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# Structure–activity relationships of key bioantioxidants in reactions with radicals in DPPH, AAPH, and Hb–$H_2O_2$ systems

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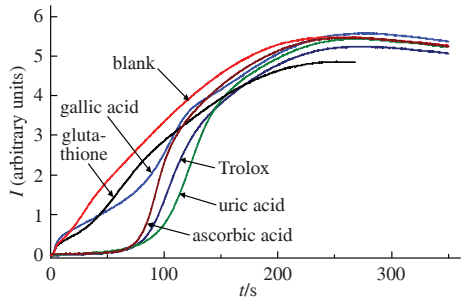
**A comparative study of three systems based on various free radicals (AAPH azo initiator, hemoglobin–hydrogen peroxide, and DPPH) was performed by examining the kinetic and analytical properties of several significant bioantioxidants treated with these radicals. Common trends in structure–activity relationships for all three methods were shown. The measured data suggest a possibility of extrapolating the results obtained from one of the three methods to another when analyzing different chemical and biological objects.**




*Keywords*: antioxidants, free radicals, chemiluminescence, luminol, DPPH, uric acid, ascorbic acid, gallic acid, glutathione, mexidol.

The current balance of antioxidants (AO) and prooxidants in organism largely determines the risks of many diseases, aging rates, tolerance to physical exertion, *etc*. The dynamics of total antioxidant activity (AOA) of blood and its components is highly informative in prognosis of various diseases progress.[1–3] Several studies have demonstrated changes of the plasma antioxidant status in patients with vibration disease, somatotropic insufficiency, and acromegaly.[4–6] In cells, among antioxidants, the highest concentrations are achieved by glutathione (GSH).[7,8] Its content in blood is in the range of 0.67–1.9 mM; in blood cells, 0.5–10 mM; and in plasma, 2–20 μM. The ascorbic acid (AA) content in plasma is in the range of 20–120 μM.

A lot of analytical methods were developed for the measurement of the antioxidant content and activity; however, to compare the results of different studies is still problematic. Therefore, studies of sensitivity of different analytical systems to antioxidants of various chemical structures and also their reaction kinetics are necessary.[9,10]

The aim of this work was a quantitative comparative study of three known methods for determining antiradical activity: two chemiluminescent (CL) model systems and a model with the use of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. To achieve this goal, the kinetic characteristics and stoichiometric inhibition coefficients of the most significant low molecular weight antioxidants† of different molecular structure present in human body (hydroxyl-containing derivatives of benzoic acid, chromane and purine derivatives, *etc*.) were studied.‡ The influence of the structure and properties of free radicals on these parameters was also analyzed.

For an antioxidant with a concentration [AO], the inhibition time of luminol oxidation can be calculated using the equation:

$$\tau = f\,[\mathrm{AO}]/R_\mathrm{i}, \qquad (1)$$

where $f$ is the stoichiometric inhibition coefficient and $R_\mathrm{i}$ is the rate of free radical initiation. The time interval $\tau$ from the initial point of the kinetic curve to the point of intersection of the tangent line applied to the CL curve at the maximum oxidation rate, with the time axis [Figure 1(*a*),(*b*)] is known as the induction period. In the case of glutathione, we drew an additional tangent to the kinetic curve before the onset of an increase in the oxidation rate, and from the point of intersection of the tangents, a perpendicular was dropped onto the abscissa axis. The measurement error of these parameters for the first device, taking into account the repeatability of the results, was no more than 15%, and for the second device it was 5%. The mechanisms of antioxidant reactions with the stable DPPH radical are described[11] previuosly.§

† Trolox [Tr, water-soluble analogue of vitamin E, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ascorbic acid (AA), uric acid (UA), gallic acid (GA), and reduced glutathione (GSH) were purchased from 'Sigma-Aldrich']. In addition to natural antioxidants, the study included the most widely used pharmaceutical antioxidant drug mexidol (2-ethyl-6-methyl-3-hydroxypyridine succinate, first discovered and synthesized at the IBCP RAS).

