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A novel automated method to measure total antioxidant response against potent free radical reactions

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Abstract

Objectives: Oxidative damage of biomolecules occurs as a result of potent free radical reactions. In this study, a novel, colorimetric and fully automated method for measuring total antioxidant response (TAR) against potent free radical reactions is described.

Design and methods: Potent free radical reactions were initiated with the production of hydroxyl radical ($OH^{\cdot}$) via Fenton reaction, and the rate of the reactions was monitored by following the absorbance of colored dianisidyl radicals. *Ortho*-dianisidine (10 mM) and ferrous ammonium sulfate (45 μM) were dissolved in KCl/HCl solution (75 mM, pH 1.8). This mixture was named as Reagent 1 and hydrogen peroxide solution (7.5 mM) as Reagent 2. The $OH^{\cdot}$, produced by mixing of R1 and R2, oxidized *o*-dianisidine molecules into dianisidyl radicals, leading to a bright yellow-brown color development within seconds. Antioxidants, present in the sample, suppressed the color formation to a degree that is proportional to their concentrations. The method was applied to an automated analyzer and analytical performance characteristics of the assay were determined.

Results: Vitamin C and Trolox, reduced glutathione, bilirubin, uric acid and (±)-catechin solutions suppressed the color formation depending on their concentrations. Serum TAR against potent free radical reactions was lower in patients with chronic renal failure (1.13 ± 0.21 mmol Trolox equiv./l) and was higher in the individuals with neonatal icterus (2.82 ± 1.18 mmol Trolox equiv./l) than in healthy subjects (1.54 ± 0.15 mmol Trolox equiv./l).

Conclusions: The easy, inexpensive and fully automated method described can be used to measure TAR of samples against potent free radical reactions.

Keywords: Antioxidant; Automated measurement; *Ortho*-dianisidine; Fenton reaction; Free radicals; Oxidative stress; Total antioxidant capacity

Introduction

Reactive oxygen species (ROS) are produced in metabolic and physiological processes, and harmful oxidative reactions may occur in organisms, which remove them via enzymatic and non-enzymatic antioxidative mechanisms. Under some conditions, the increase in oxidants and decrease in antioxidants cannot be prevented, and the oxidative/antioxidative balance shifts toward the oxidative status. Consequently, oxidative stress, which has been implicated in over 100 disorders, develops [1].

Hydroxyl radical ($OH^{\cdot}$) and its subsequent radicals are the most harmful ROS and they are mainly responsible for the oxidative injury of biomolecules. Alone hydrogen peroxide and superoxide molecules cannot directly oxidize lipids, nucleic acids and sugars. These species can lead to oxidative injury in the biomolecules indirectly by producing $OH^{\cdot}$ via Fenton reaction and/or iron-catalyzed Haber–Weiss reaction [2]. Oxidized molecules generally form new radicals leading to radical chain reactions or they are neutralized by antioxidants.

Antioxidant molecules prevent and/or inhibit these harmful reactions [3]. Serum (or plasma) concentrations of different antioxidants can be measured in laboratories separately, but the measurements are time-consuming, labor-intensive, costly and they require complicated techniques. Because the measurement of different antioxidant molecules separately, is not practical and antioxidant effects of them are additive, total antioxidant response (TAR) of a sample is measured and this is named as total antioxidant capacity [4], total antioxidant activity [5], total antioxidant power [6,7], total antioxidant status [8], TAR or their other synonyms.

Various methods have been developed to measure total antioxidant activity [4–13] and none of them is an ideal

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reference method. In general, a radical is generated in the assay, and the antioxidant response of the sample against the radical is measured. However, the oxidative potentials of the generated radicals [4,9,10] and the used Fe^{3+}–TPTZ complex [6,7] are generally weaker than those of $OH^{\bullet}$ and its subsequent radicals, which occur in biological reactions.

Recently, Koracevic et al. [5] developed a manual measurement method in which $OH^{\bullet}$ is generated via Fenton reaction. In this method, a standardized solution of Fe–EDTA complex reacts with H_2O_2 by a Fenton reaction, leading to the formation of $OH^{\bullet}$. These ROS degrade benzoate, resulting in the release of thiobarbituric acid reactive substances (TBARS). Antioxidants present in the added sample cause suppression of the production of TBARS. However, in this method, vitamin C and bilirubin, two of the most important antioxidants, are also degraded to TBARS. Any substance the oxidized product of which is degraded to TBARS cannot be measured by this method.

