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Express method for evaluating the antioxidant activity of medicinal substances and its preparations by polarography

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Abstract

The express method of medicinal substances and preparations (further substances) antiradical and antioxidant activity evaluation by polarography using a microsomal LPO model *in vitro* is presented in this work. The substances antioxidant properties have been investigated by adding Fe^{2+}-ADP inductive NADPH-dependent (enzymatic) and adding Fe^{2+}-ADP inductive ascorbate-dependent (non-enzymatic) lipid peroxidation (LPO) of microsomes from rat liver in the *in vitro* system to the polarographic cell, which activity has been recorded at oxygen absorption rate by using standard closed platinum Clark's electrode at 30 °C in 100 mmol of tris-HCl buffer, pH 7.4. It has been shown that it is possible to determine the mechanism of substances antioxidant action, which includes antiradical activity as to lipid and oxygen radicals on enzymatic and ascorbate-dependent intact microsomes LPO models by polarographic method in the *in vitro* system. It is also possible to investigate the antioxidant action features of medicinal substances, which are determined by their nature, chemical structure peculiarities, concentration and interaction in the pharmaceutical form.

Key words: enzymatic and ascorbate-dependent microsomes LPO, antioxidant activity of medicinal substances and preparations, polarographic express- method

INTRODUCTION

Antioxidant activity (AOA) of medicinal substances is characterized by a diversity of their action mechanisms that is why the role of each of them can vary significantly in different activation methods of free radical processes [1-5]. In connection with this, the choice of adequate models of free radical oxidation (FRO) for AOA preparations evaluation has the primary importance for the correct interpretation of obtained results.

Among the currently proposed methods of AOA evaluation, it is worth to mark out the methods that allow performing the evaluation of AOA preparations "integral", however they do not allow finding out the mechanism of antioxidant activity [1,2].

We have conducted the investigation of AOA preparations in model systems with certain types of radicals' generation; therefore it can give us the most complete information about antioxidant action mechanism. However, FRO activation in the cell is due not to one kind (type) of radicals, but several, so the preparation antiradical activity interpretation as to present type of radical in the cell system will not be completely correct [1,2]. A more correct approach in preparation antiradical activity mechanism clarifying as to present type of radical is to determine the total (integral) AOA of the preparation with inhibitors using that allow isolating other radicals, which also predict the additional reagents consumption. Taking into consideration the fact that in vivo the variable valence metals (especially ferrum ions) are most likely inductor of lipid peroxidation (LPO), ferrum-inductive LPO in bio membranes is a suitable model for AOA preparations studying [5,6].

It is known that not only lipid radicals are formed in the ferrum ions presence, but a large amount of reactive oxygen species (ROS) is also observed [1,6].

It should also be taken into consideration that the studied mechanism of antioxidant action of any preparation is valid only to a certain model of Fe^{2+}-ADP inductive LPO and may significantly differ from the results obtained with other methods of its initiation [1-3]. Therefore, the application of a wider (circle) variety of models and LPO systems allows studying the mechanisms of AOA different in their preparation nature more deeply.

FRO is accompanied by the oxygen absorption by microsomes [6]. The microsomes' membranes (isolated endoplasmic reticulum fraction) are a convenient object for biologically active substance (BAS) antioxidant properties screening, as both LPO systems (enzymatic and non-enzymic) stay simultaneously in membranes. Actually membranes consist 50-60% of unsaturated phospholipids, which are lipid peroxidation substrates, with overoxidation of which lipid radicals and ROS are formed [6].

The convenient polarographic method for medicinal substances antiradical and antioxidant activity determination has been used in our work.

MATERIALS AND METHODS

Herein, each sample came with a Certificate of Analysis that includes a purity determination, and all test material held purities higher than 97-98% (HPLC).

Silibor (Silymarin CAS 65666-07-1) was purchased from Pharmaceutical company «Zdorovye» (Kharkov, Ukraine). Ionol (Butylated hydroxytoluene (BHT) CAS 128-37-0) was from Sigma-Aldrich (Steinheim, Germany).

Other test compounds, such as Flacumin, Altan and Hyflarin a kind donation from the State Enterprise "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines" (SE "Pharmacopoeial Center", Kharkiv, Ukraine).