‡ The intensity of luminol chemiluminescence using the radical generation system 'hemoglobin–hydrogen peroxide' (Hb–$H_2O_2$) was recorded with a 'Lum-5773' instrument (DISoft, Moscow) at $T = 37.0 \pm 0.5$ °C according to the methodology.[12] 50 μl of Hb (15 μM, 'Sigma'), 150 μl of luminol (1 mM, 'AppliChem'), 10 μl of $H_2O_2$ (12 mM, 'Chimmed'), 2.4 ml of phosphate buffer (pH 7.4), and various volumes of the tested antioxidants (from 0.1 to 60 μl) with a concentration of 1 mM were mixed in the instrument cell. In the model system of luminol oxidation using an azo initiator (thermochemiluminescence, TCL), the reaction was also conducted at pH 7.4 and $T = 37 \pm 0.01$ °C. The TCL was recorded using the 'Minilum®' instrument (Institute of Antioxidant Therapy, Berlin, Germany) with the provided reagent kits, with a total volume of 1.5 ml in the TCL cell (www.minilum.de). The initiation rate was $1.77 \times 10^{-8}$ mol dm$^{-3}$ s$^{-1}$.

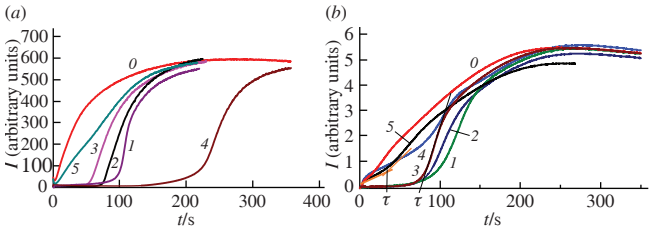



**Figure 1** Kinetic curves of chemiluminescence intensity for two models of luminol oxidation with (*a*) initiator 'Hb/$H_2O_2$' and (*b*) initiator AAPH. Curve marking: (*0*) blank experiment (without AO), (*1*) with UA, (*2*) with Tr, (*3*) with AA, (*4*) with GA, and (*5*) with GSH. The concentrations of all AOs in the CL cell were (*a*) 0.4 μM and (*b*) 0.66 μM.

Figure 1 shows the kinetic curves of chemiluminescence intensity obtained using both initiation methods. Trolox (Tr), AA, uric acid (UA), and gallic acid (GA) behave as strong antioxidants and have a pronounced induction period and a sharp exit from it, indicating that their rate constants with radicals of different structures are several orders of magnitude higher than the rate constants of these radicals interacting with luminol. Glutathione, being a weak antioxidant, has a weakly expressed induction period in 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) radical generation system and no induction period in the Hb–$H_2O_2$ system. Mexidol demonstrated a decrease in chemiluminescence intensity without any significant induction period (Figure 2).

Figure 3 shows that UA, AA, and GA also exhibit strong antioxidant properties with high reaction rate constants when they interact with stable radical DPPH. Mexidol and glutathione exhibit properties of weak antioxidants in this system: the consumption rate of DPPH is low even at relatively high concentrations of these substances.

The results obtained by the DPPH method vividly demonstrate why UA is able to fulfill function of ascorbic acid in providing blood antioxidant capacity in humans and primates which have lost the ability to synthesize ascorbic acid due to the knockout of the gene encoding gulono-lactone oxidase. Uric acid reactivity

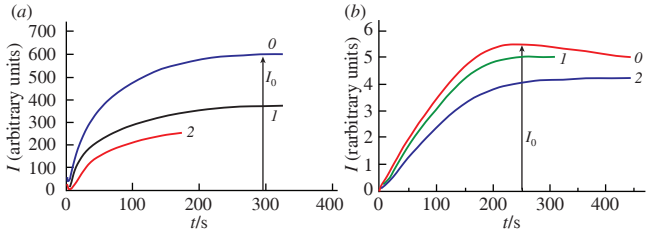



**Figure 2** Kinetic curves of chemiluminescence intensity for two models of luminol oxidation. (*a*) With initiator 'Hb/$H_2O_2$'; curve marking: (*0*) blank experiment (without AO), (*1*) with mexidol ($C$ = 8 μM), and (*2*) with mexidol ($C$ = 12 μM). (*b*) With initiator AAPH; curve marking: (*0*) blank experiment (without AO), (*1*) with mexidol ($C$ = 10 μM), and (*2*) with mexidol ($C$ = 20 μM).