In the novel method, *o*-dianisidine was used instead of benzoate and the suppression of oxidation reaction by the sample was monitored by following the change of absorbance of the dianisidyl radical instead of the measurement of TBARS which are released from the oxidized benzoate. In this way, the steps in the process were decreased, the assay period was shortened, the requirement of boiling of sample was eliminated, and fully automated measurement was easily performed by using an automated analyzer.

Methods

Chemicals

Ferrous ammonium sulfate, *ortho*-dianisidine dihydrochloride (3-3′-dimethoxybenzidine), vitamin C (L(+) ascorbic acid), bilirubin, uric acid, reduced glutathione (GSH), (±)-catechin, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), potassium persulfate, glucose, ribose, saccharose and sodium citrate were purchased from Sigma Co. and Merck Co. The water-soluble analogue of vitamin E (Trolox; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Sigma-Aldrich Chemical Co. All chemicals were ultra pure grade and type I reagent grade deionized water was used.

Samples

The novel measurement method can be performed on a wide range of complex biological fluids, including serum, plasma, semen plasma, cerebrospinal, pleural and ascites fluids, urine, simple and heterogeneous solutions of pure antioxidants, beverages and fruit juices.

Serum samples. Blood samples of healthy subjects were supplied from the individuals who were admitted to our check-up policlinic and blood bank, the individuals with neonatal icterus were recruited from the children's hospital, and the haemodialyzed patients with chronic renal failure were recruited from the haemodialyses center. They were informed. Venous blood samples obtained were collected into tubes (and heparinized tubes) and serum (and plasma) was separated from cells by centrifugation at 1500 × *g* for 10 min. Serum (and plasma) samples were run immediately or stored at −80 °C.

Antioxidants

Stock solutions (1.0 mM) of ascorbic acid, glutathione and (±)-catechin were separately prepared in saline solution (0.9% NaCl). Uric acid and solid bilirubin were dissolved in 10 mM NaOH solution. Trolox was dissolved in phosphate buffer (10 mM, pH 7.4).

Apparatus

A Cecil 3000 spectrophotometer with a temperature-controlled cuvette holder (Cecil), and an Aeroset automated analyzer (Abbott).

Assay principle of the novel method

A standardized solution of Fe^{2+}–*o*-dianisidine complex reacts with a standardized solution of hydrogen peroxide by a Fenton-type reaction, producing $OH^{\bullet}$. These potent ROS oxidize the reduced colorless *o*-dianisidine molecules to yellow-brown colored dianisidyl radicals at low pH. The oxidation reactions progress among dianisidyl radicals and further oxidation reactions develop. The color formation is increased with further oxidation reactions. Antioxidants in the sample suppress the oxidation reactions and color formation. This reaction can be monitored by spectrophotometry.

The assay principle is schematized as follows:

Reaction baseline measurement. R1 (Fe^{2+}–*o*-dianisidine + *o*-dianisidine) + R2 (H_2O_2) → Fe^{3+}–*o*-dianisidine + $OH^{\bullet}$ + *o*-dianisidine → $OH^{\bullet}$ + *o*-dianisidine → dianisidyl radical (dianisidyl$^{\bullet}$) (bright yellow-brown color development) dianisidyl$^{\bullet}$ + dianisidyl$^{\bullet}$ → further oxidation reactions of dianisidyl$^{\bullet}$ and formation of mega complex molecules (darkening of the color).

Sample measurement. Sample (reduced form) + R1 (Fe^{2+}–*o*-dianisidine + *o*-dianisidine) + R2 (H_2O_2) → Fe^{3+}–*o*-dianisidine + $OH^{\bullet}$ + *o*-dianisidine + sample → dianisidyl$^{\bullet}$ + dianisidyl$^{\bullet}$ + (oxidation of) sample (inhibition of further oxidation reactions of dianisidyl$^{\bullet}$ and mega complex formation).

Assay calibration. The suppression of the color formation is calibrated with Trolox, which is widely used as a traditional standard for TAR measurement assays, so the results in this assay are expressed as in terms of millimolar Trolox equivalent per liter.

