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Antioxidant Capacities of Ascorbic Acid, Uric Acid, α-Tocopherol, and Bilirubin Can Be Measured in the Presence of Another Antioxidant, Serum Albumin

Hiroshi Ihara,[1]* Naotaka Hashizume,[1] Toshio Hasegawa,[2] and Mitsutaka Yoshida[3]

[1]*Department of Laboratory Medicine, Ohashi Hospital, School of Medicine, Toho University, Tokyo, Japan*

[2]*Central Clinical Laboratory, Fukui Medical School University Hospital, Fukui, Japan*

[3]*Department of Environmental and Biotechnological Frontier Engineering, Fukui University of Technology, Fukui, Japan*

Human serum contains several antioxidants. The total antioxidant capacity (AOC) is the sum of all the antioxidant activities present in serum. The aim of our study was to investigate whether the AOC of bilirubin (BR), α-tocopherol (TOH), ascorbic acid (AA), and uric acid (UA) could be measured with good precision and recovery in the presence of human serum albumin (HSA). We measured the AOC of each antioxidant using a Cobas Mira S instrument (Roche Diagnostic Systems, Montclair, NJ) by measuring the inhibitory effect of a given compound on the oxidation of the radical cations of 2,2′-azino-di-(3-ethylbenzthiazoline 6-sulphonate) (ABTS) incubated with metmyoglobin and H_2O_2. The assay had a linear AOC response range of 27–2,000 μmol/L. The within- and between-day coefficients of variation (CVs) did not exceed 3.4% and 4.2%, respectively. The AOC of albumin in serum is much greater than that of BR, TOH, AA, or UA owing to the substantially greater concentration of HSA in serum. An aliquot of a solution of AA, UA, BR, or TOH was added to HSA or distilled water, and the AOC was determined. The AOC of BR, TOH, AA, and UA increased in a linear way with increasing concentrations. However, we found that the magnitude of increase in the AOC of a mixture of HSA and any of these antioxidants was lower than the sum of the AOC of HSA and any one of the following: AA, UA, BR, or TOH (all expressed in μmol/L). J. Clin. Lab. Anal. 18:45–49, 2004.

Key words: antioxidants; free radicals; total antioxidant capacity; Trolox; AAPH

INTRODUCTION

Reactions involving free radicals occur constantly in living cells, and some of these are destructive. This destruction is mitigated by certain enzymes–primarily superoxide dismutase, catalase, and glutathione peroxidase. Other, non-enzyme antioxidants include vitamin E, vitamin C, carotenoids, uric acid (UA), bilirubin (BR), and albumin (1). The antioxidant activity of these components can be measured in terms of their total peroxyl radical-trapping capacity or total AOC. It is possible to measure various types of radical generators and oxidizable compounds in serum. The usual assumption is that the total AOC of serum is the sum of the AOC of each antioxidant (2). Because albumin has three to seven times the AOC compared to vitamin E, vitamin C, and BR as a chain-breaking antioxidant in vitro (3), we wanted to determine whether the total AOC of serum is primarily accounted for by albumin. In our previous study (3), the total AOC was determined by measuring the inhibitory effect of serum on peroxy-radical-induced hemolysis of human erythrocytes. Unfortunately, this method could not measure low concentrations of AOC. We then measured the total AOC of each antioxidant by a more sensitive method (4). With the new method, we determined the AOC of vitamin C, vitamin E, BR, and UA (all at physiological concentrations) with and without the presence of serum albumin.

*Correspondence to: Hiroshi Ihara, Department of Laboratory Medicine, Ohashi Hospital, School of Medicine, Toho University, 2-17-6 Ohashi, Meguro, Tokyo 153-8515 Japan. E-mail: ihara-1@cam.hi-ho.ne.jp

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MATERIALS AND METHODS

BR and TOH were purchased from Sigma Chemical (St. Louis, MO). UA, AA, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Co. (Milwaukee, WI). We isolated HSA from pooled normal serum that was freed of AA, UA, BR, or TOH using a previously described procedure (5). The concentration of the isolated albumin was 696 μmol HSA/L.

We dissolved AA in distilled water to give a concentration of 1,130 μmol/L. We also dissolved 80 mg of TOH in 50% ethanol to give 464 μmol/L. We dissolved 80 mg of UA in 0.2 mL of 0.1 mol/L NaOH, and diluted it with distilled water to 100 mL to give 4,760 μmol/L. We dissolved 15 mg BR in 1.0 mL of 0.1 mol/L NaOH, and diluted it to 80 mL with distilled water. We then added 1.0 mL of 696 μmol/L HSA, adjusted the pH of the BR solution to 7.0, and diluted it with distilled water to 100 mL to give a 260 μmol/L solution. We prepared dilute solutions of AA, UA, BR, and TOH at five different concentrations. Then we added 0.1 mL of each compound to 0.9 mL of distilled water or 0.9 mL of the 696 μmol/L HSA. We prepared Trolox solutions (1,000 μmol/L) by dissolving 25 mg of Trolox in 0.2 mL of ethanol, and diluted them with distilled water to 100 mL.

