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A method for simultaneous determination of plasma and erythrocyte antioxidant status. Evaluation of the antioxidant activity of vitamin E in healthy volunteers

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1 Since oxygen free radicals are directly involved in a variety of pathologies such as atherosclerosis, diabetes mellitus, inflammation and/or when a deficit of defences of the organism against radicals occurs, we developed a suitable and simple method to determine both the erythrocyte sensitivity to an oxidative stress and plasma antioxidant protective capacity.

2 This test is based on the introduction at 37° C of a radical initiator, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), within an erythrocyte suspension leading to a membrane alteration and ultimately to haemolysis. The latter can be quantified by determining the lacticodeshydrogenase activity released in the medium. The erythrocyte sensitivity to haemolysis and the volume of plasma inhibiting 50% of the haemolysis were determined.

3 Intra-assay CVs were 1.9 % for erythrocyte sensitivity to oxidative stress and 3.4% for inhibitory 50% plasma volume. Inter-assay CVs for both erythrocyte sensitivity and inhibitory 50% plasma volume were 4%.

4 The reliability of this method was assessed and applied to test the protective effect of vitamin E, a well known antioxidant agent, in six healthy volunteers. Two weeks after daily administration of 500 mg of vitamin E, the mean plasma vitamin E concentration increased by 41% from 10.7 ± 2.0 mg l^{-1} before treatment ($P < 0.05$). As the vitamin E concentration increased, the mean inhibitory 50% plasma volume and the percentage of haemolysed erythrocytes decreased respectively by 29% from 3.35 ± 0.5 µl ($P < 0.05$) and 18% from 71.5 ± 3.8% ($P < 0.05$). No significative variation of these parameters was observed in six adult men without vitamin E supplementation.

5 Thus, this global and simple test permits an antioxidant status evaluation of a patient. It can be applied to various pathologies and allows the potency of new antioxidant molecules to be evaluated.

Keywords antioxidant vitamin E haemolysis lacticodeshydrogenase oxygen radicals

Introduction

Toxicity related to oxygen free radicals is observed in a variety of physiopathological states characterized either by an overproduction of these free radicals (e.g., inflammation, anoxia-reperfusion, presence of xenobiotics, transition metals) or when a deficit of defences of the organism against radicals occurs [1]. Numerous previous studies have attempted to correlate the deficit of protective systems against radicals to several pathologies associated with the abnormal production of free radical species e.g., atherosclerosis [2], diabetes mellitus [3], oxygen toxicity [4], acquired immunodeficiency syndrome [5, 6]. However, these investigations were always made difficult because of the large number of protective systems, their possible biological interferences

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and, above all, analytical difficulties. To overcome these problems, several authors have developed different methods to measure the total antiradical potential of the organism. Requiring sophisticated equipment such as luminometers [7] or specific oxygen electrodes [8–12], most of these methods were based on measuring the plasma protective capacity [8, 9, 13] or erythrocyte sensitivity to an oxidative stress [10, 11, 14], but none was able to determine these parameters simultaneously.

In the present investigation, we describe a suitable and simple method to simultaneously determine the sensitivity of erythrocytes to an oxidative stress and the plasma antioxidant protective capacity, requiring only a routine analyser present in most clinical chemistry laboratories. Briefly, this original method is based on the introduction within an erythrocyte suspension at 37° C of a radical initiator, 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) which induces a membrane alteration and ultimately haemolysis (15). This is quantified by measuring the lacticodeshydrogenase (LDH) activity in the medium. Anti-radical elements present in the plasma can inhibit AAPH-induced haemolysis and it is possible to determine a volume of plasma that inhibits 50% of haemolysis.

The reliability of this method was tested in the present investigation and applied to the assessment of the protection provided by a vitamin antioxidant therapy in healthy volunteers.