Assay reagents

Reagent 1

Clark and Lubs solution (75 mM, pH 1.8) was prepared as follows: 5.591 g of KCl was dissolved in 1000 ml of deionized water (final concentration, 75 mM). Reagent grade hydrochloric acid (36.5%, 6.41 ml) was diluted to 1000 ml with deionized water (final concentration, 75 mM). The prepared KCl solution (800 ml) was mixed with 200 ml of HCl solution under pH meter (final pH, 1.8). *Ortho*-dianisidine dihydrochloride (3.17 g) was dissolved in this solution (final concentration, 10.0 mM), and then 0.01764 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was dissolved in this solution (final concentration, 45 μM). (WARNING: *o*-dianisidine is a proven carcinogenic and toxic substance. Ensure that gloves and face mask are worn when handling this substance.). The prepared reagent was used as Reagent 1 in the novel assay. This reagent is stable for at least 6 months at 4°C.

Reagent 2

Hydrogen peroxide solution (7.5 mM) was prepared as follows: 0.641 ml commercial H_2O_2 solution (35%, Merck) was diluted to 1000 ml with the Clark and Lubs solution. The concentration of hydrogen peroxide was confirmed spectrophotometrically by absorption at 240 nm. This solution is stable for at least 1 month at 4°C.

Automated measurement

After manual spectrophotometric optimization processes, the method was applied to an automated analyzer, Aeroset. The assay format of the test is below.

Reagent 1 volume	200 μl [Reagent 1: *o*-dianisidine (10 mM), ferrous ion (45 μM) in the Clark and Lubs solution (75 mM, pH 1.8)]
Sample volume	5 μl (serum or other fluids, pure or complex antioxidant solutions)
Reagent 2 volume	10 μl [Reagent 2: H_2O_2 (7.5 mM) in the Clark and Lubs solution]
Wavelength	444 nm
Reading point	End point measurement. The first absorbance is taken before the mixing of R1 and R2 (as sample blank) and the last absorbance is taken when the reaction trace draws a plateau line (about 3–4 min after the mixing)
Calibration type	Linear

Measurement of total free sulfhydryl groups of serum samples

Free sulfhydryl groups of serum samples were assayed according to the method of Ellman [14] as modified by Hu et al. [15]. Briefly, 1 ml of buffer containing 0.1 M Tris, 10 mM EDTA, pH 8.2, and 50 μl serum was added to cuvettes, followed by 50 μl 10 mM DTNB in methanol. Blanks were run for each sample as a test, but there was no DTNB in the methanol. Following incubation for 15 min at room temperature, sample absorbance was read at 412 nm on a Cecil 3000 spectrophotometer. Sample and reagent blanks were subtracted. The concentration of sulfhydryl groups was calculated using reduced glutathione as free sulfhydryl group standard and the result was expressed as millimolars.

Measurements of albumin and total protein concentrations of serum samples

Serum albumin and total protein concentrations were measured by commercially available kits (Abbott) using an automated analyzer Aeroset (Abbott).

The improved ABTS radical cation decolorization assay

An amount of 0.384 g of ABTS was dissolved in 100 ml deionized water to make a concentration of 7 mM. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study of samples, $ABTS^{\bullet+}$ stock solution was diluted with the phosphate buffered saline (5 mM, pH 7.4) to an absorbance of 0.70 at 734 nm. After addition of 1.0 ml of diluted $ABTS^{+}$ to 10 μl of sample, the absorbance reading was taken 6 min after initial mixing [9]. Decolorization of the assay was linear with the increasing concentrations of Trolox.

Statistical analyses

ANOVA, correlation analyses and linear regression analyses were performed by using SPSS for Windows, Release 9.5 computer program (SPSS Inc.).

Results

Optimization studies

Type, concentration and pH of the buffer solution to be used in the assay

Ortho-dianisidine dihydrochloride was dissolved incompletely in acetate buffer at high pH value (pH 5.8), but it was dissolved completely at low pH value (pH 3.6). However, an amount of ferrous ions included by R1 was oxidized when serum was added to the Reagent 1 before addition of hydrogen peroxide, and hence the color formation rate was found to be lower than the expected value when acetate buffer was used. Using of glycine buffer at pH 2 prevented oxidation of ferrous ions, but pure glucose, ribose and saccharose solutions interfered the assay when the glycine buffer was used.

