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A Microplate-Based Colorimetric Assay of the Total Peroxyl Radical Trapping Capability of Human Plasma

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We developed a colorimetric assay estimating the radical-scavenging activity of human plasma. The test is based on a measure, in 96-well microplates at 450 nm, of the bleaching of carotenoid crocin by peroxyl radicals generated during thermal decomposition of 2,2′-azobis-(2-amidinopopane) dihydrochloride (ABAP). The inhibition of this bleaching is a function of the antioxidant power of substances added to incubation mixture. We determined the optimal conditions for a sensitive, rapid, and reproducible assay of 50% inhibitory capacity (IC_{50}) of a range of antioxidant substances and of plasma. Only a total of 200 μl of plasma is required in a complete dose–inhibition curve. The IC_{50} of normal human plasma resulted of 2.70 μl of plasma/250 μl assay volume. The total antioxidant capability (TAC) of plasma was defined as the reciprocal of IC_{50} and its value in a group of 19 healthy adults resulted in 0.369 ± 0.06. Intraassay and interassay coefficients of variation of plasma TAC were 6.13 and 4.80%, respectively. Measurement of samples with different uric acid concentration showed that antioxidant activity of uric acid accounts for approximately two-thirds of TAC.

Key Words: **antioxidant species; free radicals; uric acid; plasma antioxidant capability; vitamins; microplate methods; crocin bleaching assay.**

Plasma and other biological fluids abound in antioxidant molecules that scavenge free radical species to prevent or delay oxidative processes and the damage to important macromolecules, membrane lipids, and lipoproteins. A number of components have been shown to possess chain breaking antioxidant capability, including ascorbic acid, vitamin E, bilirubin, uric acid, and protein thiols (1). The relative importance of each of these antioxidants *in vivo* and the relative contributions of each antioxidant to total antioxidant capability (TAC)[2] of the biological fluid or tissue are still not well understood. According to some authors uric acid would account for almost all the antioxidant capacity of plasma (2), while others claimed that ascorbic acid is the most effective soluble antioxidant in humans, because it spares vitamin E and uric acid (3), or that sulfydryl groups represent the most expendable source of antioxidants in plasma (4, 5).

The antioxidant status of biological fluids can be assessed either by biochemical analysis of the levels of most relevant antioxidant species or by evaluating the inhibitory power of whole plasma on the oxidation of specific probes by free radical generating systems. While the biochemical analysis has the advantage of precise diagnostic value as regards the specific defects which may be concerned, the inhibitory capability of whole plasma may better reflect the true ability of natural antioxidants working in a complex mixture like plasma.

A number of methods have been developed over the past few years to measure the total antiradical potential of the organism. Most of these methods are based on the inhibition of the peroxidation reactions induced by thermal decomposition of azo-compounds. The reactions are assessed by a variety of techniques, such as oxygen consumption during lipid peroxidation (6), luminol-enhanced chemiluminescence (7, 8), fluorimetric measurement of R-phycoerythrin bleaching (9, 10), erythrocyte sensitivity to haemolysis (11), ferric reducing ability (12), and lipid peroxides generation (13). A further method is based on the bleaching of carotenoid crocin by alkoxyl radicals derived from a donor which is photolytically or thermally decomposed. The latter

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[2] Abbreviations used: TAC, total antioxidant capability; ABAP, 2,2′-azobis-(amidinopropane) dihydrochloride; TRAP, total radical-trapping antioxidant parameter; CV, coefficient of variation.

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method was developed as a spectrophotometric kinetic assay (14, 15) and has been used in food chemistry for assessing the antioxidant capability of complex mixtures and foods (16).

In the present investigation we took advantage of microplate-based technology to make the crocin-bleaching test suitable for a rapid and precise assay of a number of samples, requiring only a humidified incubator and a routine microplate reader, present in most clinical chemistry laboratories. We tested the method using several antioxidant substances, and thereafter this method was applied in order to assess the protection provided by human plasma. The use of a microplate-based method requires small plasma samples and enables performing multiple simultaneous and replicate assays that are necessary for the calculation of IC_{50}.

