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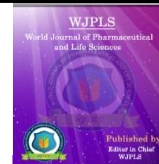

KINETICS OF INHIBITING EFFECT OF SOME WATER-SOLUBLE BIOANTIOXIDANTS AND THEIR MIXES IN CHEMILUMINESCENT MODEL SYSTEM

Dr. Natalya Sazhina*

Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, 4 Kosygin Str., Moscow, 119334 Russia.

***Corresponding Author: Dr. Natalya Sazhina**
Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, 4 Kosygin Str., Moscow, 119334 Russia.

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ABSTRACT

Definition of antioxidant activity (AOA) of various biological objects: foodstuff, medicines, drinks, blood plasma and other human biological liquids is an important task for medical and biological researches. Now for this purpose chemiluminescent (CL) methods are widely used. They are sensitive, operate and allow controlling directly kinetics of oxidation inhibition by antioxidants (AO). Biological substrates contain a wide range of antioxidants various on the chemical structure, and the total AOA is the integrated value characterizing a possibility of their combine action taking into account potential synergism. In the present work the water-soluble CL-model of free radical oxidation of the luminal initiated by the mix "hemoglobin-hydrogen peroxide" was used. Kinetics of inhibiting action of 8 water-soluble bio antioxidants and their binary mixes has been studied. The inhibition parameters for these antioxidants and their stoichiometric coefficients were determined. Evaluation of synergetic or antagonistic interaction in mixes of used AO has made. For the majority of binary AO mixes independence of CL suppression by separate AO has been revealed and more "active" AO inhibited oxidation earlier, than less "active". The exception was only for mixes of some AO which at interaction strengthened or weakened each other, showing synergetic or antagonistic effects.

KEYWORDS: antioxidant, chemiluminescence, synergism, antagonism.

INTRODUCTION

Determination of antioxidant activity (AOA) of different biological objects: foodstuff, medicines, drinks, blood plasma and other biological liquids is the important task for medico-biological researches as the inhibiting effect of antioxidants in these objects makes a contribution to the protective force of a human body in fight against oxidating stress. Specified objects, as a rule, contain a wide range of antioxidants, different on the chemical structure. In blood plasma, for example, antioxidant composition is caused by availability in it of amino acids, vitamins, hormones, enzymes, but main connections defining summary blood plasma AOA are such water-soluble components as uric and ascorbic acids, and also bilirubin, glutathione and proteins.[1] Summary AOA is the integral value characterizing a possibility of combined action of all antioxidants taking into account their potential synergism. The choice of adequate method for determination of AOA is very important for the correct assessment of results. Now chemiluminescent (CL) methods for determination of different biological substrate AOA are widely used. They are rather sensitive, operational and allow controlling directly kinetics of oxidation inhibition by antioxidants,[2,14] A large number of CL methods which are widely used in organic chemistry are based on the initiated oxidation of different hydrocarbons. As a result of peroxide radical recombination of these hydrocarbons the excited molecules of emitting light products are formed. In such systems for light gain different activators are used, and effective oxidation proceeds at temperatures of 50-80^{0}C.[3,5] These CL-models apply, as a rule, for studying of fat-soluble antioxidants.

In other CL-models for measurement of water-soluble antioxidant AOA as a chemiluminescent oxidation substrate a luminol is used.[6,14] Initiation of radicals is carried out, in most cases, or at thermo - destruction of water-soluble azo-compounds.[7,10,14] or at UF radiation of luminol,[11] or at interaction of gem-containing derivatives with hydrogen peroxide.[8,9] In the present work the water-soluble CL-model of free radical luminol oxidation initiated by a mix "hemoglobin – hydrogen peroxide" was used.[8]