Methods

Analytical procedures

Blood (5 ml) drawn on EDTA was immediately placed in an ice-bath and centrifuged for 5 min (1000 *g*; +4 C°). The plasma was isolated and erythrocytes washed three times in a sodium chloride solution (0.15 M). An aliquot of 200 µl of erythrocytes was then diluted to obtain a 2% globular suspension in the sodium chloride solution.

Table 1 summarizes the analytical procedure of the method. The globular suspension was gently shaken for 10 min: one sample without plasma for erythrocyte sensitivity and 5 samples with 2, 4, 5, 6, 8 µl of plasma for plasma protective capacity. 300 µl of AAPH solution (400 mM) (Interchim, Montluçon, France) in the sodium chloride solution, previously incubated in a water-bath at 37° C for 1 h, were placed in the erythrocyte suspension and maintained under mild agitation for 40 min at 37° C. A sample of the erythrocyte suspension was also maintained for 20 min at −80 C° and then reexposed to 37° C. LDH activity in all the samples was performed on an HITACHI 911 automate (Boehringer Mannheim, Mannheim; Germany) within 10 min. Each determination was duplicated.

Expression of the results

Erythrocyte sensitivity to radical agression LDH determination of the sample stored at −80° C corresponded to total haemolysis (haemolysis 100%). The erythrocyte sensitivity was given by the percentage of LDH measured in the suspension of erythrocyte with AAPH compared with the total haemolysis sample.

Plasma protective capacity For each volume of assayed plasma, the LDH activity was compared with the corresponding activity of the sample 'erythrocyte sensitivity' and expressed in percent of activity. The volume of plasma inhibiting 50% of haemolysis (inhibitory 50% plasma volume) was graphically determined.

Repeatability and reproducibility of the method

The intra-assay repeatability of the method was carried out by determining ($n=9$) the erythrocyte sensitivity and the plasma protective capacity on the same blood sample. An inter-assay was carried out by four different people working at the same time, using the same blood sample, but independently preparing the erythrocyte suspension, plasma dilution and the AAPH solution.

Validation of the method in healthy volunteers

We applied this method to evaluate the effectiveness of the well known antioxidant therapy, vitamin E, in six adult healthy volunteers (five men and one woman) given daily 500 mg of vitamin E (Toco 500, Pharma-2000, Buc, France) over 15 consecutive days. The test was performed and plasma vitamin E concentrations determined for each volunteer at day 0 and after 15 days of vitamin E administration. The test and

Table 1 Analytical procedure

	*Total haemolysis**	*Erythrocyte sensitivity*	*Plasma 2 µl*	*Plasma 4 µl*	*Plasma 5 µl*	*Plasma 6 µl*	*Plasma 8 µl*
Erythrocyte suspension (µl)	500	500	500	500	500	500	500
H_2O (µl)	500	0	0	0	0	0	0
NaCl 0.15 M (µl)	0	200	180	160	150	140	120
Plasma 1/10 (µl)	0	0	20	40	50	60	80
		10 min incubation (37° C)					
AAPH solution (µl)	0	300	300	300	300	300	300
		40 min incubation (37° C)					

* 20 min at −80° C

vitamin E determinations were also performed in the same conditions, in six adult men without vitamin E supplementation. All subjects provided informed written consent and the study was approved by the Antoine Béclère Hospital Ethics Committee.

Statistical methods

Statistical analysis were performed using the 'Stat view' statistical package. The data were expressed as means ± s.d. The non parametric paired Wilcoxon test was used to compare variation of plasma vitamin E concentration, erythrocyte sensitivity and inhibitory 50% plasma volume between D 0 and D 15. Moreover, correlation between changes in plasma vitamin E concentration and changes in erythrocyte sensitivity or changes in inhibitory 50% plasma volume was done using non-parametric Spearman test. Changes in plasma vitamin E concentration, changes in erythrocyte sensitivity and changes in inhibitory 50% plasma volume were defined as the difference between D 0 and D 15.

Results

Validation of the method

The intra-assay precision of the data are reported in Table 2. The CV was 1.9% for the erythrocyte sensitivity to oxidative stress and 3.4% for the inhibitory 50% plasma volume. Figure 1 illustrates the graphical determination of 50% inhibition of the plasma volume: mean value was usually 3.0 ± 0.1 µl.

