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Use of *N*,*N*-dimethyl-*p*-phenylenediamine to Evaluate the Oxidative Status of Human Plasma

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Oxidative stress has been clearly implicated in human disease by a growing body of scientific evidences. There is no ideal method for the measurement of this parameter. A possible strategy would be to measure simultaneously several biomarkers representing damage to different cellular components or, alternatively, a method able to evaluate the hydroperoxides, intermediate products of oxidation originating from different classes of molecules, such as lipids, peptides, amino acids, etc. can be used.

We are introducing a simple, rapid and inexpensive assay to measure the oxidative status of human plasma. It is based on the properties of *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD), a compound able to produce a fairly long-lived radical cation. The absorbance at 505 nm of a DMPD solution in the presence of plasma, which is proportional to the amount of hydroperoxyl compounds, is related to the oxidative status of the sample and could be expressed as hydrogen peroxide equivalents (HPE). This assay was not influenced by freezing–thawing and storage time of the plasma samples. The assay can be automated, performed in a kinetic mode, and used for routine analyses. The DMPD assay alone or in combination with analytical methods for assessing antioxidant capacity is suggested as a reliable tool to obtain information in pathologies related to oxidative stress.

Keywords: Oxidative status; Hydroperoxides; Human serum; Plasma; Radical cation

Abbreviations: CV, coefficient of variation; DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine; EDTA, ethilendiamine-tetracetic acid; EPR, electroparamagnetic resonance spectroscopy; HPE, hydrogen peroxide equivalent; HPLC, high performance liquid chromatography; M, mean; ROS, reactive oxygen species; SD, standard deviation

INTRODUCTION

Free radicals are often thought to be involved in the pathogenesis of several disorders, sometimes as causes, sometimes as effects.[1–3] In particular, chronic human disease, such as atherosclerosis, diabetes, cataract, nephropathies and more, are the processes where oxidative stress could play a relevant role.[2–5]

Oxidative stress arises when the balance between oxidants and antioxidants is tipped in favour of the former.[4] This phenomenon may be influenced by exogenous agents (such as radiation trauma, drug activation, oxygen excess), but also by endogenous ones which are associated with many pathological states including chronic inflammatory disorders, cardiovascular disease, injury to the central nervous system, connective-tissue damage, etc.[5,6] The search of a technique that demonstrates and identifies free radical species applicable to human studies is of paramount importance.

Several and different approaches in literature have been described.[7–13] Despite extensive research efforts in recent years, yielding promising results in experimental models, there is still a need for additional research on the applicability of non-invasive oxidative status measure in humans. Most of the methods developed are complex and not practical for routine clinical applications or screenings.

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The clinics, in fact, strongly need an easy tool to utilise for identifying subjects with high level of oxidative stress and analysing the effects of pharmacological treatment.

In order to satisfy this necessity, we introduced the use of the *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD), a sort of "indicator" for oxidative stress. This compound has previously been used to quantify labile sulphide in proteins,[14] to detect sterol hydroperoxides,[15] and for the measure of the antioxidant activity.[16]

Alberti *et al.*[17] recently dealt on the chemistry of a similar phenylenediamine assay. In this paper, we describe a colorimetric assay that can be utilised to determine the oxidative status of plasma or serum sample. It is based on the capability of the DMPD to give a stable coloured solution when it is transformed into its radical cation ($DMPD^{\cdot+}$).[16] When plasma is added to a DMPD solution, the coloured $DMPD^{\cdot+}$ is formed. The amount of radical cation formed is related to the oxidative status of plasma and can be expressed as hydrogen peroxide equivalents (HPE).

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals, including DMPD, were purchased from Fluka (Switzerland). L-ascorbic acids were from Aldrich (Germany). All solvents (HPLC grade) were from Carlo Erba (Italy).

Instruments

Spectrometric measures were recorded using an UV–Vis. Shimadzu 2100 apparatus (Japan). The instrument was equipped with a Peltier electronic temperature control and magnetic stirring.

Sample Preparation

The peripheral blood samples, used to set up the method, were taken from 35 healthy volunteers (F/M = 20/15; median age: 32; range: 21–40 years) after a 12-h fast. Blood samples (heparin-, EDTA- or citrate-treated and not) were centrifuged at 4000 rpm for 10 min. Plasma samples obtained were stored frozen at −20°C until assayed.

