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Comparative Reaction Rates of Various Antioxidants with ABTS Radical Cation

RICHARD B. WALKER* AND JACE D. EVERETTE

Department of Chemistry and Physics, University of Arkansas at Pine Bluff, Slot 4941, 1200 North University Avenue, Pine Bluff, Arkansas 71601

The reaction rates of several aminothiol, amidothiol, and phenolic antioxidants with ABTS radical cation were measured. Most compounds had half-lives of less than one minute. However several compounds had considerably longer half-lives. Aminothiol derivatives lacking a free thiol group, such as amifostine and RibCys, displayed longer half-lives. Reaction of these compounds with the ABTS radical cation displayed first order kinetic behavior. Of the phenolic compounds studied, chlorogenic acid and caffeic acid had the longest half-lives. Most phenolics displayed a biphasic kinetc pattern involving fast and slow steps. Some of the aminothiols also displayed this type of behavior. Glutathione disulfide was reactive toward ABTS radical cation and displayed slow kinetics. This suggests that the slow step observed with some of the aminothiols may be due to initial rapid formation of disulfide followed by slow reaction of the disulfide with ABTS radical cation. Some compounds required a considerably longer incubation time to reach end point than the six to ten minute period normally used for this assay. This suggests that, when ABTS is being used as an end point assay, a longer incubation time may be needed to obtain reliable data. When food substances are being tested using this assay, kinetic profiles should first be examined before end points are determined. This paper contains the first published data reporting antioxidant capacities of amino- and amidothiols measured by the ABTS method.

KEYWORDS: Antioxidants; polyphenols; aminothiols; thiazolidines; ABTS; antioxidant assay; kinetics; free radicals

INTRODUCTION

The Trolox equivalent antioxidant capacity (TEAC) assay is widely used in the food and nutriceutical industries to determine the antioxidant capacities of foods, beverages and nutriceutical products (*1*). The assay is based upon the ability of antioxidants to decolorize the 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation ($ABTS^{\bullet+}$), which is blue in solution and has an absorbance maximum of 734 nm. $ABTS^{\bullet+}$ is generated by reaction of ABTS with an oxidizing species (*2*, *3*). The original oxidizing species used was metmyoglobin (*2*). Later it was discovered that sodium persulfate, which is less expensive and easier to work with, works equally well. Most researchers today use this modification, which is referred to as the improved TEAC assay (*3*). The ability of various antioxidants to react with $ABTS^{\bullet+}$ is compared with that of Trolox, which is assigned a TEAC value of 1.00.

The original paper on the improved TEAC assay describes an incubation time of 6 min at 30 °C for measuring end points (*3*). However, the investigators did measure kinetics on some compounds and noted that some compounds did not reach end point in 6 min. Reports by other investigators (*4*), as well as results in our laboratory, suggest that a longer incubation time may give more reliable end point data. It has been noted that chlorogenic and caffeic acids do not reach stable end points even after one hour (*4*). This was also observed in our laboratory. TEAC values reported for certain plant polyphenols vary widely in the literature. TEAC values reported for quercetin vary from below 3 to over 6 (*5*, *6*). These variations may be related to the different incubation times used by the different investigators. In response to these observations, several investigators have examined the kinetics of reaction of $ABTS^{\bullet+}$ with antioxidants (*3*, *4*, *6*−*9*). Most plant polyphenols studied have demonstrated a biphasic kinetic pattern, involving fast and slow steps (*6*−*9*). Evidence suggests that $ABTS^{\bullet+}$ initially extracts an electron or hydrogen atom from polyphenols resulting in the formation of a semiquinone (*1*). Evidence has been presented that the semiquinone may dimerize upon reaction with $ABTS^{\bullet+}$ and that these dimers may retain antioxidant activity. It has been demonstrated recently that dimerization may contribute to the biphasic kinetic pattern seen with some phenolics (*8*). Also, recent evidence demonstrates that some polyphenols react with $ABTS^{\bullet+}$ to form covalent adducts (*7*). In some cases, these adducts retained antioxidant activity. These covalent adducts may contribute in some cases to the complex kinetic behavior which is observed.