In the works mentioned above authors defined, as a rule, AOA of blood plasma and other biological liquids, extracts of herbs, various drinks. However inhibiting

activity of individual antioxidants and their combine action practically weren't studied. Some works on kinetic research of the combine inhibiting effect of various structure antioxidants in the initiated oxidation process of lipid substrates are given in the monograph.[15] However, fat-soluble initiators at high, not physiological temperatures and a volumetric method of registration were generally used. Therefore it is expedient to investigate by operate CL-method in water-soluble oxidizing model at T=37C kinetics of the inhibiting effect for various antioxidant mixes and interaction of antioxidants among themselves, i.e. existence of their synergysm and antagonism.[16]

The purpose of work – studying of kinetics of inhibiting effect for some water-soluble bioantioxidants and their binary mixes in chemiluminescent oxidizing model system "hemoglobin (Hb) - (H_2O_2)-luminol", and the analysis of the received results.

MATERIALS AND METHODS

The following 8 substrates have been used in experiment: uric acid (UA, “Sigma”), glutathione reduced (Gl) and oxidized (Gl.ox.,” Sigma-Aldrich”), trolox (Tr, "Aldrich") - a water-soluble analog of α-tocopherol; ascorbic acid (AA, "Sigma"), gallic acid (GA, "Sigma"), mexidol (M, synthesized in IBCP RAS) and fenozan potassium (FP, synthesized in IBCP RAS). All this substances are used as oxidation inhibitors in different biological systems, food additives, medicines. As shown in Figure1, all tested substances have different structure, but possess significant well-known antioxidant properties.