The results of inter-assay precision obtained by four different technicians using the same sample, are reported in Table 3. In these conditions, the CV for both erythrocyte sensitivity and the inhibitory 50% plasma volume were 4%.

Table 2 Repeatability of erythrocyte sensitivity (haemolysis%) to the oxidative stress and plasma protective capacity (inhibitory volume 50% in µl)

Assay	*Haemolysis %*	*Inhibitory volume 50% (µl)*
1	62.6	3.10
2	63.8	2.95
3	63.9	2.90
4	64.0	2.90
5	63.9	2.90
6	61.8	3.15
7	60.5	3.10
8	63.1	3.05
9	62.2	3.10
Mean	62.9	3.01
s.d.	1.2	0.10
CV	1.9%	3.4%

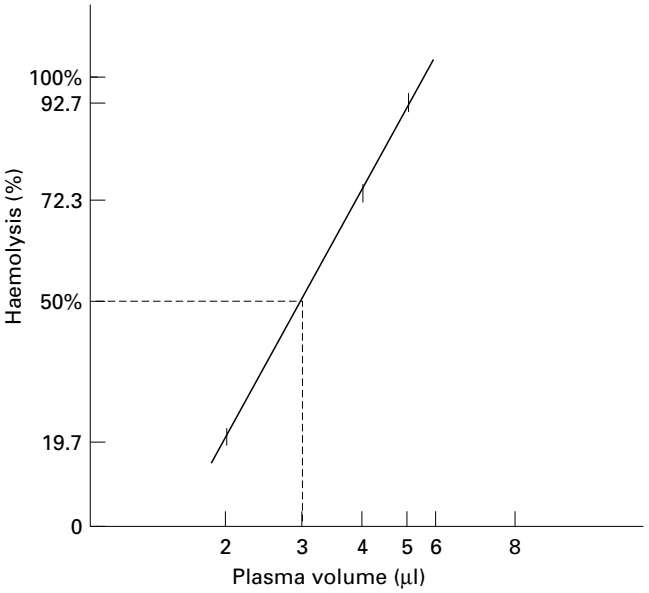

Figure 1 Graphical determination of inhibitory 50% plasma volume (semi-log scale).

Table 3 Reproducibility of erythrocyte sensitivity (haemolysis%) to the oxidative stress and plasma protective capacity (inhibitory volume 50% in µl) determined by four technicians, from the same blood sample, with their own reagents

Technician	*Haemolysis (%)*	*Inhibitor volume 50% (µl)*
1	82	3.0
2	81	3.0
3	77	3.1
4	85	2.8
Mean	81.2	2.97
s.d.	3.3	0.12
CV	4.0%	4.0%

Healthy volunteers

Fifteen days after administration of 500 mg vitamin E, the mean plasma vitamin E concentration increased by 41% from 10.7 ± 2.0 to 15.1 ± 3.9 mg l^{-1} ($P < 0.05$) in the six studied subjects. However, as illustrated in Figure 2, there were major differences between the subjects. As the vitamin E concentration increased, the mean inhibitory 50% plasma volume and the percentage of haemolysed erythrocytes decreased, respectively by 29% from 3.35 ± 0.5 µl ($P < 0.05$) and by 18% from 71.5 ± 3.8% ($P < 0.05$). Here too, important individual variations were observed between volunteers (Figure 3). Concerning patients without vitamin E supplementation, no significative variation was observed for vitamin E concentration (D 0: 9.5 ± 1.4, D 15: 10.0 ± 2.1 mg l^{-1}), erythrocyte sensitivity (D 0: 67.7 ± 7.7, D 15: 68.0 ± 9.5 %) and inhibitory 50% plasma volume (D 0: 3.17 ± 0.5, D 15: 3.24 ± 0.6 µl).