All the unfavorable characteristics mentioned were eliminated using Clark and Lubs solution pH 1.8. The ferrous

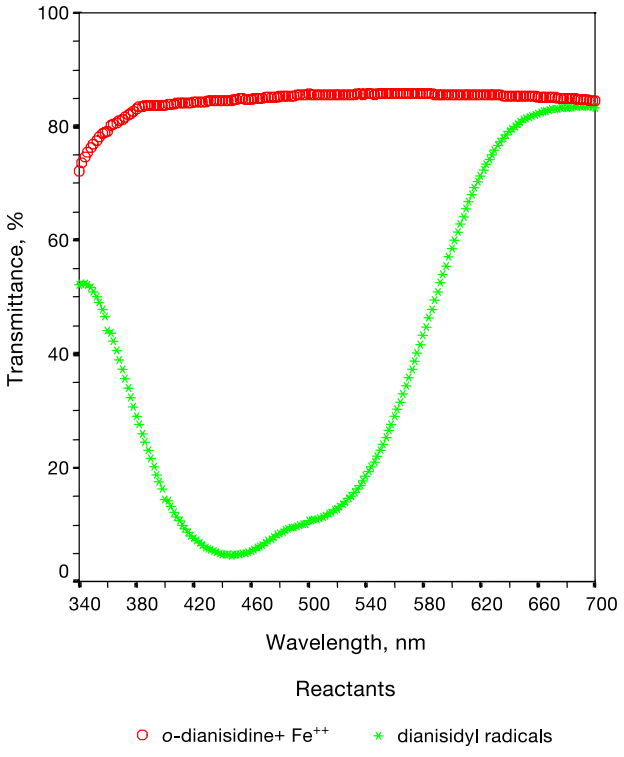

Fig. 1. Spectral transmittance curves of the reagent (*o*-dianisidine plus Fe^{2+}) and oxized *o*-dianisidine products (dianisidyl radicals and mega complexes).

ions and *o*-dianisidine molecules were protected against oxidation in the assay by using Clark and Lubs solution. The optimal pH for the assay was found to be 1.8 and the color formations were weak at lower and higher pH values than pH 1.8.

In this acidic solution, *o*-dianisidine, which is rich in electrons, protected ferrous ions from auto-oxidation for a long time, at least for 6 months at 4 °C.

Optimizations of o-dianisidine, ferrous ion and hydrogen peroxide concentrations

Optimal concentrations of the chemicals used were determined as the concentration of one chemical was changed while the concentrations of the other two chemicals were kept constant. The increases in the concentrations of *o*-dianisidine accelerated the precipitation of mega complex of dianisidyl radicals; hence, the monitoring of the changing of absorbance was difficult. The decreasing of the concentration led to a slow color formation rate and prolonged reaction time. The most appropriate concentration of *o*-dianisidine was determined to be 10 mM in Reagent 1.

The increases in the concentrations of ferrous ion or hydrogen peroxide accelerated the color formation rate; on the other hand, the upper limit of absorbance for spectrophotometric measurement was exceeded within a short time. The decreases in their concentrations decreased the reaction velocity and extended the reaction time. The optimal concentration of ferrous ion was found to be 45 μM in Reagent 1, and for hydrogen peroxide it was found to be 7.5 mM in Reagent 2.

Spectral analyses

Reagent 1, which contains *o*-dianisidine plus ferrous ions, and Reagent 2, which contains hydrogen peroxide, were clear solutions and they did not show any absorbance within the visible wavelength spectrum. After mixing of Reagent 1 and Reagent 2, a bright yellow-brown color developed within seconds and continued at least 20 min. The spectral absorbance of this color is shown in Fig. 1.

Dose-response characteristics of pure antioxidants

The dose-response characteristics of pure solutions of some endogenous and exogenous antioxidants—Trolox (a water-soluble analogue of vitamin E), vitamin C, reduced glutathione (GSH), bilirubin, uric acid and (±)-catechin—are shown in Fig. 2.

Reaction kinetics of blank and various serum samples

The reaction kinetics, which were performed in the automated analyzer, of a blank sample and serum samples of a healthy person, a patient with chronic renal failure and an infant with neonatal icterus are shown in Fig. 3.