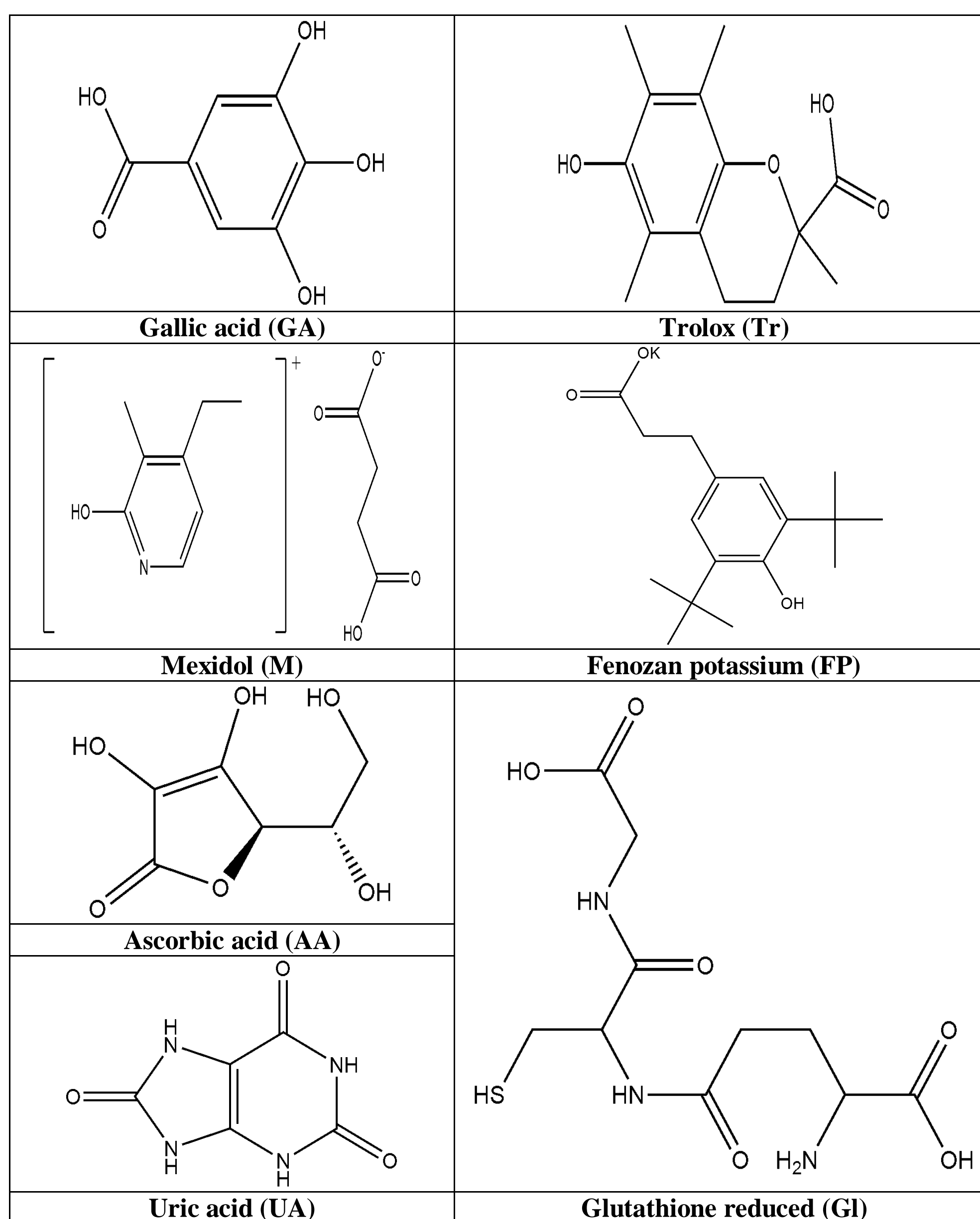

Figure 1: Structural formulas of tested compounds.

In work the water-soluble model of free radical oxidation "(Hb) - (H_2O_2) – luminol" in which generation of radicals by Hb and H_2O_2 interaction was used, and

luminol plays a role of a chemiluminogenic oxidative substance.[8] Distinctive feature of this model from other oxidation models is that the formed radicals can initiate free radical oxidation reactions in vivo as blood contains Hb and H_2O_2. Besides, all reagents are available and aren't toxic.

The kinetics and concrete schemes of reactions proceeding at interaction of Hb with H_2O_2 are rather difficult and were studied in.[17-20] So, on the one hand, interaction of H_2O_2 with metHb (commercial MetHb – Hb-Fe^{3+} is used) is accompanied by gem destruction and exit from it of iron ions which participate in education of $OH^{\bullet}$ - radical. Besides, as a result of this interaction active ferril-radicals ($Hb(^{\bullet+})–Fe^{4+}=O$) are formed. Being formed radicals initiate luminol oxidation which sequence of reactions is well-known now. In the oxidation process $L^{\bullet-}$, $O_2^{\bullet-}$-radicals are formed, a luminol-endoperoxide LO_2^{2-}, and further an aminophthalate anion in excited state $(AP^{2-})^*$ upon which transition to the main state light quantum hν is highlighted. Addition in reactionary system of bivalent ($FeSO_4$) and trivalent ($FeCl_3$) iron salts instead of Hb doesn't give a luminescence.[8] that indicates the need of presence of radical initiators with gem structure. In work.[9] authors for elimination of side effect of hemoglobin (globin) proteins used hemin in the “hemin-H_2O_2-luminol” system. In the present work replacement of hemoglobin by equivalent amount of hemin hasn't led to change of luminescence parameters.

Kinetic CL-curves were registered on “Lum-5773” device [www.chemilum.ru] at T=(37,0±0,5)°C. Initial solutions of Hb (15 µM, MetHb "Sigma"), luminol (1 mM, "AppliChem"), H_2O_2 (12 mM, “Chimmed”), phosphatic buffer (pH =7,4) were prepared. All studied antioxidants with concentration 1 mM were dissolved in buffer or distilled water. The key measured parameter characterizing antiradical activity of studied compounds was induction period τ. It decides as time from the oxidation initiation moment to a point of intersection on time axis of the tangent attached to CL-curve in the point corresponding to a maximum of its first derivative dI/dt. For antioxidants reducing CL-intensity without the expressed induction period, the measured parameter of their activity was the coefficient of intensity suppression in comparison with blank in point of his maximum (I_0/I). Statistical processing was carried out with use of standard algorithms of MS Excel programs. All results are presented in the form of average values ± standard error of an average.[21] The measurement error of all parameters taking into account repeatability of results has made no more than 15%.

