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Chemiluminescent *in vitro* Estimation of the Inhibitory Constants of Antioxidants Ascorbic and Uric Acids in Fenton's Reaction in Urine

E. L. Pavlova* and V. M. Savov

Medical Physics, Faculty of Physics, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria; fax: +359-296-252-76; E-mail: elli_pavlova@abv.bg

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Abstract—The goal of this research was to measure *in vitro* the inhibitory constants of the antioxidants ascorbic and uric acid in urine, with lucigenin enhanced chemiluminescence (CL) in Fenton's system. Maximum CL emission is registered in urine containing H_2O_2 ($5\cdot10^{-4}$ M), Fe^{2+} ($5\cdot10^{-5}$ M), EDTA ($5\cdot10^{-5}$ M), and chemical enhancer lucigenin (10^{-4} M) at pH 5.5 and 36°C. Ascorbic acid exhibits up to 4-fold stronger antioxidant effect than uric acid. The constants of antioxidant inhibition in urine were measured at concentrations 10^{-3} and 10^{-4} M: for ascorbic acid, 5.92 ± 0.04 and 24.05 ± 1.82 μmol·sec^{-1}; for uric acid, 1.60 ± 0.02 and 21.45 ± 0.97 μmol·sec^{-1}, respectively. Three phases of CL kinetics of urine are well observed: spontaneous CL (0-10 sec), fast flash of CL (10-50 sec), and latent period (50-300 sec). The antioxidant efficiency of ascorbic and uric acids in the final stage of catabolic processes in the body is discussed.

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Key words: chemiluminescence, inhibitory constant, kinetics, urine, ascorbic acid, uric acid

The processes of free radical oxidation play an important role in the metabolism and homeostasis of the organism [1]. Their activation appears to be a universal mechanism and common cause for different diseases—acute and chronic bacterial and viral infections, atherosclerosis, heart and renal failure, etc. [2]. Oxidants may contribute to progressive renal disease by specific renal hemodynamic activities, by impairing glomerular permselective properties and inducing inordinate or aberrant growth responses [3]. Chemiluminescence (CL) enables the registration of free radical concentrations in biological fluids (blood, urine, lymph, etc.) and tissues [4]. Therefore, the intensity of CL correlates to the kidneys' functional activity and metabolic concentrations and depends on the total decrease in the antioxidant concentration and increase in water-soluble lipid peroxidation products in urine [3, 5]. Two natural antioxidants, ascorbic and uric acid, are excreted in high catabolic concentrations in this biological fluid.

Russina et al. have studied the interaction between phenolic antioxidants oxidized with organic hydroperoxides by CL inhibitory analysis in model systems [6, 7].

The goal of this research was to measure *in vitro* the inhibitory constants of ascorbic and uric acids in urine with lucigenin enhanced chemiluminescence. These antioxidants react with the lipid-peroxide radicals generated in Fenton's system ($Fe^{2+} + H_2O_2$):

$$RO_2^{\cdot} + InH \xrightarrow{k} ROOH + In^{\cdot},$$

where $RO_2^{\cdot}$ is lipid-peroxide radical, InH is inhibitor, ROOH is lipid peroxides, $In^{\cdot}$ is radical of the inhibitor, and k is inhibitory constant.

MATERIALS AND METHODS

Sterilized primary morning urine samples from healthy donors (pH 5.47-6.23, 36°C) were studied. The total volume of the sample was 2 ml. The system contains Fenton's reagent ($5\cdot10^{-5}$ M Fe^{2+} + $5\cdot10^{-4}$ M H_2O_2), ascorbic acid (10^{-3} or 10^{-4} M) or uric acid (10^{-3} or 10^{-4} M), and the CL enhancer lucigenin (10^{-4} M) dissolved in dimethylsulfoxide (DMSO). The CL emission is measured automatically with LKB 1251 chemiluminometer (LKB, Sweden).

Lucigenin (N,N′-dimethyl-9,9′-biacridinum salt) and DMSO were from Aldrich (USA); EDTA and H_2O_2 from Serva (Germany); uric acid, ascorbic acid,

Abbreviations: CL) chemiluminescence; DMSO) dimethylsulfoxide; $O_2^{\cdot-}$) superoxide radical; $^{\cdot}OH$) hydroxyl radical; $RO_2^{\cdot}$) lipid-peroxide radical; ROS) reactive oxygen species; SOD) superoxide dismutase.

* To whom correspondence should be addressed.

superoxide dismutase (SOD), and catalase from Reanal (Hungary).