We measured the AOC of serum, according to the method of Miller et al. (4), with a commercially available kit (Total Antioxidant Status; Randox Laboratories, San Francisco, CA). Analysis was performed with a Cobas Mira S instrument (Roche Diagnostic System, Montclair, NJ). The method is based on the inhibition by antioxidants of the absorbance of the radical cations of 2,2′-azino-di-(3-ethylbenzthiazoline 6-sulphonate) (ABTS) at 600 nm. ABTS radical cations are formed by incubating ABTS with metmyoglobin and H_2O_2. The 1,000-μmol/L Trolox solution was used as a standard, and the final results were expressed as μmol of Trolox equivalents per liter. In the comparison study, the AOC of serum was also determined by our previously described method (3) using AAPH, a water-soluble, thermolabile free-radical generator. A 1,000-μmol/L Trolox solution served as a calibrator.

RESULTS

We determined the analytical performance of the AOC measurement by the ABTS method with the Cobas Mira S. The detection limit of AOC was 27 μmol/L, and the analysis gave a linear absorbance response at 600 nm up to 2,000 μmol/L (Fig. 1). The within-day ($n = 9$) mean and SD was $1,522 \pm 52$ μmol/L. For between-day ($n = 7$) assays, the mean and SD was 462 ± 61 μmol/L. In the AAPH method, the within-day mean and SD was $1,491 \pm 66$ μmol/L, and the between-day mean and SD was $11,464 \pm 66$ μmol/L. The usable assay range is 125–1,800 μmol/L. Although the AOC of serum determined by the AAPH method on 43 sera from volunteers was significantly correlated with that obtained by the ABTS method ($r = 0.520$, $P < 0.001$, $Sy/x = 142$ μmol/L) (see Fig. 2), the AAPH method was insensitive to small changes in the concentration of Trolox (see Fig. 1). Therefore, we chose to determine the AOC with the more sensitive ABTS method.

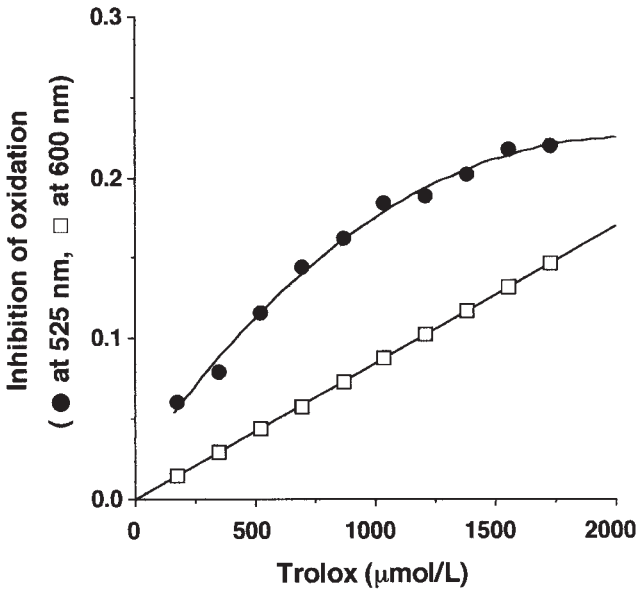

Fig. 1. Degree of oxidation inhibition as a function of the concentration of Trolox. Inhibition of oxidation was measured at 525 nm with the AAPH method (●) and at 600 nm with the ABTS method (□).

Tables 1 and 2 show that the AOC of AA, BR, and TOH in the absence of HSA increased in a linear way with their concentrations ($r = 0.882$–0.998, $P < 0.05$). The increase in the AOC per μmol/L of each antioxidant was highest for BR (2.6 μmol/L) followed in order by UA (1.5 μmol/L), TOH (1.2 μmol/L), and AA (1.1 μmol/L). The AOC of HSA was 0.5 μmol/L (Fig. 3).