RESULTS AND DISCUSSION

The received kinetic CL-curves for the used in work AO are given in Figure 2. Tr, AA, UA and GA have accurate induction period, Gl doesn't suppress a luminescence completely and has no expressed induction period (the explanation is given further) (2a). CL-curves for the oxidized Gl (Gl.ox.) coincide with blank. FP and mexidol (M) show decrease in amplitude of CL-curves without any considerable induction period even at much bigger concentration in comparison with other AO (2b). Tr, AA, UA and GA behave as the strong antioxidants. The induction period of CL at their presence can be considered as time necessary for their inactivation in the course of interaction with the radical initiators who are formed in system. For FP and M suppression of CL is caused by the speed of their interaction with intermediate radicals during luminol oxidation.

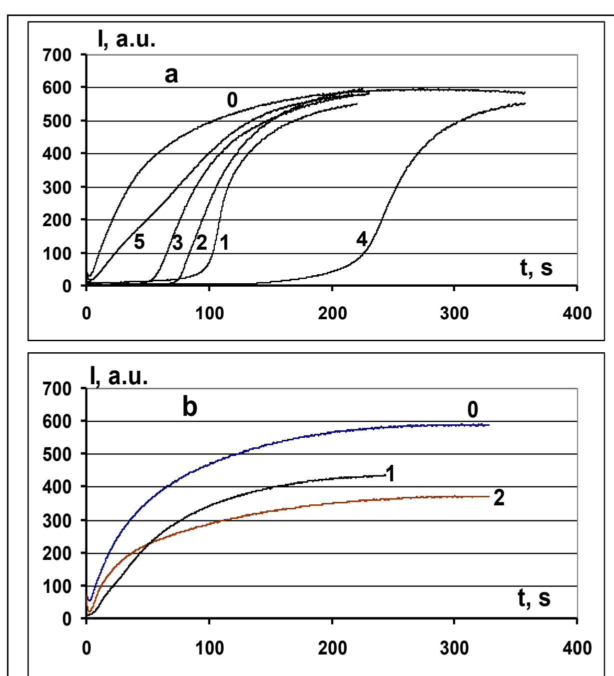

Figure 2: Kinetic CL-curves: (a) 0-blank, 1-UA, 2-Tr, 3-AA, 4-GA, 5-Gl. Concentration of all AO in CL-cell is equal 0,38 µM; (b) - 0-blank, 1-FP, 2-M, concentration in cell 7,6 µM.

I – intensity of CL.

Dependences of induction period τ on AO concentration are given in figure 3a. In the studied AO concentration range these dependences are linear and τ =k[AO], and k defines anti-radical activity of antioxidants. In fig. 3b for FP and M concentration dependences on extent of maximum CL intensity in comparison with blank (I_0/I) are presented. K = (I_0/I-1)/[AO] - amplitude inhibition coefficient is received.

Values k and K for all used AO, and also their stoichiometric coefficients of inhibition f (number of the radicals intercepted by one molecule of the AO) are presented in Table. For a trolox f=2 has been accepted.[7,9,13]