Another major class of antioxidants is the amino- and amidothiols. These include the amino acid cysteine, the endogenous tripeptide glutathione, the antihypertensive drug captopril,

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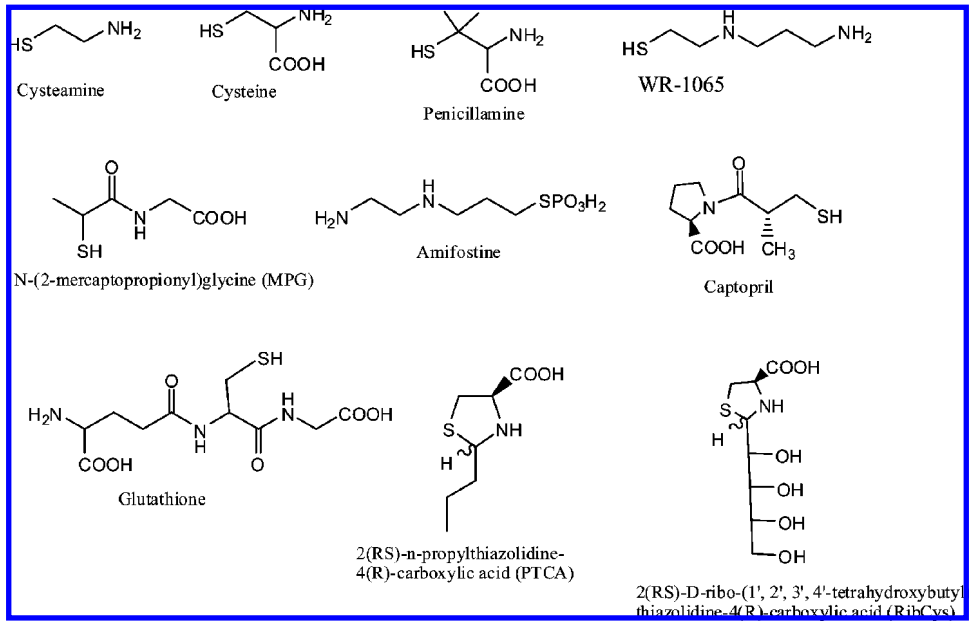

Figure 1. Chemical structures of aminothiols and amidothiols derivatives used in this study.