Using the non-parametric Spearman test, we showed a correlation ($P = 0.030$) between changes in plasma vitamin E concentration (see Statistical methods) and changes in erythrocyte sensitivity. Conversely, no correlation was found between changes in plasma vitamin E

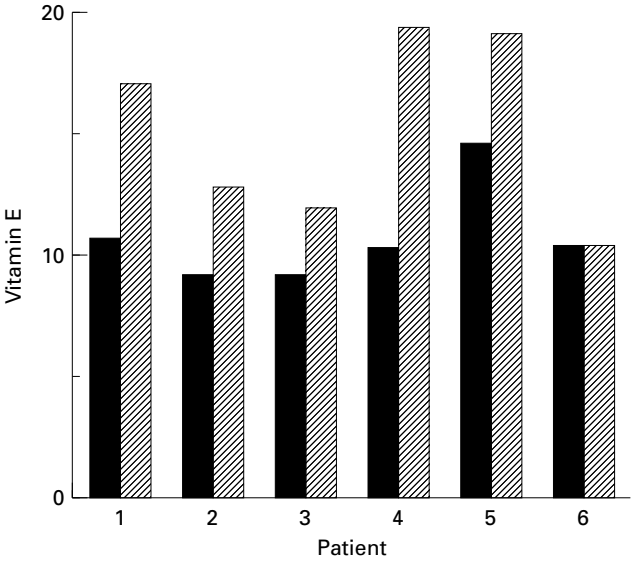

Figure 2 Individual plasma vitamin E concentrations (mg l^{-1}) before (D 0,■) and 15 days (D 15,▨) after daily administration of 500 mg of vitamin E in six healthy volunteers.

concentration and the changes in inhibitory 50% plasma volume ($P = 0.202$).

Discussion

This original method uses a single test to determine both the erythrocyte sensitivity to an oxidative stress and the plasma antioxidant protective capacity. The results demonstrate that this method is reliable and can be performed in a routine manner within 90 min in any clinical laboratory. Preliminary assays have shown that blood samples can be kept for a maximum of 8 h at $+4^{\circ}$ C before testing, without any alteration of the results. Thus, the test has to be performed the same day that the blood is collected. Considering the antioxidant capacity of the plasma, which can be quite different from one patient to another, it is important to consider that the plasma volumes required to perform the test (2 to 8 µl) allow to determine a broad range of inhibitory 50% plasma volume.

This method, using AAPH, a generator of free radicals [9, 13, 15], concerns the secondary antioxidants which directly scavenge radicals, thereby reducing the propagation phase and thus limiting the amplification of peroxidative damage (e.g., uric acid, vitamin C, vitamin E, proteins ...) [8, 9]. This generator of free radicals has been previously used in several methods using an oxygen electrode [8] or a luminometer [7] for measuring the plasma 'Total Radical Trapping Antioxidant Potential' (TRAP). TRAP is increased in preeclampsia [7] and decreased in children with cystic fibrosis [16], in acute myocardial infarction [9] and in uncontrolled insulin dependent diabetes mellitus [17]. More recently, a new free radical generator system has been proposed with incubation of a coloured radical, 2,2-azino-di-(3-ethylbenzthiazoline sulphonate) or ABTS with a peroxidase (methmyoglobin) and H_2O_2 resulting in the production of blue green radical ABTS+. The plasma protective capacity is evaluated by quantifying the decrease of the coloured radical (Total antioxidant status kit, Randox, Crumlin, U.K) [18].