All solvents and reagents, while from various suppliers, were of the highest purity needed for each application.

The polarographic method of substances antiradical and antioxidant activity evaluation is to determine the substances inhibition effectiveness of medicinal preparations, which have antioxidant activity of inductive Fe^{+2}·ADP NADPH-dependent (enzymatic) and inductive Fe^{+2}·ADP ascorbate-dependent (non-enzymatic) LPO of intact animals microsomes in the *in vitro* system. The method principle is based on the rate suppression of oxygen absorption at enzymatic and ascorbate-dependent by LPO-microsomes different in nature substances of with a variety of action mechanism.

With the help of the polarography method, antiradical activity, interconnection can be investigated: the structure *vs.* antiradical and antioxidant activity of individual substances of medicinal preparations and antioxidant activity of medicinal preparations on their basis.

Method of microsomes isolation

The liver microsomes of white male rats weighing 200-240 g have been isolated by the Kamath method [7] in a medium containing 0.25 mol of sucrose and 20 mmol of tris-HCl buffer, pH 7.4. The obtained microsomes precipitation has been suspended in a medium containing 125 mmol KCl and 20 mmol tris-HCl buffers, pH 7.4.

The protein final concentration in the microsome suspension, which was determined by the Lowry method [8], was 40-60 mg/ml. All procedures have been carried out at +2÷+4 °C. The isolated microsomes were maintained at +2÷+4 °C for no more than 5 hours.

Method of medicinal substances and preparations antioxidant activity determination by polarographic method

The antioxidant properties of the medicinal substances and medicinal preparations (further substances) have been investigated by adding Fe^{2+} ADP inductive NADPH-dependent (enzymatic) and adding Fe^{2+} ADP inductive ascorbate-dependent (non-enzymatic) LPO of microsomes from rats liver in the *in vitro* system, the activity of which has been recorded due to oxygen absorption, into the polarographic cell (electrolyzer).

The oxygen rate consumption by microsome suspension has been determined by the polarographic method with using Clark-type standard closed platinum electrode at 30 °C in 100 mmol of tris-HCl buffer, pH 7.4 [9].

Initially, the activity of the enzymatic and ascorbate-dependent LPO of microsomes has been recorded, then the antioxidant activity of substances investigated (the effect on the enzymatic and ascorbate-dependent LPO of microsomes) has been studied by adding them to the polarographic cell to microsomes.

To the medium of polarographic cell with volume of 1,0 ml, which contains 100 mmol of tris-HCl buffer, pH 7.4, the 1.0-1.5 mg of microsome protein, 4 mmol of ADP, 1.0 mmol of NADPH or 0.8 mmol sodium ascorbate were added consecutively, in 1-2 minutes – 0.012 mmol of $Fe(NH_4)_2{\cdot}(SO_4)_2{\cdot}6H_2O$ was added, then substances were added by titration. Substances which were insoluble in distilled water or in 100 mmol of tris-HCl buffer with pH 7.4 have been dissolved in a mixture of borax or alcohol. The used concentrations of borax and alcohol solutions, in which the substances were dissolved, did not affect the activity of microsomes LPO.

Subsequently, the substances concentrations that inhibit microsomes respiration up to 50% (ID_{50}) have been calculated. The smaller the ID_{50} value, the more active substance.

RESULTS AND DISCUSSION

The determination of substances concentration values that causes microsomes LPO inhibition (definition ID_{50}) is carried out as follows. After reaching the established stationary rate of oxygen consumption at microsomes LPO, gradually (with the interval of 0.5-1.0 min) a solution of the investigated substance is added into the polarographic cell, increasing its concentration to achieve a distinct respiration inhibition. The initial concentration of the investigated substance is chosen empirically, so that the inhibition of breathing will not be instantaneous, but will have gradual (translational) character. When using non-aqueous solvents, it is necessary to state a test sample, adding to microsomes the equivalent quantities of pure organic solvent.

Subsequently, the substances concentrations that inhibit microsomes respiration to 50% have been calculated. The respiration rate before adding the first portion of the investigated substance and the respiration rate on each of the linear parts of the curve are calculated (Fig. 1).