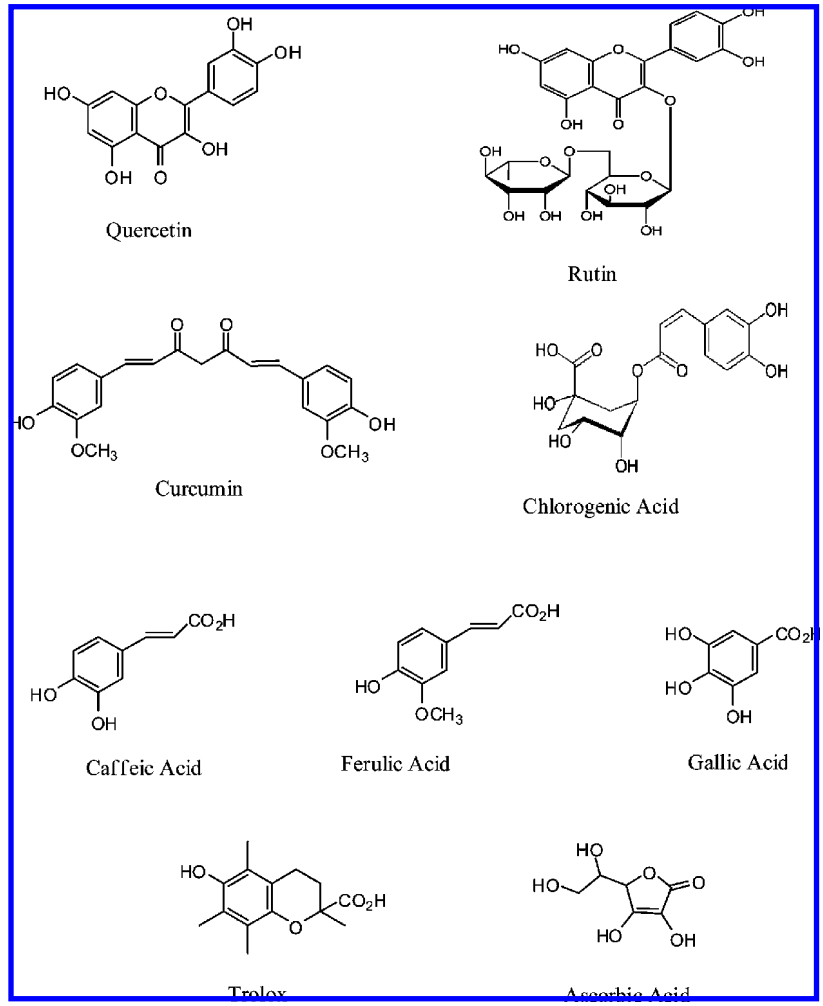

Figure 2. Structures of ascorbic acid and phenolic compounds used in this study.

the antiarthritic drug penicillamine, the antiurolithic drug *N*-(2-mercaptopropionyl)glycine (MPG, Tiopronin) and the radioprotective drug amifostine. Although not widely present in foods, they are recognized as antioxidants which may be beneficial to health. Some compounds of this class are present in garlic (*10*). Among these are *S*-allyl-cysteine, *S*-ethyl-cysteine and *S*-propyl-cysteine. *N*-Acetylcysteine (NAC) is a common over-the-counter nutritional supplement which is marketed for its antioxidant properties. It is interesting that, despite the known antioxidant properties of these compounds, $ABTS^{\bullet+}$ data for most amino- and amidothiols have not been published to this date. Therefore, we felt that it may be of interest to readers to

Table 1. TEAC Values for Compounds Tested

compound	TEAC value	compound	TEAC value
quercetin	4.95	glutathione	1.09
rutin	3.30	PTCA	1.04
curcumin	2.89	ascorbic acid	1.00
gallic acid	2.60	Trolox	1.00
chlorogenic acid	2.00	captopril	0.90
caffeic acid	1.76	*N*-acetylcysteine	0.88
amifostine	1.60	MPG	0.53
ferulic acid	1.56	WR-1065	0.50
penicillamine	1.54	cysteine	0.43
RibCys	1.14	cysteamine	0.40

report the antioxidant capacity of the best known amino- and amidothiols using the $ABTS^{\bullet+}$ method.

The thiazolidine derivatives 2(*RS*)-(D-ribo-(1′,2′,3′,4′-tetrahydroxybutyl)thiazolidine-4(*R*)-carboxylic acid (RibCys) (*11, 12, 14*) and 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid (PTCA) (*13*) have demonstrated favorable cytoprotective properties and are of interest to researchers. Therefore they have also been included in our study. RibCys is currently being developed into an antioxidant nutritional supplement. Thiazolidines are condensation products formed by reaction of a 1,2-aminothiol with an aldehyde or ketone. They undergo enzymatic or nonenzymatic hydrolysis in aqueous media to yield their original components (*15*). Therefore, RibCys and PTCA may be considered prodrugs of cysteine (*11*−*15*).

In this study, we measured the rates of reaction of seven plant polyphenols, Trolox, ascorbic acid, and ten amino- and amidothiol derivatives with $ABTS^{\bullet+}$. End point values were also measured, allowing a 30 min incubation time at 37 °C. Rate constants and half-lives were calculated for RibCys, PTCA and amifostine.

The purpose of this study was to examine the kinetic behavior of reactions between common antioxidants and $ABTS^{\bullet+}$ with special emphasis on the aminothiols, amidothiols and their derivatives. With the exception of glutathione (*16*), the data concerning the reactivity of the amino- and amidothiols with $ABTS^{\bullet+}$ have not been previously reported in the literature. We measured the kinetic profiles of several phenolic compounds to serve as a basis of comparison with those observed for the aminothiols and amidothiols.