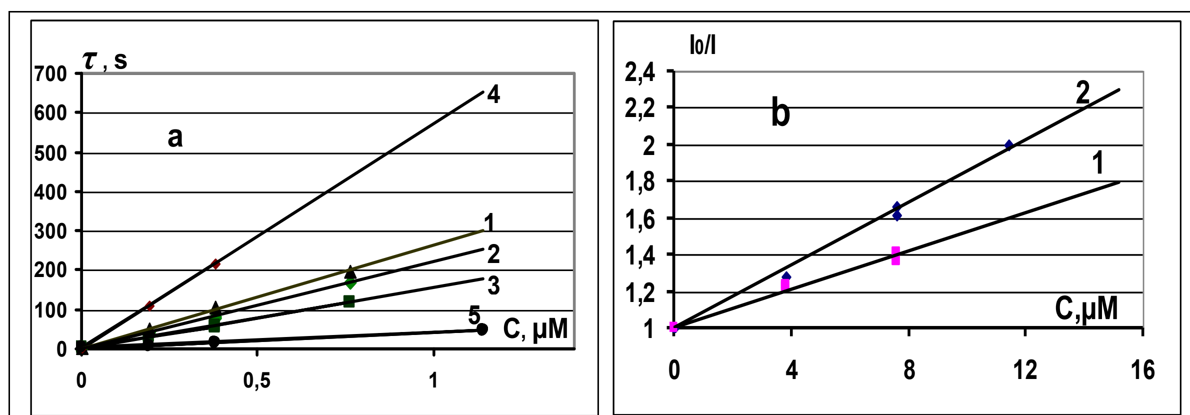

Figure 3: (a) - dependence of induction period τ on concentration C for: 1-UA, 2-Tr, 3-AA, 4-GA, 5-Gl and (b) - extent of CL amplitude suppression for (I0/I) on C for 1-FP and 2-M.

Table 1: Antiradical activity k, amplitude intensity suppression coefficient K and stoichiometric coefficients of inhibition f for studied AO.

Antioxidant (AO)	k, $s \cdot M^{-1}$	K, M^{-1}	f
Trolox	$(2,23\pm0,12)\cdot10^8$		2,0
Uric acid	$(2,65\pm0,16)\cdot10^8$		2,35±0,20
Ascorbic acid	$(1,55\pm0,17)\cdot10^8$		1,39±0,15
Gallic acid	$(5,41\pm0,29)\cdot10^8$		4,84 ±0,34
Glutathione	$(0,42\pm0,06)\cdot10^8$		0,38±0,06
Mexidol		$(8,5\pm0,9)\cdot10^4$	
Fenozan potassium		$(5,2\pm0,7)\cdot10^4$	

Stoichiometric coefficients of inhibition for UA, AA were defined by many authors [7-9, 12-14]. The values f, closest to our values, were received in work.[13] for CL-model with the azo-initiator (ABAP). In this work f=0,44 for compounds of SH-group has been also defined, however the kinetics of CL hasn't been investigated. As already it has been noted earlier, kinetic CL-curves for Gl (Fig. 2, curve 5) markedly differ from CL-curves for other AO, without well expressed induction period. It demonstrates what Gl interacts with the formed intermediates during of oxidation, changing its rate and kinetics. As shown in work.[22] some thiols, including Gl, can restore hydroperoxides (LOOH) which are formed at oxidation, and this restoration can pass through radical stages RSH+LOOH → $RS^{\bullet}$ + $RO^{\bullet}$ + H_2O, forming additional source of radicals and changing process of inhibition. In our case H_2O_2 is this hydroperoxide, acquiring $HS^{\bullet}$ and $OH^{\bullet}$ - radicals, and strengthening luminol oxidation. Therefore induction period isn't accurately expressed, and stoichiometric inhibition coefficient f for glutathione has rather low value.