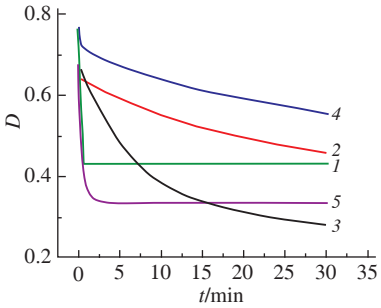



**Figure 3** Kinetic curves of changes in optical density of the DPPH solution in ethanol at a wavelength of 517 nm upon interaction with antioxidants: (*1*) $1.3\times10^{-5}$ M AA, (*2*) $4.9\times10^{-2}$ M mexidol, (*3*) $9\times10^{-6}$ M GA, (*4*) $9.4\times10^{-5}$ M GSH, and (*5*) $1.3\times10^{-5}$ M UA.

as a hydrogen atom donor, although inferior to that of ascorbic acid, significantly exceeds the reactivity of phenolic antioxidants.

The linear concentration dependences of the registered analytical parameters for all investigated AO and radical generation systems are presented in the Online Supplementary Materials. For the CL models, angular coefficients of the regression lines can be calculated according to the equation:

$$k = f/R_i. \quad (2)$$

The highest tangent of the regression line slope (see Online Supplementary Materials) was 572 s dm$^3$ μmol$^{-1}$ for GA. For UA, Tr and AA, $k$ values are 265, 223, and 155 s dm$^3$ μmol$^{-1}$, respectively. In the case of AAPH free radical generation (*b*), $k$ were 125, 113, 87, 76, and 55 s dm$^3$ μmol$^{-1}$ for UA, Tr, AA, GA, and GSH. According to numerous studies, the stoichiometric coefficient of Trolox is used as a universal 'reference' in different model systems and is equal to 2.[13–15] It allows one to calculate the initiation rate of the Hb–$H_2O_2$ system, $R_i$, at the given experimental temperature and pH values, which was found to be $9\times10^{-9}$ mol dm$^{-3}$ s$^{-1}$.

Another way to evaluate stoichiometric inhibition coefficients for GSH consists of calculating the area between the kinetic curve of GSH and the blank curve, corresponding to the time from the origin to the point of intersection of these curves, which corresponds to the depletion of glutathione in the system. The desired value is obtained by dividing this area by a similar square for the Tr curve at its same concentration and multiplying the result by the $f$ value for Tr which is equal to 2 [equation (3)]:

$$f_{GSH} = 2(S_{bl} - S_{GSH})/(S_{bl} - S_{Tr}), \quad (3)$$

where $S_{bl}$, $S_{GSH}$, and $S_{Tr}$ are squares under blank, GSH, and Tr curves, respectively.

Stoichiometric inhibition coefficients calculated according to formulas (2) and (3) are presented in Table 1. It is not possible to calculate the stoichiometric inhibition coefficient for mexidol in CL systems due to the absence of an induction period. Therefore, a kinetic parameter $K$ was calculated for it, which expresses the relative decrease in CL normalized to the concentration of AO [equation (4)]:

$$K = (I_0/I - 1)/C. \quad (4)$$

For mexidol, which does not have an induction period, comparing the kinetic parameters $K$ provides information about the ratio of free radical inhibition rate constants. It is evident that mexidol exhibits a higher inhibition rate constant to radicals generated in the Hb–$H_2O_2$ system.

For UA and AA which constitute the basis of blood antioxidant capacity, the $f$ values are close for each antioxidant in all three model systems used. Gallic acid showed a 3.8 times higher inhibition coefficient in the Hb–$H_2O_2$ model compared to the azo initiator generation system. The first result is close in value to the

§ Different volumes of aqueous AO solutions were mixed with ethanol to a final volume of 0.8 ml and quickly added to 2.4 ml of $8.1\times10^{-5}$ M DPPH solution in ethanol with stirring. The kinetic curve of the optical density decrease was recorded using a 'Specord M40' spectrophotometer (Carl Zeiss Jena, Germany) equipped with a computer and Soft Spectra 5,0 software at a wavelength of 517 nm in 1 cm thermostated cuvette at 298 K. The final point used for calculating the stoichiometric inhibition coefficients was measured after 30 min of the reaction start. The volume of the added AO solution was varied to ensure that the DPPH radical conversion degree at the end of the experiment was in the range of 15–70%. For glutathione, an additional series of experiments was conducted with the registration of the final optical density value after 24 h of mixing the reagents. The DPPH concentration during the experiments was determined based on the molar extinction coefficient of 12 350 dm$^3$ mol$^{-1}$ cm$^{-1}$.