MATERIALS AND METHODS

2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and *N*-(2-mercaptopropionyl)glycine (MPG) were purchased from Sigma-Aldrich Company, St. Louis, MO. Rutin and chlorogenic acid were contributed by Dr. Stephen Grace of the Department of Biology at the University of Arkansas at Little Rock. Amifostine and WR-1065 were donated by the National Cancer Institute. 2(*RS*)-(D-Ribo-(1′,2′,3′,4′-tetrahydroxybutyl)thiazolidine-4(*R*)-carboxylic acid (RibCys) was donated by CellGevity, Inc., Torrance, CA. 2(*RS*)-*n*-Propylthiazolidine-4(*R*)-carboxylic acid (PTCA) was synthesized in our laboratory by a method reported in the literature (*13*). All other materials were purchased from Fisher Scientific Company, Houston, TX.

$ABTS^{\bullet+}$ Assay. Stock solutions of ABTS (5.00×10^{-4} M) and sodium persulfate (6.89×10^{-3} M) in PBS (pH = 8.0) were prepared. Sodium persulfate stock solution (1.0 mL) was added to ABTS stock

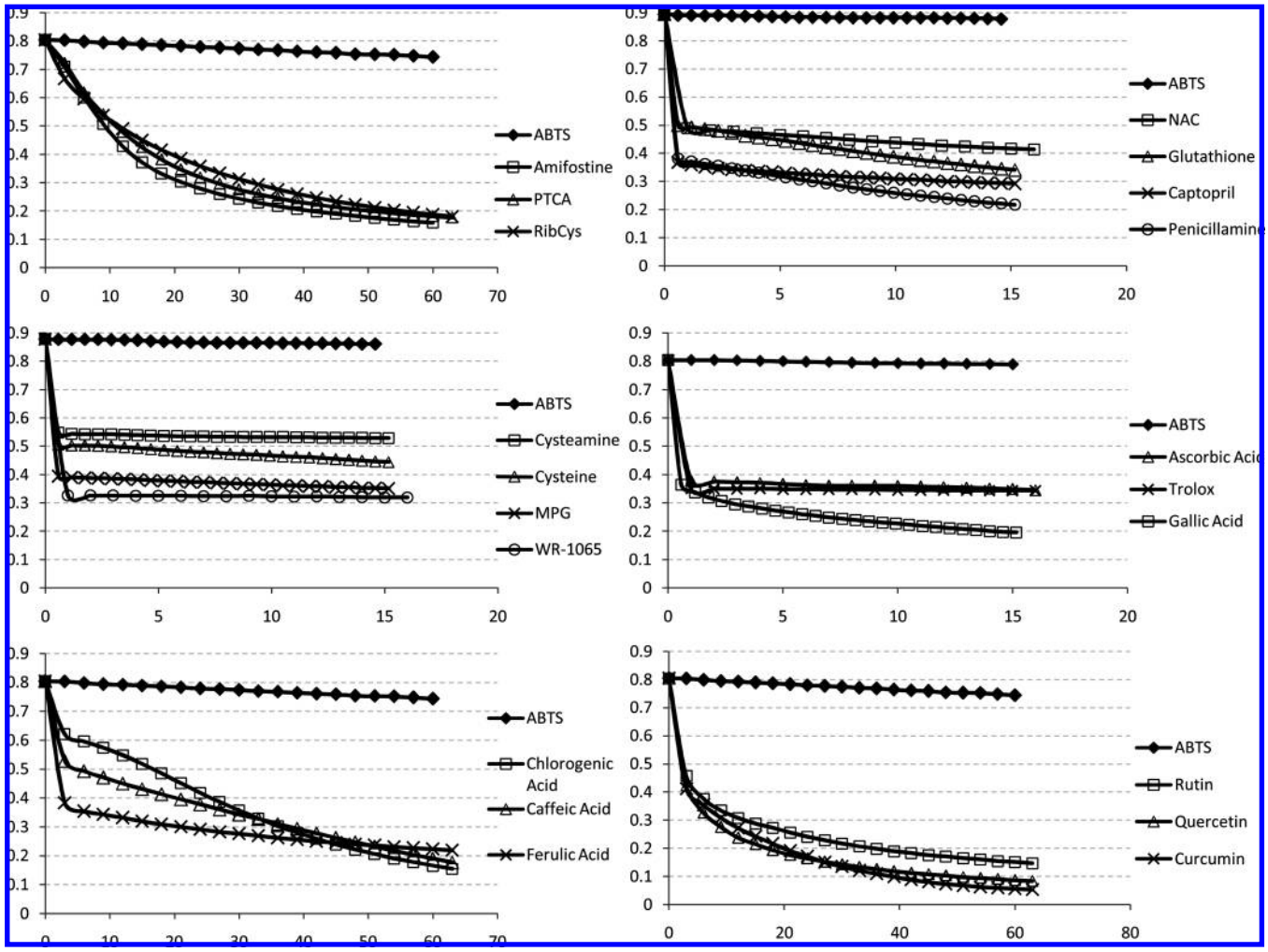

Figure 3. Time course of the absorbance changes for the reaction of the compounds used in this study with $ABTS^{\bullet+}$.

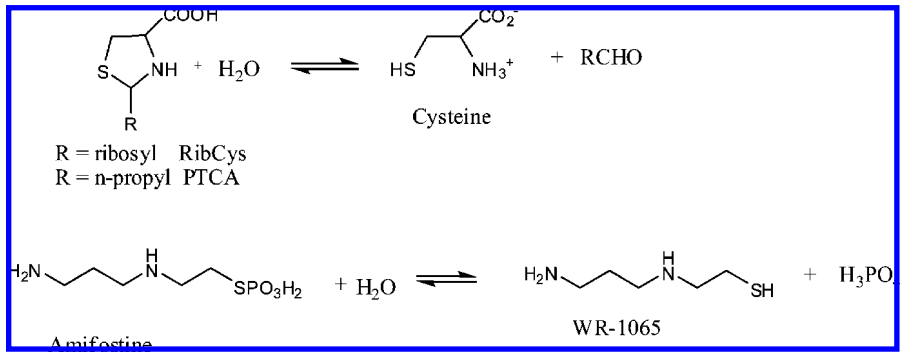

Figure 4. Hydrolysis of "masked thiol" compounds. "Masked thiols" are defined as compounds capable of undergoing hydrolysis to yield a thiol.

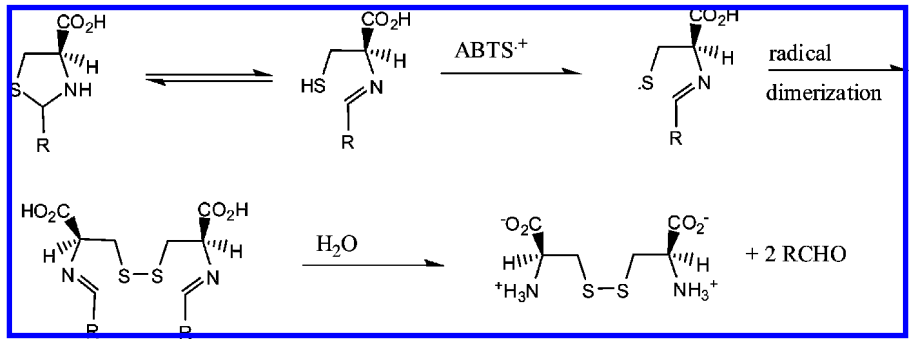

Figure 5. Probable mechanism of reaction of thiazolidines with ABTS$^{\bullet+}$.