MATERIALS AND METHODS

Materials

2,2′-Azobis-(2-amidinopropane) dihydrochloride (ABAP) was from Polysciences, Inc (Warrington, PA) and was dissolved in 10 mM phosphate buffer, pH 7.4, at a concentration of 5 mg/ml just before use; saffron and all other chemicals were purchased from Sigma Chemical (St Louis, MO). Crocin was isolated from saffron by water/methanol extraction after repeated extraction with ethyl ether to eliminate possible interfering substances (14–16). Five hundred milligrams of saffron was suspended in 20 ml of ethyl ether, the mixture was gently stirred for 2 min, and then the ethyl ether extract was discarded. This procedure was repeated for a total of three times, then the residual ether was evaporated in air. Saffron was suspended in 15 ml of 30% methanol (v/v) in distilled water and stirred for 5 min at room temperature. The extract was collected and filtered through a 0.45-μm Millipore filter. The extract was diluted fivefold with 10 mM phosphate buffer, pH 7.4, and the concentration of crocin was adjusted to 25 μM with addition of the same buffer, using the coefficient ϵ_{443} = 89,000 M^{-1} cm^{-1}, reported for crocin in aqueous solution (17). Crocin was protected from direct light throughout the preparation procedure, and the stock solution was stored in 10-ml aliquots at −20°C in the dark for a maximum of 2 months.

Microplate Assay

This assay is based on the competition of a parallel reaction, where a donor of peroxyl-radicals, such as ABAP, bleaches carotenoid crocin. At the same time the antioxidant inhibits bleaching by trapping formed radicals. In the standard assay (final volume, 250 μl/well), the 96-well microtiter plates with flat-bottom wells were supplemented with 100 μl of crocin solution and 50 μl of antioxidant or plasma. The external wells of the microplates were not utilized because we sometimes noted that in those wells the reaction rates were less reproducible, probably due to small temperature differences. The plates were then brought to 37°C for 10 min. The bleaching reaction of crocin was started by the addition of 100 μl of freshly prepared and prewarmed (5 min at 37°C) ABAP (5 mg/ml, final concentration 2 mg/ml) and the reaction took place at 37°C in a humidified thermostat. Blanks with crocin in the absence of ABAP (substituted by 100 μl of 10 mM phosphate buffer, pH 7.4) were run to rule out interference by small changes of absorbance due to the spontaneous decay of crocin absorbance (<2%) in the absence of the free radical donor during assay. The plates were read at time intervals with a microplate reader (Reader 400, SLT Labs Instruments) at 450 nm, and the absolute value of the difference in the o.d. of ABAP-free blank minus the o.d. of samples containing ABAP was taken as the value of crocin bleaching (Δo.d.).

The antioxidant activity of the test samples was calculated on the basis of the percentage of inhibition of crocin bleaching according to the formula

$$\% \text{ inhibition} = \frac{\Delta\text{o.d. total (no antioxidants)} - \Delta\text{o.d. in the presence of sample}}{\Delta\text{o.d. total (no antioxidants)}} \times 100.$$

This value defines the response of the system and is proportional to the antioxidant capability of the sample. The antioxidant power of the sample was characterized as the dose of the substance (or the volume of plasma) which caused a 50% inhibition of crocin bleaching (IC_{50}). Using plasma as the test sample, we found it useful to express the TAC as the reciprocal of IC_{50} ($TAC = 1/IC_{50}$) because by this way the TAC value is directly proportional (and not inversely proportional as in the case when IC_{50} is used) to the actual antioxidant power of the test sample.

Blood Samples

Whole blood was collected after an overnight fast between 8.00 and 10.00 h in K_2 EDTA tubes[3] for biochemical investigations and immediately centrifuged (3000g × 10 min) to obtain plasma. For TAC measurement, plasma was used immediately or stored at

[3] Blood can be collected also in heparin tubes (10–20 international units/ml blood). We have recently observed that TAC of heparin-anticoagulated blood is about 30% higher than TAC of K_2 EDTA-anticoagulated blood. Other results are not affected by the anticoagulant used.