Constants are reported as mean ± standard deviation of the mean. Microsoft Excel 2000 and Microcal Origin 5.0 were used for calculations and statistics.

RESULTS AND DISCUSSION

The experimental data show that the spontaneous CL intensity of urine is extremely low (Fig. 1, bar *1*). The luminescence of urine is due to the dark decomposition of long-living intermediates produced by oxidation of biological molecules. The intensity of CL depends on the activation of free radical oxidation processes in Fenton's system as well as on pH (5.2-6.7), temperature, and the concentration of generated reactive oxygen species (ROS) [8, 9]. Maximum CL emission is registered in urine containing H_2O_2 ($5\cdot10^{-4}$ M), Fe^{2+} ($5\cdot10^{-5}$ M), EDTA ($5\cdot10^{-5}$ M), and chemical CL enhancer lucigenin (10^{-4} M) at pH 5.5 and 36°C.

The addition of H_2O_2 results in 3-fold higher CL emission in urine (Fig. 1, bar *3*). Fenton's reagent increases the signal up to 3.5-fold in comparison to the control level (Fig. 1, bar *4*). This effect is a result from the generated hydroxyl radicals ($^{\cdot}OH$), which interact with the catabolic free fatty acids in urine forming $RO_2^{\cdot}$.

It is well known that the chemical CL enhancer lucigenin reacts specifically with superoxide radicals ($O_2^{\cdot-}$) in ROS-generating systems (Figs. 1 and 2) [4]. It results in 8-fold increase in the CL signal compared to the emission of urine [4, 9]. The chelator EDTA ($5\cdot10^{-5}$ M) maximizes sharply the light emission — up to 14-fold in comparison to the metabolic levels. This is due to the strong catalytic properties of the Fe^{2+}/EDTA complex (Fig. 1, bar *8*). For instance, the inhibitory effect of the antioxidant enzymes superoxide dismutase (SOD) (20 IU/ml) and catalase

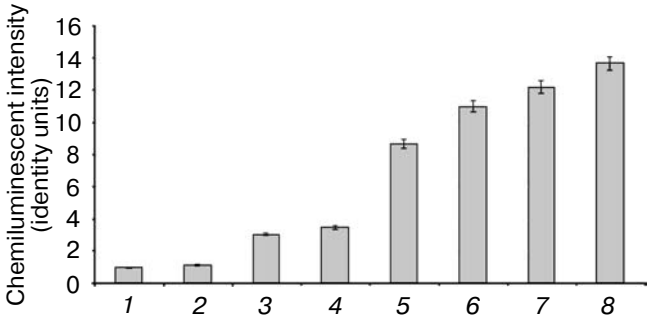

Fig. 1. Chemiluminescence of urine in systems generating reactive oxygen species: *1*) urine (pH 5.5, 36°C); *2*) urine + Fe^{2+} ($5\cdot10^{-5}$ M); *3*) urine + H_2O_2 ($5\cdot10^{-4}$ M); *4*) urine + H_2O_2 + Fe^{2+}; *5*) urine + lucigenin (10^{-4} M); *6*) urine + lucigenin + H_2O_2; *7*) urine + lucigenin + H_2O_2 + Fe^{2+}; *8*) urine + lucigenin + H_2O_2 + Fe^{2+}-EDTA ($5\cdot10^{-5}$ M).

Antioxidant constants of uric acid and ascorbic acid registered with enhanced chemiluminescence in urine and Fenton's system (urine pH 5.5, 36°C; lucigenin, 10^{-4} M; H_2O_2, $5\cdot10^{-4}$ M; Fe^{2+}, $5\cdot10^{-5}$ M; EDTA, $5\cdot10^{-5}$ M)

Antioxidant	Concentration, M	k, μmol·sec^{-1}
Ascorbic acid	10^{-3}	5.92 ± 0.04
Ascorbic acid	10^{-4}	24.05 ± 1.82
Uric acid	10^{-3}	1.60 ± 0.02
Uric acid	10^{-4}	21.45 ± 0.97

(15 IU/ml), respectively, is 90 and 70% [9]. This confirms the role of ROS ($O_2^{\cdot-}$, $^{\cdot}OH$) for the generation of CL.

The oxidative "burst" in the organism is a result from the activated free radical processes, including many simultaneous and interdependent reactions as well as a decrease in the reducing equivalents in the system [8]. The ROS activated processes lead also to free radical lipid peroxidation products through the intrarenal adenosine catabolism of xanthine [10].