When AA, UA, BR, or TOH was added to HSA, the AOC of this mixture increased in a linear way with the concentration of each antioxidant ($r = 0.974$–0.999, $P < 0.05$) for all. The AOC in the mixture was lower than the sum of the AOC of HSA and AA, UA, BR, or TOH. The recoveries of the AOC for AA, UA, BR, and TOH were on average 94%, 87%, 99%, and 94%, respectively. We found that the aqueous solutions of AA, BR, and TOH were unstable in the absence of HSA; therefore, the AOC of these antioxidants gave

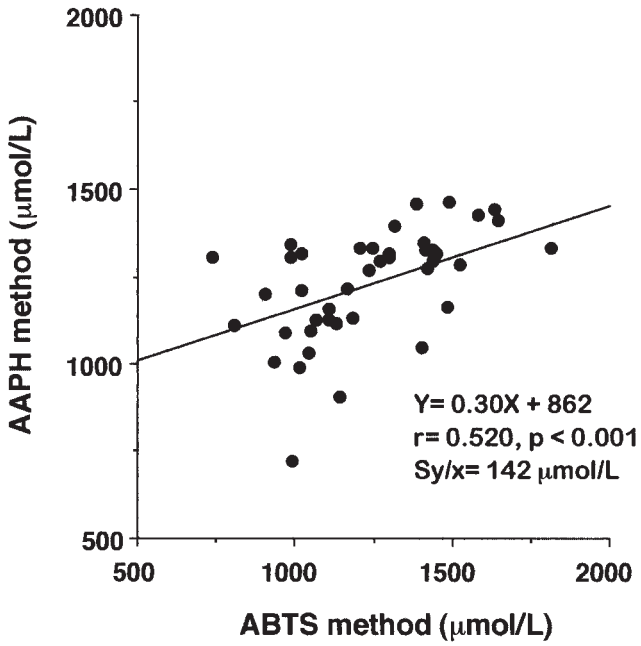

Fig. 2. Correlation of total AOC of serum in 43 sera by the AAPH and ABTS methods ($r = 0.520$, $P < 0.001$, $Sy/x = 142$ μmol/L). The least-squares equation is $Y = 0.30X + 862$.

TABLE 1. Antioxidant capacity (AOC) of hydrophilic antioxidants

Antioxidant, μmol/L	AOC in μmol/L without added HSA	AOC in μmol/L with added 626 μmol/L HSA	Recovery (%)
Ascorbic acid			
0	0	313±4	
14	9±10	316±9	98
29	35±12	322±11	95
57	66±15	352±11	93
114	122±16	391±25	90
			Mean, 94%
Uric acid			
0	0	294±7	
178	284±19	509±9	88
238	379±19	621±8	92
357	575±21	729±18	84
476	728±3	852±14	83
			Mean, 87%

The values are the mean±SD of three experiments. The recovery (%) was calculated as follows: Recovery = 100 × (AOC with added 626 μmol/L HSA)/[(AOC without added HSA) + (AOC of 626 μmol/L HSA without added antioxidant)]. The reference range for ascorbic acid in our laboratory is 40–80 μmol/L. The reference range for uric acid is 226–446 μmol/L for men and 143–345 μmol/L for women.

TABLE 2. Antioxidant capacity (AOC) of lipophilic antioxidants

Antioxidant in μmol/L	AOC in μmol/L without added HSA	AOC in μmol/L with added 626 μmol/L HSA	Recovery (%)
Bilirubin			
0	0	294±2	
3	21±1	309±1	98
6	31±3	317±7	97
13	41±11	336±2	100
26	83±11	376±6	99
			Mean, 99%
α-Tocopherol			
0	0	280±18	
6	12±6	285±15	98
12	34±5	290±10	92
23	57±21	310±8	92
46	64±19	325±6	95
			Mean, 94%

The values are the mean±SD of three experiments. Recovery (%) was calculated as follows: Recovery = 100 × (AOC with added 626 μmol/L HSA)/[(AOC without added HSA) + (AOC of 626 μmol/L HSA without added antioxidant)]. The reference ranges for bilirubin and α-tocopherol in our laboratory are 4–17 μmol/L and 11–46 μmol/L, respectively.