The effect of storage was evaluated by measuring each plasma sample after up to one week of storage at 4°C or at −20°C with daily freezing–thawing of sample. To establish the reproducibility of the method, the same set of samples was measured for five times in five consecutive days. Anticoagulants were added to the blood at the concentration they are normally used in clinical practice.

From 2 of the 35 subjects, 4 blood samples has been taken in order to analyse potential differences in the oxidative state during the daylife.

In two of the serum sample was assessed the separate contribution of ascorbic acid, glutathione, uric acid and bilirubin by adding increasing amounts of each antioxidant (within the physiological range) to serum. Standard solutions of each antioxidant were prepared dissolving them in deionised water. Five microlitres of the antioxidant solutions at different concentrations were mixed with 20 μl of plasma and then added to the mixture containing DMPD and buffer. The concentrations tested were between 0.5 and 2 mM for glutathione (mean physiological value 3–4 mM) and between 0.05 and 2 mM for ascorbic acid (mean physiological value 0.01–0.04 mM).[18]

Measurement of the Oxidative Status

A 100-mM solution of DMPD was prepared dissolving 209 mg of DMPD dihydrochloride salt in 10 ml of deionised water. The 0.1-M DMPD solution can be stored at −20°C for more than 1 month. Its title is stable up to 12 h in an ice-bath in the dark. The assay was performed in a plastic tube adding 20 μl of DMPD (final concentration 1 mM) and 10 μl of the plasma sample to 2 ml of 0.1 M acetate buffer at pH 4.8. The mixture was incubated at 37°C for 75 min. The formation of the coloured DMPD radical cation was monitored reading the absorbance at 505 nm.

Definition of Unit of Measure for Oxidative Status

To express oxidative status of the samples analysed, 5 μl of a solution of $FeCl_2$ 2.52 mM and 5 μl of H_2O_2 at different concentrations (from 0.041 to 40.6 μM) were added to a plastic tube containing 20 μl of a PBS buffer solution. After 2 min, the content of the plastic tube was transferred to 2 ml of a 1-mM solution of DMPD in buffer acetate pH 4.8 (final Fe^{2+} concentration 6.2 μM, final H_2O_2 concentration between 0.1 and 0.0001 μM). The colour development was related to the H_2O_2 concentration and a calibration curve was built up.

Statistics

Five replicate values for each tested sample yielded a mean (M), a repeatability standard deviation (SD), and a coefficient of variation (CV = SD/M, in %). The data were statistically analysed by two-tailed *t*-test for unpaired data using a SPSS/PC + 8 statistical package (SPSS Inc. Chicago, IL).

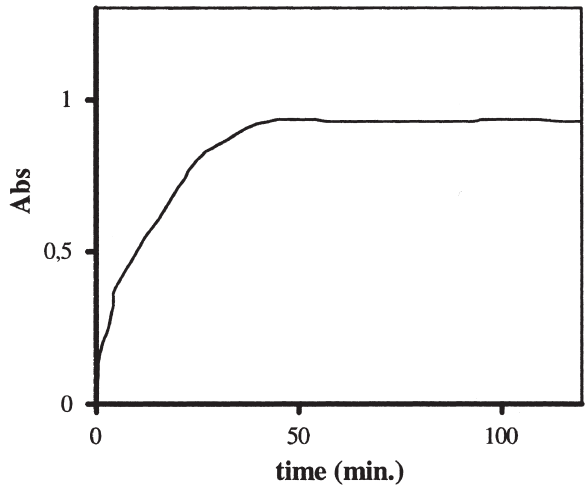

FIGURE 1 Time course of the $DMPD^{+}$ formation.