Linearity

Serial dilutions of the Trolox solution were made. The upper limit of the linearity in the assay was 6.0 mmol Trolox

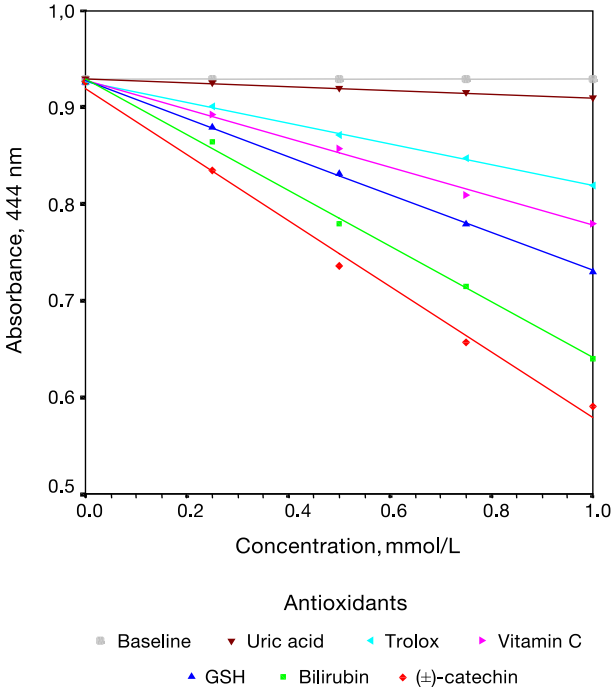

Fig. 2. Dose-response characteristics of pure solutions of the antioxidants.

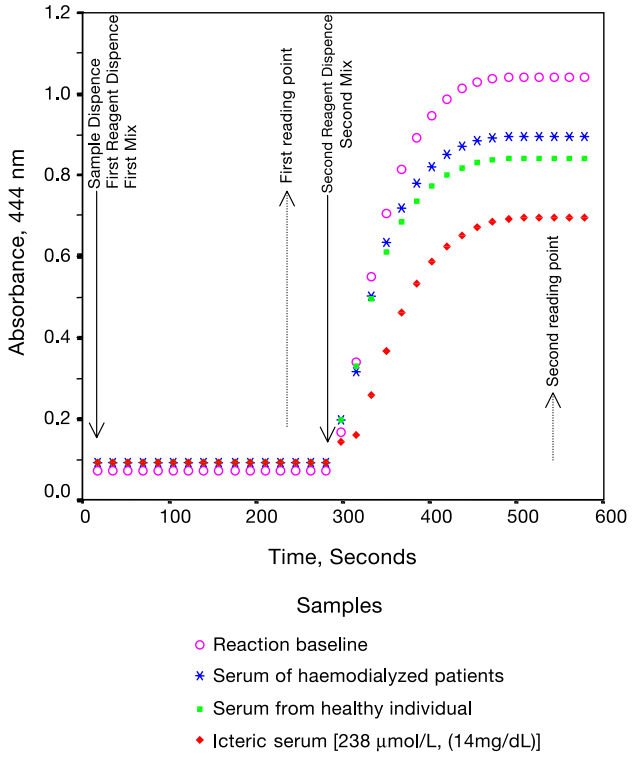

Fig. 3. Reaction kinetics of various serum samples.

equiv./l. In the regression analyses, the r value was 0.99 ($P < 0.001$ Sy/x = 0.23), the slope was 7.051 ($P < 0.001$ Sy/x = 0.206), and the intercept was −7.636 ($P < 0.001$ Sy/x = 0.370). On the other hand, dilution of serum did not affect the novel assay.

Analytical recovery

The percent recovery of the novel method was determined by addition of 1 mM vitamin C and GSH to serum samples. The mean percent recovery of added vitamin C and GSH was 98–101%.

Analytical sensitivity

As the slope of the calibration line, analytical sensitivity was found to be 0.11 Absorbance/Amount, [AX $(mM)^{-1}$].

Lower detection limit

Detection limit of the method was determined by evaluating the zero calibrator 10 times. The detection limit, defined as the mean TAR value of zero calibrator + 3 SD, was 0.09 mmol Trolox equiv./l.