solution (99.0 mL). The mixture was stored in the dark for 16 h. This produced a solution of ABTS$^{\bullet+}$ which gave an absorbance of approximately 0.85 at 734 nm. 10.0 mM stock solutions of test compounds were prepared. Ethanol was the solvent for all polyphenols, as well as *N*-acetylcysteine, captopril, Trolox and PTCA. Water was used for all other compounds. Several dilutions were prepared for each compound. Dilution strengths were dependent upon the relative antioxidant capacity of each compound. Three replicates of each dilution were measured by adding 20 μL of each dilution to cuvettes containing 2.5 mL of ABTS$^{\bullet+}$. Cuvettes were incubated at 37 °C in a dry bath for 30 min, and absorbances were read at 734 nm using a BioRad SmartSpec 3000 spectrophotometer. Plots of absorbance versus concentration were prepared using Sigma Plot. TEAC values were measured by comparing the slopes of plots obtained for each compound compared to that of Trolox.

Kinetic Measurements. Stock solutions of ABTS$^{\bullet+}$ and test compounds were prepared as described above. Dilutions of test compounds were made in such a manner that the final concentrations would be the same as the last three points on the graphs used to obtain TEAC values. Five replicates of each dilution were prepared by adding 10 μL of each dilution to five wells of a 96-well plate. 190 μL of ABTS$^{\bullet+}$ was rapidly added to each well using a multichannel pipet. Absorbance readings at 734 nm were taken every minute for one hour using a kinetics program on a BioTek Synergy NT-1 96-well plate reader. Readings were taken at 37 °C. Data was exported to Excel and absorbance versus time graphs were prepared.. For RibCys, PTCA and amifostine, first order ($\ln(A_\infty - A)$ versus time) plots were prepared. Rate constants were determined from the slope of the plots. Half-lives were determined using the formula $T_{1/2} = \ln 2/k$.

RESULTS AND DISCUSSION

Chemical structures of all compounds tested are shown in **Figures 1** and **2**. End point data for all compounds tested are shown in **Table 1**. Absorbance versus time graphs for all compounds tested are shown in **Figure 3**.

The data indicate that all amino- and amidothiol derivatives which have a free thiol group displayed fast reaction kinetics with ABTS$^{\bullet+}$. The three aminothiol derivatives tested which have a masked thiol group (amifostine, PTCA, RibCys) demonstrated slower kinetics. These three compounds are capable of undergoing hydrolysis to yield a free aminothiol. This is shown in **Figure 4**. The aminothiol produced upon hydrolysis of amifostine is WR-1065, while hydrolysis of RibCys and PTCA yield cysteine. The observation of slower reaction rates for these compounds suggests that the compounds must first undergo hydrolysis before being reactive toward ABTS$^{\bullet+}$. However, the half-life of hydrolysis of RibCys has been reported to be 1.7 days under similar conditions to those used in our experiments (*14*). This is considerably longer than the 14.1 min half-life measured in our laboratory for the reaction of RibCys with ABTS$^{\bullet+}$. Therefore, a more likely mechanism for the reactivity of RibCys toward ABTS$^{\bullet+}$ involves abstraction of a hydrogen atom from the ring opened tautomeric iminothiol forming thiyl radicals, which then couple to form an iminodisulfide. The iminodisulfide would be expected to undergo rapid hydrolysis to yield cystine and ribose. Also PTCA probably follows a similar mechanism. See **Figure 5**.

Amifostine displays a similar kinetic profile to those displayed by the thiazolidines RibCys and PTCA. This compound has been shown to be stable in aqueous solution in the absence of the enzyme alkaline phosphatase (*17*). Therefore its reactivity toward ABTS$^{\bullet+}$ cannot be explained in terms of initial hydrolysis. This mechanism must be determined by further experimentation.

We also observed that the end point values for the various aminothiols differed considerably. The antioxidant capacity measured for penicillamine was over three times that observed for cysteine, despite the similarity in the chemical structures of the two compounds. Also, the TEAC value measured for amifostine is over three times as high as that measured for its dephosphorylated form, WR-1065. Although phosphate cannot be easily oxidized, its presence in the molecule is somehow rendering the molecule more reactive toward ABTS$^{\bullet+}$. The thiazolidine derivatives RibCys and PTCA had TEAC values over twice that which was observed for cysteine. The reasons for these differences are unknown and need to be probed by further experimentation.