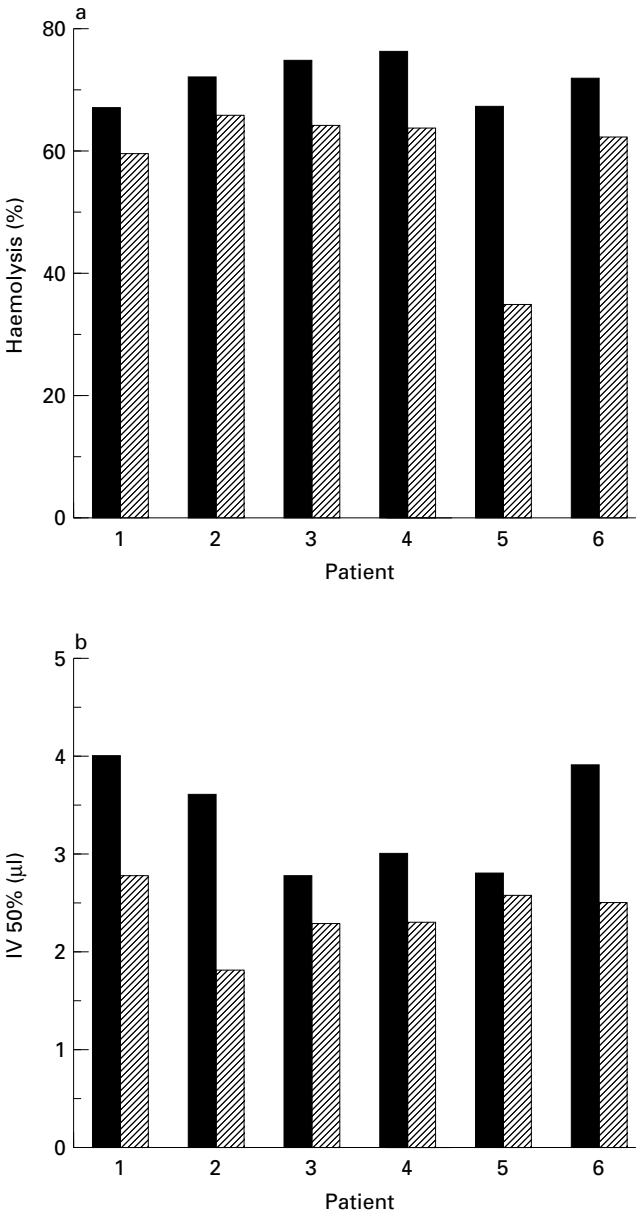

Figure 3 Individual erythrocyte sensitivity a) (haemolysis %) b) and inhibitory 50% plasma volume (IV50%) determined before (D0,■) and 15 days (D15,▨) after daily administration of 500 mg of vitamin E in six healthy volunteers.

Most of the previously published methods have been devoted to quantifying the erythrocyte sensitivity to an oxidative stress: determination of lipoperoxidation [10, 19, 20], cell deformability [14] or haemoglobin released following haemolysis [11]. In the present experimental conditions, we found that AAPH also induced some transformation of haemoglobin in methaemoglobin and therefore induced a systematic underestimation of the results. Consequently, we chose to quantify the resulting haemolysis by measuring LDH activity which, in our conditions, is not sensitive to the effect of AAPH.

With this method, we were able to demonstrate that

daily administration of vitamin E for 2 weeks significantly decreased the erythrocyte sensitivity and simultaneously increased the plasma protective capacity against oxidant agents. The rather close correlation between changes in vitamin E and erythrocyte sensitivity found in the present study can be explained by the fact that vitamin E is practically the only antioxidant of the erythrocyte membrane [21]. Conversely, there was no correlation between changes in vitamin E and plasma protecting capacity, since vitamin E is only one among numerous other antioxidants in the plasma [22]. This lack of correlation confirms the study of Mulholland & Strain [13] showing that the antioxidant activity of a molecule (ascorbic acid and alpha tocopherol) is not directly correlated to its concentration but also depends on its additive or antagonistic interactions with other antioxidant agents.

In conclusion, the proposed analytical method permits a simultaneous evaluation of the erythrocyte sensitivity to a radical oxidative stress and the capacity of the plasma to decrease it. This method is sensitive and reliable and only requires equipment for LDH determination. This global and simple test allows an evaluation of the antioxidant status of a patient. It can be applied to various pathologies and allows an evaluation of the potency of a new antioxidant molecule or drug.

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