Precision and storage

To determine the precision of the novel measurement method, we assayed three levels of sera. A serum pool that had high TAR was obtained from samples of infants with neonatal icterus. The serum pool with medium TAR was obtained from healthy persons. The serum pool with low TAR was obtained from normal pregnant women. Within- and between-batch precisions calculated for each of the three sera pools were lower than 3%. Serum TAR was not affected by storage at 4 °C for 1 day and −80 °C for 3 months.

Interference

Hemolyzes interfered the assay. Bilirubin did not. EDTA and citrate inhibited color formation. Heparin and oxalate did not interfere the assay.

TAR values of plasma samples were 15% higher than those of serum samples of the same individuals. The difference between plasma and serum TAR values was significantly correlated with the difference between total protein levels of plasma and serum samples ($r = 0.62$, $P = 0.002$; $n = 25$). Fibrinogen, which is an antioxidant protein [16], is present only in plasma and not in serum. The determined difference between serum and plasma TAR values may originate from the fibrinogen.

The effect of lipemia was examined by mixing a specimen with increased triglyceride and TAR levels proportionally with a specimen with low triglyceride and TAR concentrations. A triglyceride concentration of up to 15.8 mM (1400 mg/dl) had no effect on recovery. Uremic plasma samples did not interfere the assay.

Relative antioxidant responses of the antioxidant components of serum against potent free radical reactions and their estimated contributions to the TAR of serum

Relative antioxidant responses of the individual antioxidant components of serum and their estimated contributions to total antioxidant capacity of fresh fasting serum are shown in Table 1. As seen in Table 1, the major antioxidant component of serum against potent free radical reactions is proteins.

Table 1
Relative antioxidant responses against potent free radical reactions of the individual antioxidant components of serum and their estimated contributions to total antioxidant response of fresh fasting serum

Antioxidant component of serum	Relative activity	Serum concentration (µM)	Estimated contribution to TAR of serum (mmol Trolox equiv./l)	Estimated contribution to TAR of serum (%)
Total –SH	1.82	303–525	0.753	48.89
Vitamin C	1.36	28–85	0.077	5.00
Uric acid	0.19	150–470	0.059	3.83
Vitamin E	1.00	12–45	0.029	1.88
Bilirubin	2.64	3–17	0.026	1.69
Others	–	–	0.596	38.71
Total			1.540	100

TAR: Total antioxidant response against potent free radical reactions.

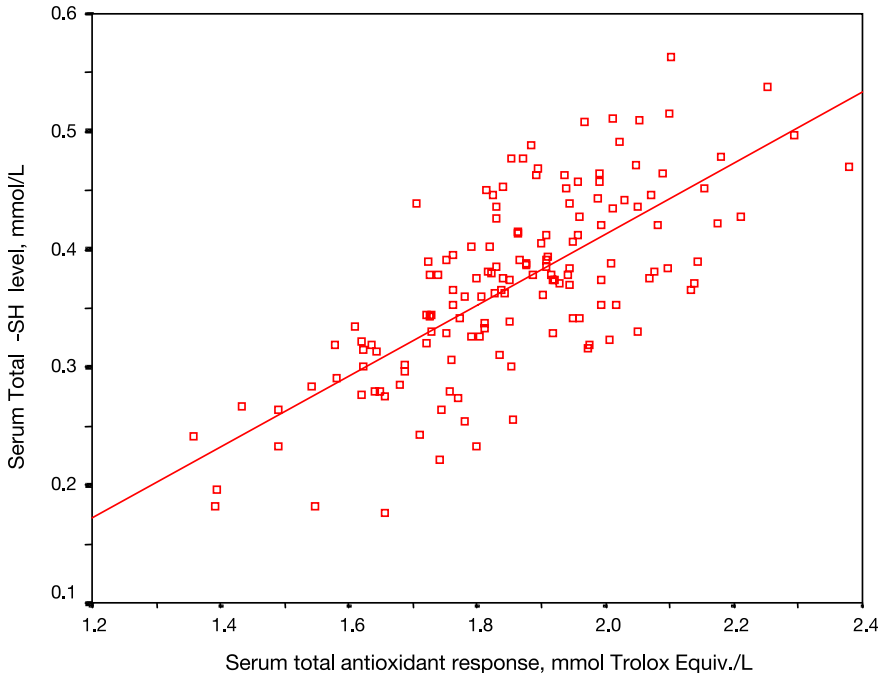

Fig. 4. The relationship between serum TAR against potent free radical reactions and serum total free sulfhydryl group levels ($r = 0.714$, $P < 0.0001$; $n = 145$).