−80°C until analysis. Preliminary experiments showed that this procedure had no effect on TAC when compared with fresh plasma, at least within 1 month. Plasma was serially diluted in physiological saline to achieve the desired final dose, before adding it to the assay mixture. A total of 200 μl of each plasma sample was used for preparing serial dilutions necessary to determine TAC by a complete dose–inhibition curve, performed in duplicate. We noted that the best precision of the assay was achieved when 10 plasma dilutions (corresponding to final volumes of 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 10 μl of plasma) were distributed in the plate using the rows from B to G of the microplate and utilizing row H for blanks. In this way, the TAC of three plasma samples can be assayed in duplicate in a single microplate.

The control, healthy individuals were non-smoker volunteers aged 21–43 years. They weren't on any medication, including vitamin supplements, 10 days prior to the experiment. Informed consent to participate in the study was obtained after a clear explanation of its experimental nature. Plasma samples of hypouricemic and hyperuricemic patients were taken from samples in the hospital laboratory and utilized immediately after the determination of requested analytical parameters. The only variable considered for inclusion in the study was uricemia.

Uric acid assay was performed by an autoanalyzer DAX 96 (Bayer S.p.A.), using an enzymatic method based on the specific uricase-catalyzed oxidation of uric acid to allantoin (18). This reaction was monitored indirectly by coupling a Tynder reaction (19).

The intraassay repeatability of the method was carried out by determining (n = 6) TAC on the same plasma, and an interassay was carried out by four different people working at the same time, using the same sample, but independently preparing the plasma dilution and the reagents.

Statistical Methods

Statistical analysis was performed using the Sigma Plot for Windows (Jandel Scientific Corporation) statistical package. The data were expressed as means ± SD. IC_{50} was determined by regression analysis of dose-dependence inhibition curves and by using the regression results (x) at $f(x) = 50$ from the curve which best plotted the experimental data.

RESULTS

Validation of the Method and Application to Standard Antioxidants

Figure 1 reports the absorption spectrum of the crocin extract, in the absence and in the presence of ABAP. The spectrum showed an absorbance peak at 440–443 nm and a shoulder at 464 nm, exactly as reported for pure crocin (16, 17). A peak of optical absorption was present also in the UV region of the spectrum, indicating that the extract contains components other than crocin. However, Fig. 1 shows that these contaminants did not interfere with the measurement of the absorption peak of crocin, which was almost totally quenched after addition of ABAP.

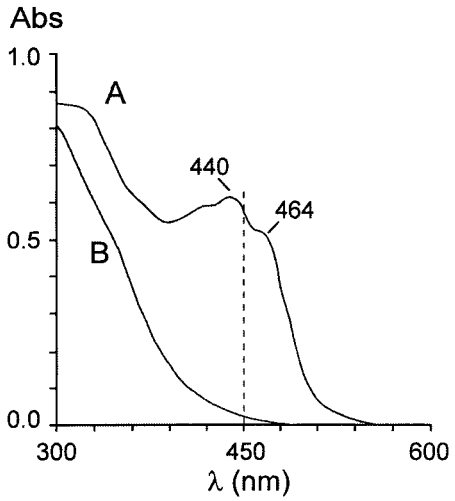

FIG. 1. Spectral changes associated with oxidation of crocin by the free radical initiator ABAP. Absorption spectra were recorded by a double-beam spectrophotometer (Kontron Uvikon 860) using phosphate buffer in the reference cuvette. (A) Crocin (7 μM) in 10 mM phosphate buffer, pH 7.4, in the absence of ABAP. (B) Spectra taken after incubation of both sample and reference solutions with 2 mg/ml ABAP for 60 min at 37°C.

Microplate readers, working with filters, usually read at 450 nm and not at 440 nm, but from Fig. 1 it can be seen that the absorption of crocin at 450 nm is only 6% lower than the maximum, indicating that microplate reading is suitable for sensitive assays. Using microplate reading as described under Materials and Methods, a linear relationship between crocin concentration and absorbance at 450 nm was obtained in the range between 1 and 20 μM crocin ($y = 0.0963x$, $r^2 = 0.99$) (data not shown). Figure 2 shows the kinetics of crocin bleaching obtained by thermal decomposition of ABAP at 37°C. After a slight initial delay, a linear decrease in absorbance was observed, independent of the initial concentration, until 0.1–0.2 o.d. was reached, i.e., until the sufficient amount of crocin was available. On the basis of these results, an initial concentration of crocin of 10 μM (optimal range: 8 to 12 μM) was chosen as the most suitable for sensitive and reproducible assays.