For this purpose, the right-angled triangles were constructed on the recording respiration curve, as it is shown in Fig. 2.

The values of ΔO, that is, O_2 consumption for each curve segments (ΔO_0, ΔO_1, ΔO_2, …, ΔO_n), were determined, due to oxygen solubility, the scale width and the horizontal cathetus value. 1 ml of incubation medium contains 250 nmol of oxygen (constant value); cell volume – 1.0 ml; scale width – 100 units; width of the cathetus – 20 units. Consequently:

$$\Delta O = \frac{250\ \text{nmol} \cdot 1.0 \cdot 20}{100} = 50\ \text{nmol}\ O_2 \cdot t\ .$$

Then the amount of microsomal protein introduced into the cell has been determined and the microsomes respiration rate has been calculated on each curve segments according to the formula:

$$V = \frac{\Delta O}{\text{mg}_{\text{protein}} \cdot \text{min}}\ .$$

The degree of respiration inhibition for each of the successively increasing concentrations of the investigated substances has been calculated in the same way as previously. If in calculation results, a point, which

corresponds 50% of the inhibition degree will not be determined, then a diagram is built, that illustrates the dependence of the respiration inhibition on the added substance concentration on the basis of which ID_{50} is determined.

As an example, the antioxidant properties on the model of enzymatic and ascorbate-dependent LPO intact rat microsomes were investigated, in vitro polyphenolic preparations: Altan tablets, Piflamin tablets, Flacumin granules, Silibor granules, 1% solution for injection of Hyflarin and their active substances.

In the Silibor granules, the active substance of silibor contains flavolignans – silibinin, silidianin, silicrystin and flavonols – taxifolin and quercetin; in Flacumin granules – the active substance flacumin contains flavonols – quercetin, myricetin and kaempferol. Pectin, citric acid, sorbic acid and sugar are included to the composition of granular preparations as auxiliary substances

Altan tablets contain altan substance (tannins of elohotannins group) and auxiliary substances (potato starch, sugar powder, calcium stearate).

Piflamin tablets contain piflamin substance, the main active substances of which are represented by polyphenols – flavonoid aglycones – luteolin, quercetin, kaempferol with their glycosides and hydroxycoric acids – ferulic acid and p-hydroxycoric acid). The BAS complex of piflamin substance also contains amino acids, guanidines, oligosaccharides, macro- and microelements. Auxiliary substances (milk sugar, calcium stearate, microcrystalline cellulose, magnesium stearate, titanium dioxide, methyl cellulose, tropeoline 0).

The active substance of Hyflarin preparation (1% solution in ampoules) is flavonoglycoside giprozidum.

The table shows the antioxidant properties of substances of medicinal substances and its preparations.

Polyphenols, in comparison with each other, did not equally inhibit the LPO microsomes, while polyphenols inhibited equally both enzymatic and ascorbate-dependent LPO. The equal effect of inhibition by LPO enzymatic and ascorbate-dependent substances confirms the same kinetics and stoichiometry of peroxidation, which are stimulated by NADPH and ascorbate [6]. Altan substance showed the most antioxidant activity, which exceeded the activity of other plant polyphenols in 2-27 times, which effect decreased in the such range as: Altan> Hyflarin> Flacumin> Silibor> Piflamin. Low antioxidant activity of Piflamin is due to low polyphenols content in the substance (table). The antioxidant activity of the investigated polyphenolic substances was in 2-50 times higher than the classical Ionol antioxidant in the" in vitro" system.

Medical preparations (Altan tablets, Piflamin tablets and Hyflarin 1% solution in ampoules) as well as their active substances equally inhibited the intact microsomes LPO, while Silibor granules, Flacumin granules in terms of their active substance in comparison with the last to a greater extent inhibited intact microsomes LPO. In this case, not only flavonoid substances showed antioxidant effect, but also auxiliary substances, synergist – substance: organic acids and sugars. The last regenerate antioxidants that lost electrons during the interaction with free radicals, increasing the real antioxidants efficiency [10]. The most pronounced synergizing antioxidantion effect is shown for Flacumin granules, where in terms of the substance; they are in 5 times exceeded the antioxidant activity of a single flacumin substance in intact microsomes (Table).