**Table 1** Kinetic parameters ($K$) and stoichiometric inhibition coefficients ($f$) for radical generation by the Hb–$H_2O_2$ system, AAPH, and DPPH.

| Anti-oxidant | $K$/μM$^{-1}$ | | $f$ | | |
|---|---|---|---|---|---|
| | Hb–$H_2O_2$ | AAPH | Hb–$H_2O_2$ | AAPH | DPPH |
| Trolox | – | – | 2.0 | 2.0 | 2.0 |
| UA | – | – | 2.4±0.2 | 2.2±0.1 | 2.2±0.1 |
| AA | – | – | 1.4±0.1 | 1.5±0.1 | 2.0±0.1 |
| GA | | | 5.1±0.3 | 1.3±0.1 | 4.8±0.3 |
| Glutathione | – | – | 0.70±0.1[a] | 1.0±0.1[b]<br>0.9±0.1[a] | 0.13±0.01[c]<br>1.0±0.1[d] |
| Mexidol | $8.5\times10^4$ | $3.4\times10^4$ | – | – | $(6.0\pm2.1)\times10^{-4}$ |

[a] By square. [b] By $\tau$. [c] 30 min exposition. [d] 24 h exposition.

stoichiometric inhibition coefficient obtained by us for GA when interacting with the stable radical DPPH (4.8). In styrene free radical oxidation initiated by azobisisobutyronitrile (AIBN),[16] a stoichiometric inhibition coefficient of 1.0 was obtained for GA, which is close in value to the result for the AAPH radical generation system in this study. As we can see from Figure 1(*b*), GA shows not full CL inhibition in the case of AAPH initiator. Thus, significant differences in the values of the stoichiometric inhibition coefficient of GA when using different free radical initiators are presumably related to the much higher reactivity of alkylperoxy radicals in comparison with ferryl [Hb($^{\bullet+}$)–$Fe^{4+}$=O] and superoxide radicals formed in the case of the Hb–$H_2O_2$ system. In addition, GA in a weakly alkaline medium (pH 7.4) has several groups with a negative charge, which may contribute to electrostatic attraction to cationic ferryl radicals. Full CL inhibition in the Hb–$H_2O_2$ system by GA indicates that OH$^{\bullet}$-radicals do not form in it. As for the DPPH system, stability of this radical makes it possible to reach deep conversions of antioxidants by long reaction time including consumption of their oligomerization products. Thus, we see a high stoichiometric inhibition coefficient of GA.

The stoichiometric inhibition coefficient for GSH in DPPH test is equal to the AAPH CL method if 24 h exposition is used. Stoichiometric inhibition coefficient estimation by the induction period and simultaneously by the method of squares showed no significant difference between these calculation methods.

As shown previously,[17] some thiol compounds, including glutathione, can reduce the hydroperoxides formed during oxidation according to equation (5) forming an additional source of radicals. This reaction shows the reason of the lower GSH stoichiometric inhibition coefficient in the Hb–$H_2O_2$ CL system compared with other two methods.

$$RSH + ROOH \rightarrow RS^{\bullet} + RO^{\bullet} + H_2O \quad (5)$$

It should be noted that using a 30-min DPPH test or a Hb–$H_2O_2$ CL test with induction period estimation can lead to the sufficient GSH underestimation during the multicomponent object analysis due to the low rate constant of GSH interaction with radicals of different structures despite a high GSH concentration in cells and its role as a universal redox buffer. This factor should be taken into account when interpreting research results.

The model system based on the use of the DPPH radical is interesting because it allows easy determination of one-electron oxidation energy contribution to antioxidant inhibitory effectiveness (*via* homolytic and heterolytic mechanisms).

In summary, the obtained data suggest a possibility of comparing the results obtained by different methods. This is particularly relevant in view of the fact that chemiluminometers are highly specialized equipment, which, unlike spectrophotometers, are available in a limited number of laboratories. Also it may be noted that the AAPH CL method has a physicochemical nature close to the widely used but labor-intensive ORAC (oxygen radical absorbance capacity) method.[18] The DPPH method has gained wide popularity due to its simplicity, rapidity and high reproducibility of results.

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*Online Supplementary Materials*

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.7466.