Three phases of CL kinetics are well observed when ROS react with urine (Fig. 2): 1) spontaneous CL (0-10 sec); 2) fast flash of CL (10-50 sec); 3) latent period (50-300 sec). This type of kinetics is totally altered after the reaction with an antioxidant. The interaction between ascorbic or uric acid and $RO_2^{\cdot}$ radicals sharply decreases the emitted light.

The inhibitory constants are calculated according to the CL kinetics by the equation [7]:

$$[(dI/I_0)dt]_{t=0} = -2k[InH]_0,$$

where $[(dI/I_0)dt]_{t=0}$ is tanα, the tangent of the angle between the kinetic straight line and the ordinate in the first period of fast emission; $[InH]_0$ is the concentration of the antioxidant (Fig. 2).

The experimental results confirm that these two antioxidants are more effective at concentrations close to physiological levels (table). Ascorbic acid has up to 4-fold stronger antioxidant effect at 10^{-3} M and about once better efficiency at lower (10^{-4} M) concentration when it is compared to uric acid. Ascorbic acid reacts as a pro-oxidant at higher concentrations and low content of metal ions (Cu^+/Cu^{2+}, Fe^{2+}/Fe^{3+}) [11].

The link between metabolism, biochemical activity, and the concentrations of uric and ascorbic acids is well established [10, 11]. This estimation is due to the fact that high levels of ascorbic acid in the body result from the maximal formation rate of uric acid according to the xanthine–xanthine oxidase pathway in patients with renal failure [10]. The non-pathological concentrations of

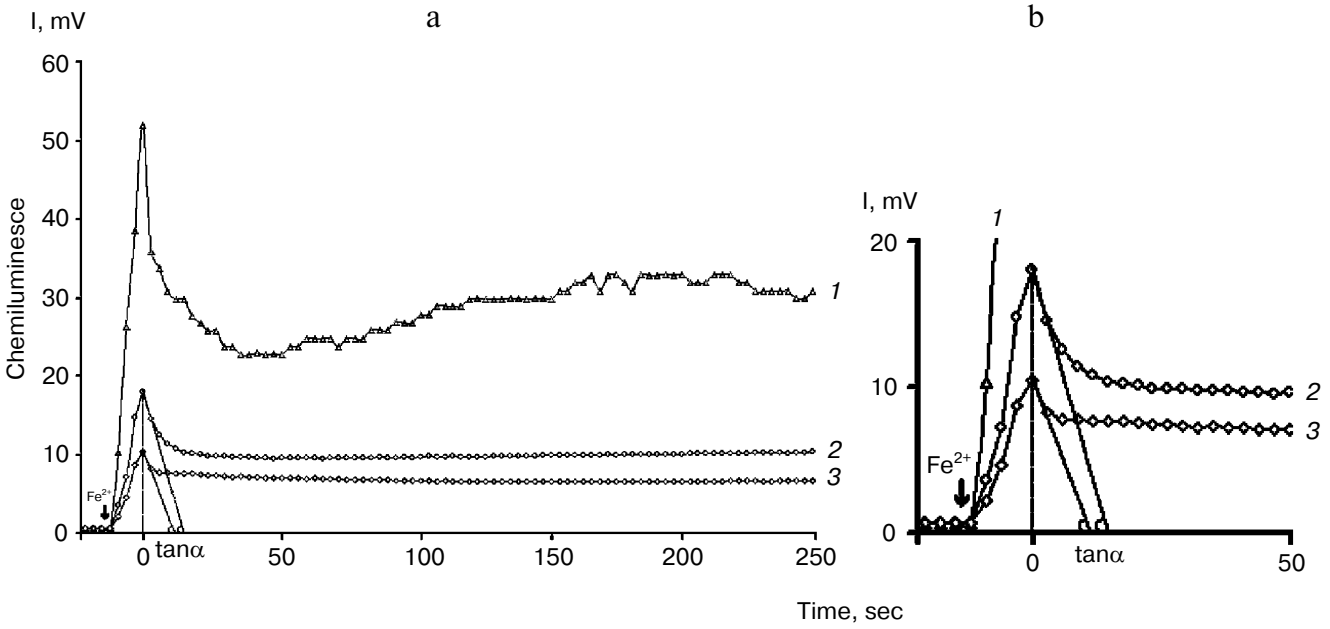

Fig. 2. Chemiluminescent kinetics of urine in Fenton's system (urine pH 5.5, 36°C; lucigenin, 10^{-4} M; H_2O_2, $5 \cdot 10^{-4}$ M; Fe^{2+}, $5 \cdot 10^{-5}$ M; EDTA, $5 \cdot 10^{-5}$ M) (a) and the antioxidant effect of ascorbic and uric acids (10^{-4} M) (b): *1*) urine + lucigenin + H_2O_2 + Fe^{2+}-EDTA; *2*) urine + lucigenin + H_2O_2 + Fe^{2+}-EDTA + ascorbic acid; *3*) urine + lucigenin + H_2O_2 + Fe^{2+}-EDTA + uric acid.