We determined the antioxidant IC_{50} of six well-known scavenger substances, namely, ascorbic acid, a major soluble defence against free radicals in body fluids (3), Trolox C, a water-soluble analogue of vitamin E (1); propylgallate and chlorpromazine, two syn-

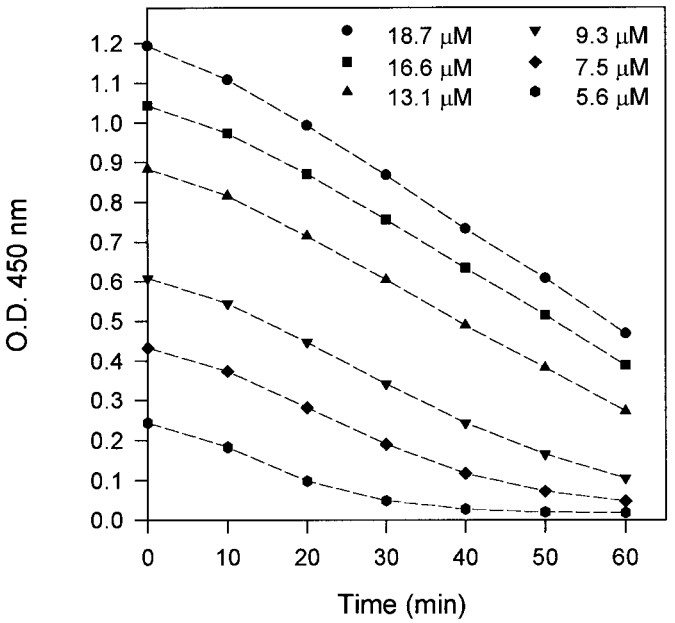

FIG. 2. Decrease of absorbance of various solutions of crocin over 1 h after induction with 2 mg/ml ABAP. The concentrations of crocin in the assay mixture are also reported.

thetic antioxidants which showed high scavenging rate constant with hydroxyl radicals in previous reports (14); and hydroquinone and uric acid, two natural antioxidant compounds, the latter of which is probably the most important antioxidant molecule of plasma in healthy subjects (1, 2). These data (Fig. 3) showed that ascorbic acid is the most powerful of the tested compounds, with an IC_{50} of 0.7 μg/ml, followed by propylgallate (0.85 μg/ml), uric acid (1.37 μg/ml), and Trolox C (1.38 μg/ml). Chlorpromazine and hydroquinone showed an IC_{50} one order of magnitude higher than that of the four previously mentioned substances.

TAC of Human Plasma

Figure 4 illustrates the dependence of the inhibition of crocin bleaching on the volume of plasma, in a population of 19 healthy subjects. The test appears to be very sensitive, because almost complete inhibition was achieved by 10 μl of plasma and IC_{50} was 2.70 $\pm$ 0.49. The TAC of normal human plasma (expressed as the reciprocal of IC_{50}) resulted in 0.37 $\pm$ 0.06.

The intraassay precision of the test was determined by repeating the measurement of the same plasma sample six times, utilizing two plates simultaneously by the same technician. In these conditions, the coefficient of variation (CV) of TAC resulted in 6.1%. The interassay precision was determined by four different technicians using the same sample: CV of TAC resulted in 4.8 %.

Since it has been suggested that the major component of antioxidant power of plasma is uric acid (2), we assessed TAC in subjects with different uricemia values. As shown in Fig. 5, TAC was correlated with uric acid concentration, but the line of correlation corresponding to zero uric acid crossed the TAC value around 0.12. Since the normal value of TAC is 0.369, this indicates that most antioxidant activity is due to uric acid, but a significant part—approximately one third—is due to other mechanisms.

