transition period until 7th June 2000.

Conclusion

In conclusion, from the clinical point of view the market supplied our investigations with easy and cheap instrumentations suitable to detect the redox target by means of divalent-trivalent metals reactivity read through photometric instruments: the tests have been challenged against other more cumbersome and have been proven reliable in different pathological conditions and acute and chronic illnesses.

In vitro diagnostics 98/79 EC Requirements of the European Parliament and of the Council of 27/10/1998	**BAP**	**FORD**
CE certification	The manufacturer has the IVD (in vitro diagnostic) mark	The manufacturer has the IVD and Self Testing Certification (CE0344)
Clear IVD intended use	Only on the external cardboard box	On the external cardboard box, on the blister's labels and instructions for use as required by the directive
EN 980-European Standard for medical and IVD graphical symbols on labelling	None. The manufacturer does not comply to the CE Directive .	Graphical symbols have been applied as explicitly required by the CE Directive for the following information: • Expire date; • Manufacturer; • Temperature; • Biological symbol; • Batch number; • IVD symbol; • Catalogue number (REF); • Dispose of after use (do not reuse)

Even if these point-of-care tests represent an aspecific redox imbalance perspective their easy and quick use suggest a very wide range of potential clinical use, either in emergency, or in routine laboratory.

The main advantages of these procedures is the very small amount of blood required, and the chance to compare the results with classic lab exams, in order better qualify, by comparison, the redox profile of different diseases and to improve the therapeutic effectiveness with proper antioxidant drugs.

In this perspective, the contemporary dosage of antioxidant potential is quite necessary, even if we lack of detailed informations about the specific compound to be supplied.

In one of these measuring device (intended to testing FORD and FORT) is enclosed also the uric acid level, whose antioxidant potential has recently been emphasized; this parameter adds more informations to the clinical picture of redox-imbalance, focusing the attention of investigators, on nucleic acid metabolism, and the reflex of their tunover on general antioxidative status of the patient.

Finally, even if we know the role of the oxidative balance in some pathogenetic diseases, the point of care dedicated instruments highlight by comparison with other lab exams a potential coresponsibility and some therapeutic options.

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