ascorbic acid in urine vary about $1.54 \cdot 10^{-4}$ M as hyperdoses are easily excreted. Ascorbic acid acts as a co-substrate in chemical reactions, makes easier the cleavage of Fe^{2+} from ferritin, and takes part in the oxidation of proline and hydroxylation of lysine amino acid residues [12-14]. In fact, higher concentrations of ascorbic acid in the body support glucocorticoid synthesis, which protects optimal levels of uric acid [11]. On the other hand, uric acid inhibits the generation of hypochlorite radicals ($^{-}$OCl). It successfully "switches off" ROS such as $O_2^{\cdot-}$ and $^{\cdot}$OH in living systems [10]. Both low molecular weight compounds are very good reducers, which defines their very good antioxidant properties. The physiological concentration of uric acid in urine is $0.3 \cdot 10^{-6}$ M. About 80% of this weakly water-soluble antioxidant is excreted out as the body catabolizes about 20%. Uric acid appears as a final product of purine metabolism. It acts as a better donor of electrons than ascorbic acid.

The urinary CL is a result from the total excretion of oxidized biomolecules and could provide a valuable index of systemic oxidative stress. In fact, the calculation of the inhibitory constant is an antioxidant efficiency estimation about the final stage of catabolic processes in the body. The CL analysis defines the molecular mechanisms that are directly linked to the light emission of urine. This method is a proper and simple way of free radical estimation. It does not require special preparations of the urine samples. This approach for inhibitory analysis and calculation meets all demands for express methods evaluating the interaction between generated ROS and antioxidants.

REFERENCES

1. Vladimirov, Yu. A., Azizova, O. A., Deev, A. I., Kozlov, A. V., Osipov, A. N., and Roshchupkin, D. I. (1991) *Advances in Science and Technology. Biophysics* [in Russian], Vol. 29, VINITI, Moscow, pp. 1-252.
2. Hancock, J. T., Desikan, R., and Neill, S. J. (2001) *Biochem. Soc. Trans.*, **29**, 345-350.
3. Haugen, E., and Nath, K. (1999) *Blood Purif.*, **17**, 58-65.
4. Vladimirov, Yu. A., and Sherstnev, M. P. (1989) *Advances in Science and Technology. Biophysics* [in Russian], Vol. 24, VINITI, Moscow, pp. 1-177.
5. Figatner, J. J. (1986) in *Biochemiluminescence in Agriculture* [in Russian], Moscow, pp. 54-57.
6. Russina, I. F., and Gagarina, A. B. (1986) in *MOIP: Theoretical and Methodical Bases of Biochemiluminescence* [in Russian], Nauka, Moscow, pp. 38-40.
7. Russina, I. F. (1975) *Dokl. Akad. Nauk SSSR*, **224**, 646-649.
8. Farkhutdinov, R. R., Kantinkov, S. A., and Arkhmadeev, R. L. (1986) *Lab. Delo*, **5**, 263-267.
9. Pavlova, E. L., Lilova, M. I., and Savov, V. M. (2002) *Annuaire de L'universite de Sofia "St. Kliment Ohridski", Faculte de Physique*, **95**, 57-69.
10. Sumbaev, V. V., and Yasinskaya, I. M. (1997) *Ukr. Biokhim. Zh.*, **69**, 116-120.
11. Mitch, W. E., Johnson, M. W., Kirshenbaum, J. M., and Lopez, R. E. (1981) *Clin. Pharmacol. Ther.*, **29**, 318-321.
12. Vasil'eva, O. V., Lyubitsky, O. B., Gus'kova, T. A., Glushkov, R. G., Medvedev, O. S., and Vladimirov, Yu. A. (1999) *Vopr. Med. Khim.*, **4**, 1-6.
13. Krasowka, A., David, R., Szkapiak, K., Oswiecimska, M., Witek, S., and Lukaszewicz, M. (2001) *Cell. Molec. Biol. Lett.*, **6**, 71-81.
14. Avramchik, O. A., Korotkova, E. I., Plotnikov, E. V., Lukina, A. N., and Karbainov, Y. A. (2005) *J. Pharm. Biomed. Anal.*, **37**, 1149-1154.