One aspect which might be of particular importance is the potential interference of bilirubin with the assay, because this substance absorbs at 450 nm. However, in this assay system plasma is markedly diluted and bilirubin absorbance becomes negligible in patients with plasma bilirubin <3 mg/dl. In fact, the absorbance reading at time 0 of 10 μl of a plasma sample (maximum dose used in determination of IC_{50}) containing 3.5 mg/dl of total bilirubin resulted in only 0.045 o.d. units (normal values, 0.015 $\pm$ 0.014 o.d. units).

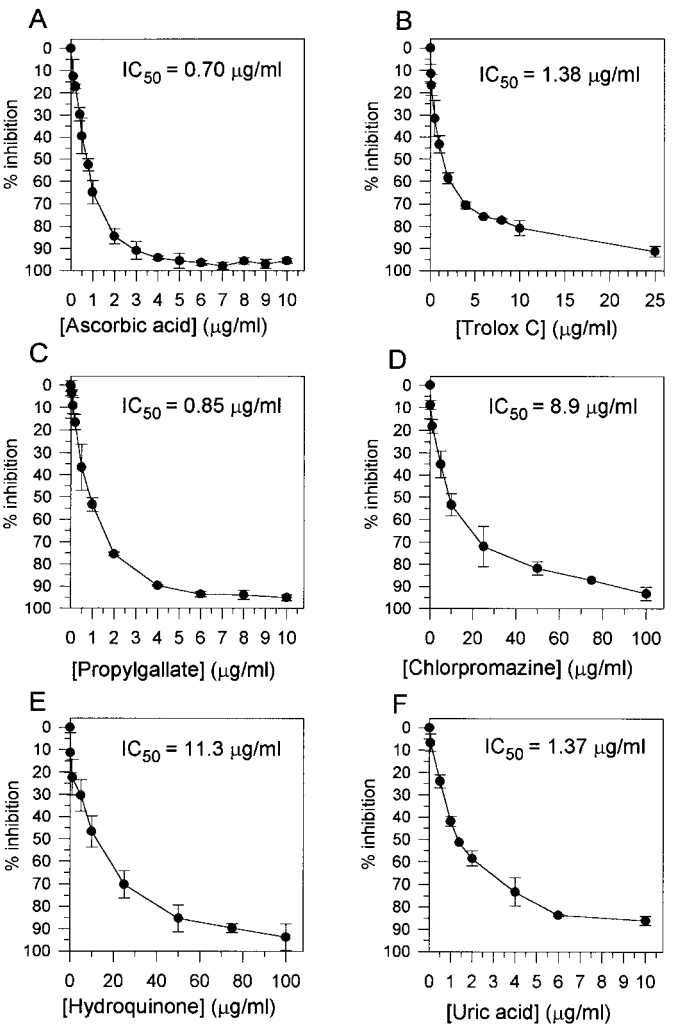

FIG. 3. Dose–inibition curves of six antioxidant compounds.

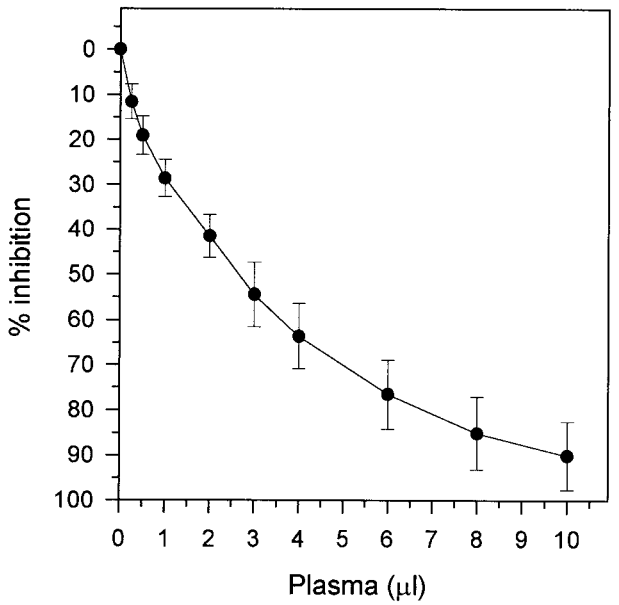

FIG. 4. Dose–inhibition curve of human plasma. The values represent the average ± SD of separate determinations from 19 healthy subjects.