Antiradical (ARA) and antioxidate (AOA) activities of bioantioxidants depend on structure of their molecules.[23] (Fig. 1). In particular, for phenol AO - from durability of OH-bond (D_{OH}), from length of this bond, from character of the deputy in ortho- and para-position, extent of shielding of hydroxyl groups in a benzene ring, ortho-effect of two hydroxyl groups or hydroxyl and carbonyl groups.[24] Shielding of OH-group leads to essential increasing in activity of antioxidants in comparison with unshielded phenol. Tret-butil deputies in ortho-configuration increase electronic density on OH-group, decreasing her dissociation energy D_{OH}.[24,25] High antiradical activity of gallic acid is explained by existence in her molecule three electro-donor OH-deputies, durability of OH-bond or her bond dissociation energy D_{OH}=347 of kJ/mol.[25] For trolox dissociation energy of the weakest OH-bond is higher.[25] therefore ARA under the same conditions is less. In literature there isn't enough information on dissociation energy of not phenol AO. Uric acid doesn't belong to phenol compounds and has rather low potential of two-electronic electrochemical oxidation (≈0,65 B.[26] and his stoichiometric inhibition coefficient is rather high. Ascorbic acid is monosaccharide derivative and a strong reducer. Characteristic structure feature of ascorbic acid is the presence in her molecule group –C=C-, which defines high biological activity and oxidation-reduction properties.[24] However her ARA and stoichiometric coefficient are less, than for Tr and UA. In the presence of oxygen ascorbic acid is oxidized quickly with formation of dehydroascorbin acid that complicates work with her. Glutathione – an important coenzyme for glutathione-peroxidase activity and also provides protection of sulfhydryl groups of proteins against oxidation, itself at the same time being oxidized to a disulfide. However, as it has been told above, the inhibiting activity of Gl in oxidizing models with hydroperoxides is much less because of ability of Gl to form with them additional free radicals. A mexidol and a

fenozan potassium are more active in reactions with peroxil radicals, than with radical initiators. They belong to spatial complicated phenols and are as the majority of monophenols, rather weak inhibitors.

ARA and AOA values received in real work well correlate with similar parameters for same compounds measured by other methods.[9,14,27,28] For example, when comparing our ARA values with coefficients of electrochemical oxidability received by amperometry for the same AO, the correlation coefficient has made r=0,97.[28]

For studying of interaction between certain AO during inhibition of luminol oxidation kinetic CL-curves for various combinations of binary mixes of AO (1:1) have been received. Some of them are given in Figure 4. Concentration of all AO, except a mexidol and a fenozan potassium, was identical separately for AO and in their mixes. In the majority of mixes antioxidants act independently from each other, and curves for mixes move to the right (a, b, c). The first more active AO are spent. For mix AA+Gl (c) it is well visible that Gl begins to inhibit oxidation after "working off" of AA. Mix AA+Glox. has shown synergetic effect, i.e. AA strengthens her action in the presence of oxidized glutathione (d). Perhaps, AA partially restores Gl.ox. A mexidol and a fenozan potassium begin "to work" with other AO only after the end of induction period of "stronger" AO (e, f).