## References

1 I. Popov and G. Lewin, *Luminescence*, 1999, **14**, 169; https://doi.org/10.1002/(SICI)1522-7243(199905/06)14:3<169::AID-BIO539>3.0.CO;2-K.
2 I. N. Popov, G. Lewin, A. K. Anosov, A. A. Markin and B. V. Morukov, *Bull. of RSMU*, 2013, **2**, 69 (in Russian); https://www.elibrary.ru/item.asp?id=24329472.
3 I. N. Popov and G. Lewin, *Biophysics*, 2013, **58**, 669; https://doi.org/10.1134/S0006350913050138.
4 M. V. Faassen, M. S. Pankratova, N. N. Molitvoslovova, A. A. Baizhumanov, S. S. Kovalenko, A. I. Yusipovich, G. V. Maksimov and V. A. Peterkova, *Probl. Endokrinol.*, 2015, **61** (2), 8 (in Russian); https://doi.org/10.14341/probl20156128-11.
5 M. S. Pankratova, A. I. Yusipovich, M. V. Vorontsova, S. S. Kovalenko, A. A. Baizhumanov, E. Yu. Parshina, T. Yb. Shiryaeva, G. V. Maksimov, V. A. Peterkova and I. I. Dedov, *Probl. Endokrinol.*, 2012, **58** (5), 10 (in Russian); https://doi.org/10.14341/probl201258510-15.
6 N. N. Malyutina, A. F. Bolotova, R. B. Eremeev, A. Z. Gilmanov and D. Y. Sosnin, *Med. Tr. Prom. Ekol.*, 2019, **59**, 978 (in Russian); https://doi.org/10.31089/1026-9428-2019-59-12-978-982.
7 O. V. Trineeva, *Razrab. Regist. Lek. Sredstv*, 2017, no. 4, 180 (in Russian); https://www.pharmjournal.ru/jour/article/view/515/510.
8 O. G. Tishkova, L. V. Dikareva and D. D. Teply, *Astrakh. Med. Zh.*, 2023, **18** (1), 27 (in Russian); https://doi.org/10.29039/1992-6499-2023-1-27-38.
9 J. S. Mendonça, R. C. Avellaneda Guimarães, V. A. Zorgetto-Pinheiro, C. Di Pietro Fernandes, G. Marcelino, D. Bogo, K. de Cássia Freita, P. Aiko Hiane, E. Silva de Pádua Melo, M. L. Brandão Vilela and V. Aragão do Nascimento, *Molecules*, 2022, **27**, 3563; https://doi.org/10.3390/molecules27113563.
10 I. G. Munteanu and C. Apetrei, *Int. J. Mol. Sci.*, 2021, **22**, 3380; https://doi.org/10.3390/ijms22073380.
11 Yu. O. Teselkin, I. V. Babenkova, O. B. Lyubitsky, G. I. Klebanov and Yu. A. Vladimirov, *Vopr. Med. Khim.*, 1997, **43**, 87 (in Russian); https://www.elibrary.ru/item.asp?id=22536849.
12 V. A. Volkov and V. M. Misin, *Kinet. Catal.*, 2015, **56**, 43; https://doi.org/10.1134/S0023158415010139.
13 E. A. Lissi, M. Salim-Hanna, C. Pascual and M. D. Castillo, *Free Radical Biol. Med.*, 1995, **18**, 153; https://doi.org/10.1016/0891-5849(94)00117-3.
14 E. L. Bastos, P. Romoff, C. R. Eckert and W. J. Baader, *J. Agric. Food Chem.*, 2003, **57**, 7481; https://doi.org/10.1021/jf0345189.
15 J. T. Uotila, A. L. Kirkkol, M. Rorarius, R. J. Tuimala and T. Metsä-Ketelä, *Free Radical Biol. Med.*, 1994, **16**, 581; https://doi.org/10.1016/0891-5849(94)90058-2.
16 I. Tikhonov, V. Roginsky and E. Pliss, *Int. J. Chem. Kinet.*, 2009, **41**, 92; https://doi.org/10.1002/kin.20377.
17 E. A. Mengele, D. A. Krugovov and O. T. Kasaikina, *Russ. Chem. Bull.*, 2015, **64**, 846; https://doi.org/10.1007/s11172-015-0943-1.
18 M. V. Voronkov, V. A. Volkov, V. V. Volkov, V. S. Romanova, I. G. Plashchina and E. V. Sidorsky, *Mendeleev Commun.*, 2023, **33**, 823; https://doi.org/10.1016/j.mencom.2023.10.028.

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