DISCUSSION

The relative insufficiency of antioxidant defences is critical to the development of oxidative stress in patients affected by a number of diseases, including HIV infection (20, 21), neurodegeneration (22), diabetes mellitus (5, 23–25), angina (26, 27), preterm births (28), and probably in aging (29, 30). These diseases are characterized either by an overproduction of free radicals or are established when a deficit of defences of the organism against radicals occurs. The primary defence against oxidative stress in extracellular fluids rests with low-molecular-weight antioxidant molecules, including ascorbic acid, vitamin E, uric acid, thiols, and bilirubin (31). The levels of these antioxidants are suitable not only as a protection against oxidation but may also reflect consumption during acute oxidative processes.

Methods that have been developed for the measurement of the antioxidant power of fluids are all essentially inhibition methods. A free radical species is generated, there is an end point by which the presence of the radical is detected, and the scavenging activity of the added sample inhibits the end point. Methods vary greatly as to the radical which is generated and the end point that is used. Wayner *et al.* (1, 6) developed a test to measure plasma antioxidant activity, which has become one of the most widely used. These authors introduced the acronym TRAP, meaning total radical-trapping antioxidant parameter (TRAP). Their test is based on the measure of oxygen consumption during thermal decomposition of ABAP, a water-soluble azo compound which yields peroxyl radicals at a known and constant rate. A major problem with the original TRAP assay method lies in the oxygen electrode end point, which is too lengthy (up to 2 h per sample) to permit analysis of large numbers and/or multiple doses of samples. The TRAP assay was modified by other authors with respect to the end point (5, 7, 8, 10) or the source of peroxyl radicals (11). The existence of many different methods indicates that all of them have potential advantages and shortcomings, and a series of reports dealing with these methodological problems can be found elsewhere (6, 28, 29, 32, 33).

In the present study the TAC plasma was measured by the inhibition of crocin bleaching, which was adapted to microplate assay. We set up a method based on the following criteria: (a) by utilizing a known and previously validated free radical generating system (ABAP) (5, 7) and end point (crocin bleaching) (14–16), (b) by utilizing colorimetric measurement of sufficient sensitivity in order to use small plasma samples, and (c) by utilizing flat-bottom 96-well microplates, which are widely used in all laboratories and allow simultaneous assay of several samples. The latter point is particularly important because, due to the complex competition reactions occurring in the assay system, a precise determination of antioxidant activity requires a dose–inhibition curve and determination of IC_{50}, a step that is very time-consuming using either polarometric measurement of oxygen or conventional spectrophotometry and fluorimetry. This time-limiting step has until now prevented large-scale clinical application of TAC assay.

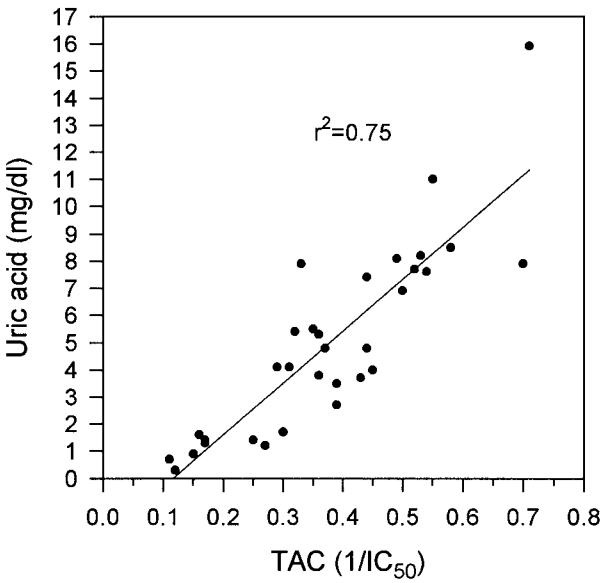

FIG. 5. Correlation between TAC and uric acid concentration in plasma samples recruited on the basis of their different uric acid concentrations.