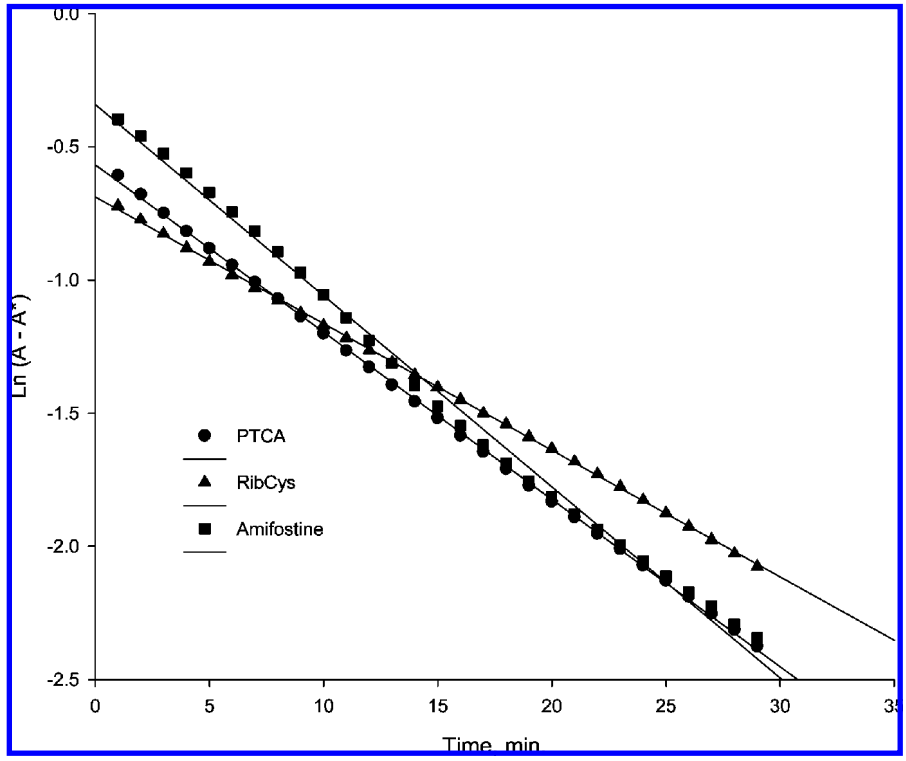

Figure 6. First order kinetics graphs of masked aminothiols. *A** = absorbance recorded when reaction has reached end point.

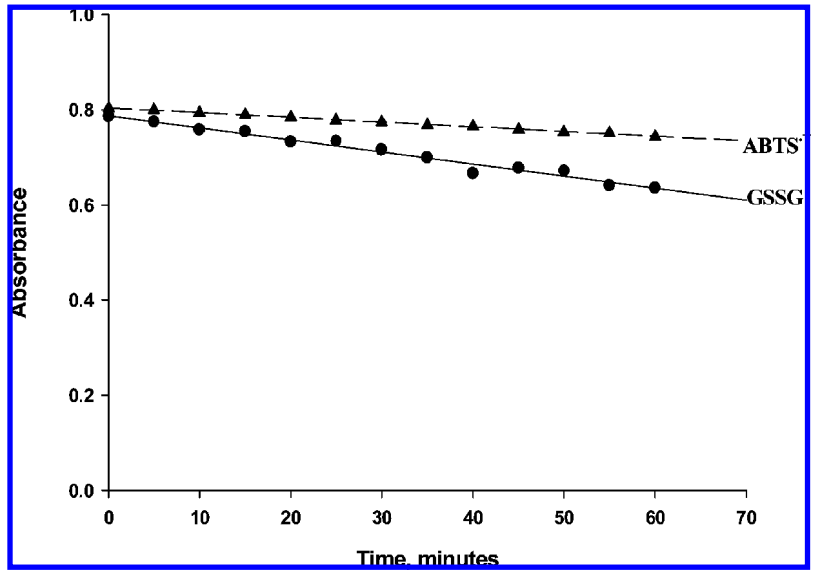

Figure 7. Reaction rate of glutathione disulfide (GSSG) with $ABTS^{\bullet+}$.