The relationship between serum TAR against potent free radical reactions and serum total free sulfhydryl groups

As seen in Fig. 4, there was a significant correlation between serum TAR against potent free radical reactions and the amount of serum total free sulfhydryl groups ($r = 0.714$, $P < 0.0001$; $n = 145$).

The relationship between the novel method and the improved ABTS$^{\bullet+}$ decolorization assay

To compare the novel method with the improved ABTS$^{\bullet+}$ decolorization assay, 200 serum samples from patients selected to include a wide variety of pathologic conditions were studied. There was a significant correlation

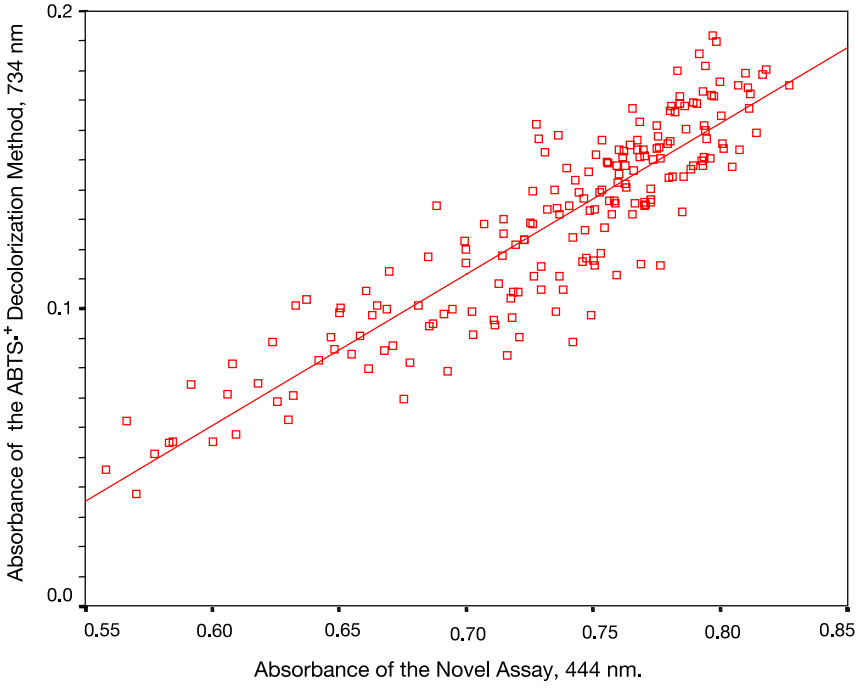

Fig. 5. The relationship between the novel assay and the improved ABTS radical cation decolorization method ($r = 0.875$, $P < 0.0001$; $n = 200$).

between the novel method and the improved $ABTS^{\bullet+}$ decolorization assay ($r = 0.875$, $P < 0.0001$; $n = 200$) (Fig. 5).

Reference interval

To determine the reference interval for serum TAR, serum specimens from 109 healthy individuals (57 women, 52 men 17–41 years old) were assayed. The reference ranges were 1.20–1.81 mmol Trolox equiv./l for women and 1.24–1.84 mmol Trolox equiv./l for men; the difference was not significant ($P = 0.142$).

The results for healthy subjects and the patients

Serum TAR against potent free radicals was found to be significantly lower in haemodialyzed patients with chronic renal failure (1.13 ± 0.21 mmol Trolox equiv./l, $n = 31$) and it was significantly higher in individuals with neonatal icterus (2.82 ± 1.18 mmol Trolox equiv./l, $n = 27$) than in healthy subjects (1.54 ± 0.15 mmol Trolox equiv./l, $n = 80$) according to ANOVA test ($P < 0.05$).