It is worth noting that using this procedure a complete dose–inhibition curve with 10 points in duplicate assays can be performed using a total of 200 μl of plasma, taking into account also the serial dilution procedures.

Pure crocin is not commercially available and complete purification is very time-consuming (17). In these assays, we used a crude extract of crocin from saffron after extensive ethyl ether washing for the following reasons: (a) the absorption peak of crocin is at 440–443 nm, a wavelength where other components present in the mixture do not interfere with optical measurement; (b) as shown in Figs. 1 and 2, the quenching of crocin by free radicals generated during thermal decomposition of ABAP is complete and follows an almost linear rate in the time interval chosen for the measure, indicating that possible contaminants do not inhibit the crocin oxidation; (c) several purification steps reduce the yield and may cause loss of crocin (due to the well-known lability of carotenoid pigments) without significant advantage in the test procedure; and (d) crude methanol extracts of crocin have been successfully employed by others for the colorimetric determination of antioxidants by competition kinetics (14–16).

One aspect which might be of particular importance is the hydro- or lipophilicity of the substances under investigation. Our method is applicable only to water-soluble antioxidants, because testing of strongly hydrophobic compounds would require different incubation conditions and/or addition of detergents, which would probably change the competition reactions.

Another problem with the use of competition methods is the standardization in different laboratories and the comparison of results obtained using different experimental conditions and end points. This problem may be partially solved using internal standards produced by known amounts of a reference antioxidant (usually Trolox C) (2, 8, 16, 18, 28). In our assay system, the normal value of IC_{50} is 2.7 μl of plasma/250 μl of test volume, which corresponds to 1.38 μg/ml (= 0.345 μg/250 μl of test volume) of Trolox C (Fig. 3). On these bases, it can be calculated that the normal antioxidant capacity of 1 L of plasma, assayed by our method, is $0.345/2.7 \times 10^6 = 127.7$ mg of Trolox equivalents.

As with other methods based on the same principle, our test does not allow identification of a specific defect in antioxidants in clinical settings, but has to be regarded as a screening assay of biologically protective substances of plasma. In any case, the potential advantages of measuring total antioxidant activity are the following: (a) The total antioxidant activity is due to the activity of several natural antioxidants (6, 25), and therefore measurement of all these compounds is time-consuming; (b) many antioxidants may be as yet undiscovered (8); (c) the total activity may be greater than the sum of the activities of individual antioxidants because of cooperative interactions and reciprocal regeneration (10, 19, 25); and (d) other factors present in serum (lipoproteins, iron, and polyols) may influence the free-radical-scavenging activity of antioxidants. For example, it has been demonstrated that when abnormalities of metal chelation exist, ascorbic acid reducing properties paradoxically transform it into a prooxidant (34).

Good correlation with uric acid (Fig. 5) showed that hypo- and hyperuricemia are respectively associated with a decrease and increase of TAC and that patients with almost no uric acid in their blood have only one-third of normal TAC. This value is in agreement with the measurements obtained by Wayner and co-workers (6), according to whom uric acid accounts for 35–65% of TRAP and with a recent report where uric acid has been shown, by a different method, to contribute to approximately two-thirds of the total antioxidant activity of normal human plasma (24). The concentration of uric acid of plasma in men is 25–80 μg/ml and 15–60 μg/ml in women (35), a concentration that is in the same order of magnitude of its IC_{50} of pure substance determined with this method (Fig. 3). In fact, given that 1.37 μg/ml in an assay volume of 250 μl corresponds to 0.342 μg and that this amount has the same inhibitory capacity of 2.7 μl of normal human plasma, we have the figure of $0.345/2.7 \times 1000 = 126$ μg /ml as the IC_{50} of uric acid if all the assay volume was plasma. On the other hand, as we have shown, a substantial portion of TAC depends on other molecular mechanisms, whose relative relevance has not been studied in this work.

Thus, this global and simple test permits an antioxidant status evaluation in very small plasma samples. It can be applied to various pathologies and allows comparison with reference compounds or new antioxidant drugs to be evaluated by dose–inhibition curves.

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