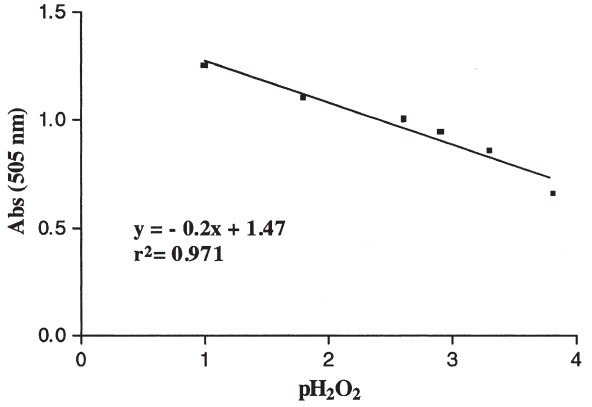

FIGURE 2 Reference curve obtained using different concentrations of H_2O_2 in the presence of 6.2 μM Fe^{2+}.

RESULTS

Standardization of Method

Hydroperoxides are fairly stable molecules under physiological conditions, but their decomposition is catalysed by transition metal ions. Both Fe^{2+} and Fe^{3+} are effective catalysts for the reaction for the degradation of those compounds.[7] In human serum, metal ions are released from the transport proteins in acidic medium and hydroperoxides readily decompose in alkoxy and peroxy radicals, which are able to convert *N*-alkylated *p*-phenylenediamines into the corresponding coloured radical cation.[17] In particular, the reactions occurring in our experimental condition are described in Scheme 1:

a) $ROOH + Fe^{2+} \rightarrow RO^{\bullet} + OH^{-} + Fe^{3+}$

b) $ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + OH^{-} + Fe^{2+} + H^{+}$

c) $ROOH + Fe^{3+} \rightarrow RO^{\bullet}\,[Fe(IV)=O]^{2+} + H^{+}$

d) $RO^{\bullet} + DMPD \rightarrow RO^{-} + DMPD^{\bullet+}$ (purple color)

e) $ROO^{\bullet} + DMPD \rightarrow ROO^{-} + DMPD^{\bullet+}$ (purple color)

As shown in Fig. 1, the reaction of the DMPD in serum sample is completed in 60 min at 37°C. After that, the value of absorbance at 505 nm remains constant for more than 30 min. Hence, a time of 75 min was selected to record the value at the endpoint of the colorimetric reaction. Also the increase in absorbance in the first 7 min is fairly linear and can be related to the oxidative status of the sample[17] and with the integrated spectral intensity of the signal obtained by EPR for the radical cation.[17]

In order to define a unit of measure for oxidative status, we decided to relate it to hydrogen peroxide concentration. A reference curve (Fig. 2) was created as described in the above section. The equation of this curve allowed us to compare the value of absorbance to the equivalent of H_2O_2 (HPE). The unit of measurement is pH_2O_2 ($-\log$ of H_2O_2 concentration, expressed in micromolarity) and its defined as the oxidative status of a sample having an absorbance equivalent to that of a 0.1 μM solution of hydrogen peroxide in analogous experimental conditions. The lower the HPE, the higher is the oxidative status.

If the absorbance value of a sample higher than 1.25 Abs units is obtained, it is recommendable to dilute the sample in water as appropriate, and then to repeat the test.

Analogously, in the automated assay (d-ROMs®), oxidative status is expressed using a conventional arbitrary unit, called CARR unit.[17] One CARR unit corresponds to the colour development caused by a H_2O_2 solution at a concentration of 0.08%.

The 0.1-M DMPD solution can be stored at −20°C for more than 1 month. Its title is stable up to 12 h in an ice-bath in the dark.

Measurements performed on samples stored at different time-lengths and temperatures demonstrated that the oxidative status of the sample had not changed either at −20°C or at 4°C up to six days. A marginal increase of the absorbance was observed at −20°C, probably owing to the freezing–thawing of samples.

The DMPD assay can also be utilised for plasma sample. The values of oxidative state obtained are slightly lower than those of serum sample (see Table I),

TABLE I Effect of anticoagulants on oxidative status in plasma. Values obtained on serum taken as 100%

Sample	Heparin (%)	EDTA (%)	Citrate (%)
1	+6	−57	−44
2	+5	−59	−44
3	+4	−68	−50
4	+7	−62	−46
5	+10	−57	−42
6	+12	−57	−42

depending on the anticoagulant chosen. Plasma obtained using EDTA or citrate always yielded lower values, whereas heparined plasma could be considered as a substitute to serum sample. On the other hand, the addition of ferrous ion to a plasma sample from 30 to 300 μM did not increase the oxidative status value.