Discussion

In the novel assay, most potent free radicals are produced and they oxidize to colorless *o*-dianisidine molecules to bright yellow-brown colored dianisidyl$^{\bullet}$. The potent free radical reactions, starting with $OH^{\bullet}$, do not end in only a one-step reaction; generally, they continue, even forming a free radical chain reaction. Antioxidants prevent the prolongation of these oxidation reactions and the increasing of color formation in various steps.

Suppression of the bright yellow-brown color formation is the base of the novel assay. Reaction kinetic trace of the novel assay can be divided into three phases. In the first phase, *o*-dianisidine is oxidized and the absorbance increases linearly. In the next phase, the oxidation is completed and the line draws a plateau. In this area, the color intensity is at its highest point. In the last phase, the color changes from brown to black and insoluble precipitates are formed. The plateau phase was obtained by the optimization of the *o*-dianisidine, the ferrous ion, the hydrogen peroxide concentrations, and the pH and the molarity of the Clark and Lubs solution.

Serum contains various antioxidant molecules. Proteins constitute the main antioxidant component of serum. Free sulfhydryl groups of proteins are mainly responsible for antioxidant response of them. Free sulfhydryl groups of serum practically belong to proteins because the serum concentration of linoleic acid, which also has –SH groups, is very low, and the contribution to serum total free sulfhydryl level is negligible. It was calculated that proteins compose about 49% of the measured serum TAR against potent free radical reactions in healthy subjects (Table 1). On the other hand, a most significant correlation was found between TAR levels and total –SH contents of serum (Fig. 4).

It is well known that vitamin C directly scavenges radicals present in the aqueous compartment. In this study, it was shown that vitamin C delays and suppresses potent free radical reactions. Vitamin C constitutes about 5% of the measured serum TAR against potent free radical reactions in healthy subjects (Table 1).

Recent studies have shown that bilirubin is a strong physiological antioxidant that may provide important protection against atherosclerosis, coronary artery disease, and inflammation [17]. It has also been suggested that bilirubin might play a particularly crucial role in protecting the neonate from oxidative damage [18]. We found that the infants with neonatal icterus have higher serum TAR against potent free radical reactions than do healthy adult subjects. In this study, it was shown that bilirubin is a strong antioxidant molecule against potent free radical reactions (Fig. 2). The total bilirubin content of serum constitutes about 1.69% of the measured serum TAR value in healthy subjects (Table 1).

The reaction characteristics of uric acid against potent and weak radicals and oxidants are completely different. It gives only one electron to $ABTS^{\bullet+}$ [4,9] or Fe^{3+}–TPTZ complex [6,7], which are weak oxidants. The formula structure of uric acid is not changed after its electron loss. However, it has been shown that uric acid is converted to uric acid radicals while uric acid is reacted with potent free radicals, and the uric acid radicals produced oxidize vitamin C [19]. In other studies, it has been well demonstrated that uric acid is not a strong antioxidant against potent free radical reactions, and that it cannot prevent potent free radical reactions [20,21]. Potent free radicals convert the formula structure of uric acid to allantoin, and if the potent free radical reactions are prolonged, the allantoin is converted to urea [22].

In patients with chronic renal failure, lipid peroxidation, protein methylation and DNA oxidation levels are highly increased, and thus potent free radical reactions are dominant. These oxidative products of biomolecules are produced via only potent free radical reactions, not weak radicals or oxidants. In this study, we found by the novel method that the patients with chronic renal failure had lower serum TAR against potent free radical reactions than did healthy subjects.

The protective roles of tea, fruits and vegetables, which are rich in antioxidant polyphenolic compounds, against cancer and atherosclerosis have been demonstrated [23,24]. It has been reported that a single dose of tea increases plasma total antioxidant capacity [25]. In our study, the antioxidative power of (±)-catechin, which is abundantly present in tea, was determined and compared with those of other known antioxidants. It was determined that the antioxidant response against potent free radical reactions of (±)-catechin is higher than those of vitamin C, Trolox and others (Fig. 2).

The novel assay is linear up to 6 mmol Trolox equiv./l, its precision values are lower than 3%, and it is not affected by bilirubin interference. The method developed is significantly correlated ($r = 0.875$, $P < 0.0001$; $n = 200$) with the improved $ABTS^{\bullet+}$ decolorization assay, which is a recently developed total antioxidant capacity measurement method.

The method developed can be used to measure the TAR of samples against potent free radical reactions.

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