Another observation is that, despite very fast initial kinetics, glutathione, penicillamine and captopril do not fully reach end point at one hour. This would suggest that a biphasic kinetic pattern may be involved, similar to the behavior noted for some phenolic compounds. The possibility exists that, for aminothiols which seem to reach end point, such as WR-1065, cysteine and cysteamine, the slow step is still present, but is too slow to be detected under the conditions of the experiment.

In light of the evidence that dimerization may contribute to the biphasic kinetic pattern of some polyphenols, we tested the hypothesis that disulfides may display some reactivity toward $ABTS^{\bullet+}$, and that this may contribute to the biphasic pattern seen with some aminothiols. Glutathione disulfide demonstrated slow reactivity with $ABTS^{\bullet+}$. This suggests that the biphasic kinetic pattern observed for some aminothiols may be due to fast initial reaction of the thiol with $ABTS^{\bullet+}$ to form the disulfide, followed by slow reaction of the disulfide to form other products. See **Figure 7**.

What was observed for the phenolic compounds was consistent with that reported by other investigators. Trolox exhibited the fastest kinetics. It reached end point within several seconds. Ascorbic acid behaved similarly. Chlorogenic and caffeic acids demonstrated the longest half-lives.

Quercetin, although its initial rate was relatively fast, did not yield a stable end point even after one hour. This may account for the wide range of TEAC values reported for this compound by various investigators. Allowing a 30 min incubation period at 37 °C, we obtained a TEAC value of 4.97, which is close to the most commonly accepted value of 4.7 reported by Rice-Evans et al. (*18*).

The chemical structure of chlorogenic acid compared to that of caffeic acid might lead one to suspect that the abnormally

slow rate observed for chlorogenic acid may be related to the presence of the quinic acid moiety. Therefore, we measured the antioxidant capacity of quinic acid using the TEAC method. Quinic acid showed no measurable antioxidant activity even after four days. Possibly the quinic acid group in the chlorogenic acid molecule may provide steric hindrance to the access of $ABTS^{\bullet+}$ to the phenolic center.

The first order kinetics plots for amifostine, RibCys and PTCA are shown in **Figure 6**. It is evident from these plots that the data fit the first order kinetic model. Since the initial concentrations of test compounds and $ABTS^{\bullet+}$ used are similar ($\approx 10^{-4}$ M), one might expect second order kinetics. However two observations may be made. A multistep mechanism is proposed for reaction of the thiazolidines with $ABTS^{\bullet+}$ which is shown in **Figure 5**. The equilibrium between thiazolidine and ring-opened iminothiol tautomer strongly favors the thiazolidine (*13*). Therefore, concentrations of the reactive iminothiol would be very low with respect to $ABTS^{\bullet+}$. This would favor pseudo first order kinetics.

The half-lives measured for the reaction of these three compounds with $ABTS^{\bullet+}$ were amifostine, 10.04 min, RibCys, 14.14 min and PTCA, 11.00 min.

In summary, we observed that most of the antioxidants studied displayed a complex kinetic pattern of reaction with $ABTS^{\bullet+}$. Some did not reach stable end points at one hour. Our findings confirm the observations of other investigators that, to obtain clearer information concerning antioxidant capacities of food products and chemical compounds, kinetic profiles should first be established, then end point measurements should be taken at a time when the reaction has reached, or at least neared, the end point. Our study is unique in that it confirms the reactivity of aminothiols and amidothiols toward $ABTS^{\bullet+}$, and kinetic and end point data for several pharmacologically important compounds of this class are reported.

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