Single antioxidants were added to plasma prior to the measurement in order to ascertain how the principal antioxidant compounds of plasma contribute to the colorimetric reaction of DMPD. Results showed that the increased concentration of ascorbic acid and reduced glutathione up to five fold their physiological level, did not influence the measurement of plasma oxidative status.

The DMPD assay proved to be highly reproducible and precise. Replicate measures on the same serum or heparined plasma sample showed a within-assay coefficient of variation (CV) less than 0.5% (HPE = 4.20 ± 0.2, $n = 8$) and a between-assay CV less than 2.9% (HPE = 4.25 ± 0.1, $n = 8$).

Measure of oxidative status on plasma sample taken from 35 apparent healthy subjects gives an HPE value of 4.1 ± 0.8.

Taking four blood samples during a day from the same subject ($n = 2$), the variation of HPE was within 10%, without any statistically significant difference.

DISCUSSION

In serum or plasma samples, the amount of hydroperoxides represents a good index of free radical attack because it is indicative of intermediate oxidative products of lipids, peptides and amino acids. Hydroperoxides, in presence of free iron traces, produce several secondary reactive radical species, which can be measured by a spectrophotometric assay.

We have introduced a handy assay to measure the oxidative status of human plasma or serum through the indirect estimate of the hydroperoxide levels. A coloured radical cation is originated from oxidation of the DMPD itself by alkoxy and peroxy radicals derived from the iron-induced decomposition of hydroperoxides.

Results demonstrate that both time and temperature of the sample storage does not influence the reaction, while all the substances with a significant absorbance at 505 nm may be a potential source of positive interference. For this reason, haemolysed samples were not utilised.

The addition of exogenous antioxidants, within the physiological range, to plasma samples before measurement has no incidence. This result is not surprising and indicates that these antioxidants are not able, in the assay condition, to shift the equilibria described in Scheme 1 (reactions d and e).

An inhibitory effect was, on the other hand, exerted by EDTA and citrate and it is related to their iron-chelating ability. This action prevents iron participation in the Fenton reaction and therefore, the formation of peroxyl and hydroxyl radicals (Scheme 1: reactions a–c).

This evidence has raised the question of the usefulness of this assay for people having low blood iron levels. Actually our data (not shown) indicate that HPE value of subjects having hyposideremic anaemia do not differ from that of healthy subjects and that the addition of iron to their plasma do not alter the measure. Therefore, we can assume that the iron amount present in anaemic subjects having low Fe^{2+} level is still sufficient to speed up the colorimetric reaction. Therefore, the limiting rate of the colorimetric reaction is only the amount of peroxyl and hydroperoxyl radicals present.

Although the assay is very reproducible for the same subject during the day and the CV, both inter and intra-assay, results are very low, the value of HPE measured on healthy subjects is highly variable (4.1 ± 0.8). This value distribution is very similar with that observed by Alberti *et al.*[17] using their method, operating in kinetic mode during the second minute of the colour development of a compound analogue to the DMPD.

A wide range of methods are currently used to assess antioxidant or oxidative capacity of plasma samples by measuring the oxidative damage, its prevention or the assessing of radical scavenging. Anyway, at present, it is still not possible to determine the absolute value of the antioxidant or oxidative potential of plasma. In fact, the measurement of these parameters is dependent on the method used and each method has a different sensitivity for the various substances present in plasma. It was recently demonstrated that there is no correlation among the absolute values of antioxidant activity obtained on the same samples using different analytical methods.[19] Moreover, most of the methods proposed in literature can test only a restricted class of compounds. In this paper, we propose an alternative method able to measure a larger class of primary oxidation products.

Although this method lacks specificity, however, it could provide a wider spectrum of analysis of free radical damage and for this reason could be a useful test, eventually coupled with other assays evaluating different parameter, to assess the oxidative state in clinical practises.

In conclusion, the DMPD method has the potential for a wide-scale use in clinical diagnosis and some ongoing trials are using this methodology. In fact, a large screening of ROS levels, together with the measure of plasma antioxidant ability, should be

considered to evaluate health status, particularly in people belonging to risk-categories such as smokers, obese, diabetics and hypertensives. In this view, the assay was automated allowing to obtain in few minutes the measure of the oxidative status.

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