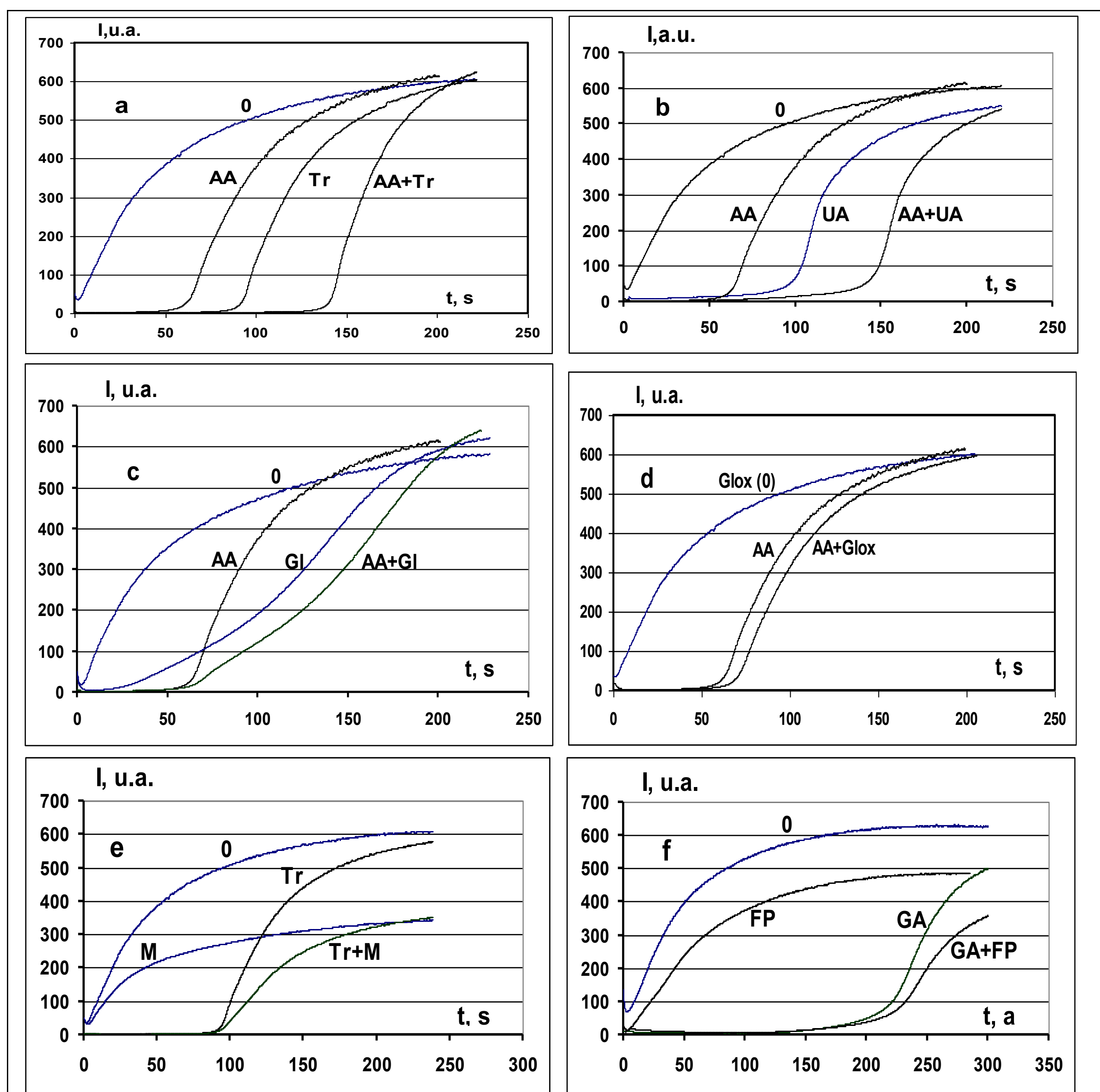

Figure 4: Kinetic curves of CL-suppression by some AO and their binary mixes. Concentration of AA, GA, Tr, UA in all figures - 0,38 µM, Gl – 0,76 µM, mexidol – 7,6 µM (e), FP – 3,8 µM (f), Glox. – 10 µM (d).

In fig. 5a for various couples of AO the comparative values of induction periods τ_c, calculated as the sums τ for certain AO with identical concentration ($\tau_c = \tau_i + \tau_j$), and measured values τ_m for binary mixes with the corresponding calculation and measurement errors are given. The error τ_c was calculated as sum error of two values.[21] error for τ_m – proceeding from repeatability of results.