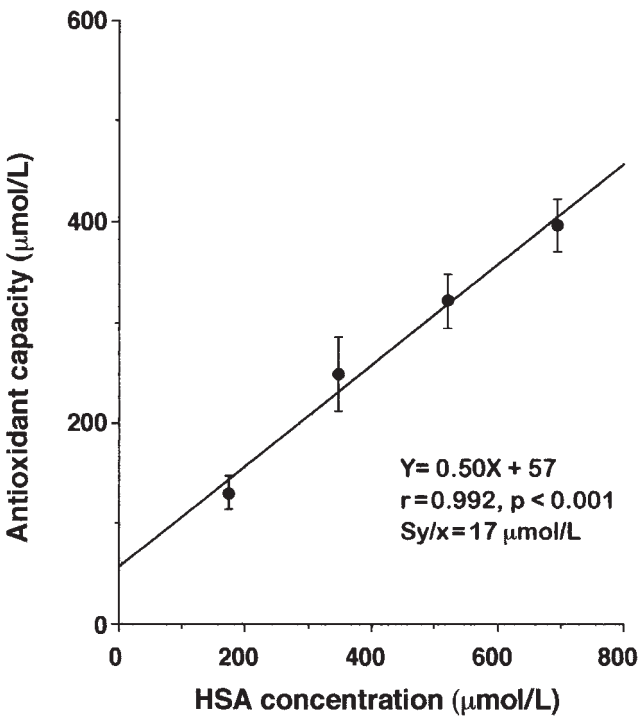

Fig. 3. Plot of the AOC observed by the ABTS method as a function of the HSA concentration. The line drawn has a slope of 0.5, and the reference range in our laboratory for serum albumin is 514–735 μmol/L. Plots show the mean±SD of three experiments.

lower values and higher SDs compared to the specimens prepared with HSA. The AOC of aqueous solutions of AA, BR, and TOH was stable for no more than 30 min.

DISCUSSION

By comparing the AOC measurements obtained with the ABTS and AAPH procedures, we found that the ABTS method provided acceptable analytical precision, and the sensitivity was satisfactory for low concentrations of AA, UA, BR, and TOH. The AOC values obtained by our previous AAPH method could not respond to small changes in the concentration of an antioxidant. Therefore, we chose to study the effect of HSA on the total AOC of serum by the ABTS method. We concluded that the insensitivity of the AAPH method resulted from the use of intact erythrocytes. AAPH-derived radicals first oxidize the phospholipid membrane of erythrocytes, and then oxidize the TOH present in the membrane. Antioxidants that were added to the reaction mixture inhibited this oxidation with a time delay. We also concluded that ABTS was oxidized directly by H_2O_2, which was competing with externally added antioxidants.

Using this sensitive ABTS method, we investigated the effect of HSA on the total AOC of serum. The results indicate that AA, UA, BR, and TOH can trap the ABTS radical cations with or without the presence of HSA. Our ranking of the AOC of BR, UA, TOH, AA, and HSA in the order of their AOC activity is in agreement with previous reports (1–4). Furthermore, the AOC of AA increased with increasing concentration of AA from 14 to 114 μmol/L. This correlates with Wayner et al.'s report (6) that AA at concentrations of 1–100 μmol/L acts as an antioxidant, and AA at $>1{,}000$ μmol/L undergoes auto-oxidation and behaves like an oxidant.

The AOC of the mixture of HSA with AA, UA, BR, or TOH increased in parallel with the increasing concentration of each antioxidant. As expected, the AOC of the mixture was greater than the original AOC of HSA. This means that the low AOC of AA, UA, BR, and TOH in serum were detected with good precision by the ABTS method. Small increases of the AOC by these antioxidants could still be detected in the presence of physiological concentrations of albumin (i.e., the AOCs of the AA, BR, TOH, and UA were not swamped by the albumin). Although HSA showed the lowest AOC in terms of μmol/L, HSA contributed the most to the total AOC of serum, because its physiological concentration in serum is 10–40 times that of AA, BR, and TOH. The AOC of HSA is provided by sulfhydryl groups of human mercaptalbumin present in HSA (7,8). UA has a higher AOC/μmol compared to albumin, but the concentration of UA in serum is much less than that of albumin. UA and albumin have physiological concentrations in serum of about 300 and 700 μmol/L, respectively.

However, the AOC of the HSA mixture and each of the described antioxidants was less than the sum of the AOC of the individual components. Niki et al. (9) reported that the rate of consumption of TOH as an antioxidant was suppressed by UA when the oxidation of methyl linoleate micelles was initiated by AAPH. Sato et al. (10) also showed that the rate of consumption of TOH was suppressed by AA when low-density lipoprotein was oxidized by AAPH. In addition, Stocker et al. (11,12) reported that BR could compete with TOH and UA for peroxyl radicals. We found that the individual antioxidants did not exert their maximum AOC in the mixture of two antioxidants or more (i.e., the total AOC in serum directly measured by the ABTS method would not necessarily be equal to the sum of AOC of all antioxidants present in serum). We also found that the low AOC of AA, UA, BR, and TOH in serum was not overwhelmed by the stronger AOC of HSA. Therefore, we conclude that with this new method, one can measure the AOC of low-concentration analytes in the presence of a much larger concentration of albumin.

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