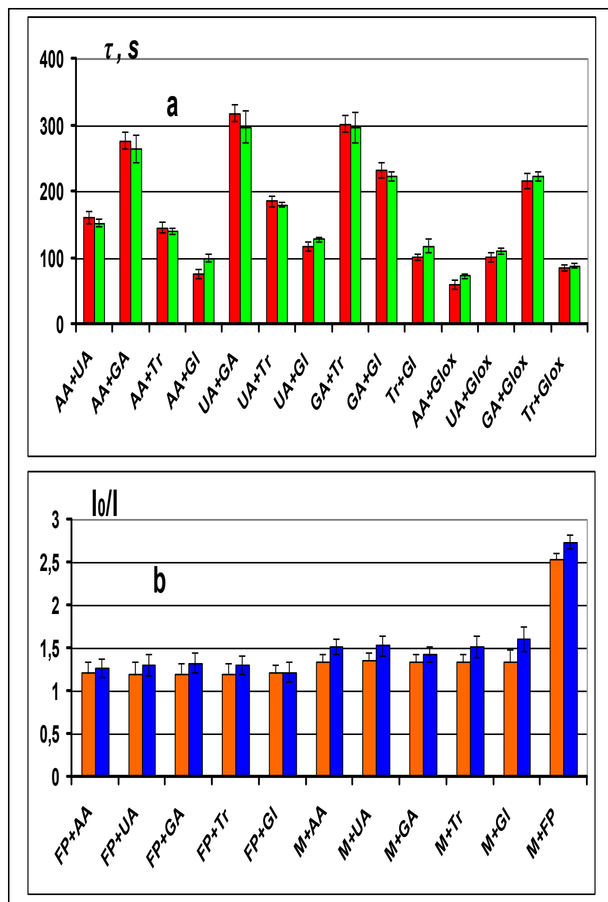

Figure 5: (a) - Calculated (by additivity) induction periods $\tau_c = \tau_i + \tau_j$ (left columns) and experimentally measured τ_m (right columns) for various AO couples. Concentration of all AO is equal 0, 38 µM, the oxidized glutathione Glox. - 10 µM. (b) – The suppression of CL-maximum amplitude (Io/I) by binary mixes of FP and M with other AO. Left columns – calculation of Io/I by additivity for various mixes, right – experimentally measured Io/I for their mixes. Concentration of FP and M is equal 3,8 µM, for others AO – 0,38 µM.

It is visible (a) that within measurement errors for the majority of mixes calculated values coincide with measured that is explained by independence of luminol oxidation inhibition by components of these mixes. In these cases additivity is observed. The exception is made by mixes of AA, Tr, UA with Gl and also AA with Glox. These mixes show synergetic effect, most likely, because of "abnormal" behavior of Gl during oxidation. In mix AA+ Glox., perhaps, occurs partial reduction of Glox. by ascorbic acid. The same effect was observed at electrochemical oxidation of this mix.[28]

Fig.5 b shows decrease of maximum amplitude of CL (Io/I) by binary mixes of FP and M with other AO. Left columns – calculated (by additivity) values according to table for FP and M, right – experimentally measured values (Io/I) for various AO couples. For the majority of mixes, within measurements and calculation errors, decrease of CL-amplitude happens only due to action of FP and M, however some AO (AA, UA, Gl) with mexidol strengthen this action. Mexidol and FP show synergetic effect, interacting with each other [16]. For majority of mixes for M and FP with other AO small (to 10%) increase in the measured induction periods τ_m in comparison with calculated on additivity τ_c is observed. An exception have shown two couples “M+AA” and “FP+AA” at which τ_m have decreased approximately twice (from 60 to 35s) that demonstrates strong influence of FP and M on oxidation inhibition of luminol by ascorbic acid (antagonism).

CONCLUSION

The kinetics of the inhibiting effect of some water-soluble bioantioxidants and their binary mixes in oxidizing model system "hemoglobin (Hb) - (H_2O_2)-luminol" is studied. Luminol oxidation inhibition parameters for these AO and their stoichiometric coefficients are determined. The feature of CL suppression by glutathione explained with education of intermediate radicals at interaction of glutathione with hydroperoxides is revealed. Comparison of the inhibiting activity of studied antioxidants with their antiradical activity received by other methods is carried out.

For the majority of binary mixes of the used AO independence of CL suppression by certain AO has been noted, and more "active" AO inhibited oxidation earlier, than "not active". The exception was made by mixes of some AO: AA + Gl, UA + Gl, Tr + Gl, AA + Glox., FP + M. These antioxidants at interaction have strengthened each other, i.e. have shown synergysm. For mixes AA+FP and AA+M antagonism was observed – the induction period for them has decreased twice in comparison with the induction period for AA that demonstrates her interaction with FP and M and their competition during luminol oxidation inhibition.

The received results can be used in medico-biological researches.

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