The marked antiradical activity of Altan, containing the tannins of the elohotannin group, is due to the high content of OH-groups in its molecules in comparison with other substances and their relative position, the molecules structure peculiarities, which determine the antioxidant activity [11]. Hydroxyl groups of elohotannins that interact with lipid peroxide radicals, inhibit effectively FRO in rat liver microsomes [12].

Thus, Altan tablets and Flacumin granules are related to the most potent antioxidant preparations investigated in the work.

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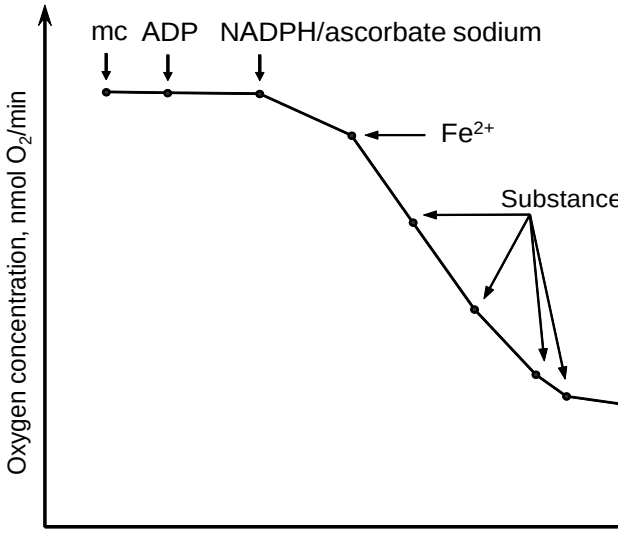

Figure 1. The titration of microsomes respiration by investigated substance solution

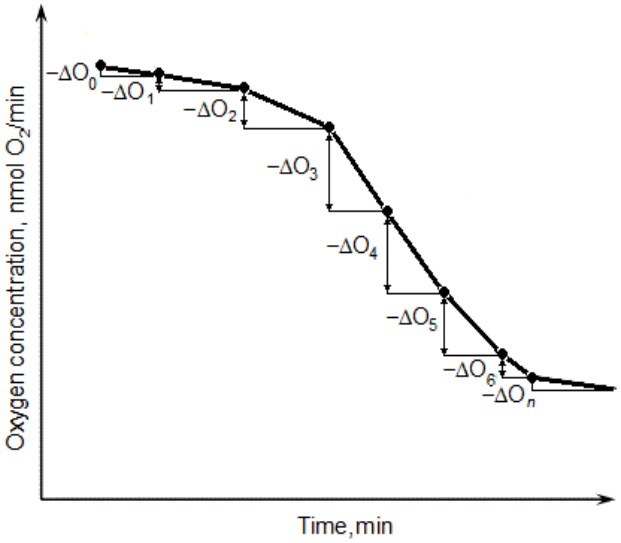

Figure 2. The calculation of respiration rate

Table. Influence of medicinal substances and its preparations on the LPO of rats liver microsomes in the *in vitro* system, n = 6

Medicinal substances and its preparations	ID_{50} μg/ml	
	Intact microsomes	
	Enzymatic LPO	Ascorbate-dependent LPO
Silibor substance	65.0±6.0	64.0±6.0
Silibor granules	29.3±2.7*	30.7±2.7*
Flacumin substance	50.0±4.8	50.0±4.7
Flacumin granules	11.4±1.0*	9.2±0.9*
Altan substance	10.0±0.8	11.0±0.9
Altan tablets	10.0±0.8	10.0±0.9
Piflamin substance	260.0±25.0	270.0±26.0
Piflamin tablets	255.0±25.0	260.0±25.0
Hyflarin substance	20.0±1.5	19.0± 1.4
Hyflarin 1% solution in ampoules	20.0±1.5	20.0± 1.5
Ionol	500.0±50.0	490.0±45.0

Notes: a) ID_{50} – the solutions concentration of medicinal substances and its preparations, in μg/ml, which inhibits the processes of microsomes LPO to 50%.

b) * – probability in comparison with the substance, $p < 0,05$.

c) *n* – number of observations.

CONCLUSIONS

The inhibition degree by LPO substances that have antioxidant properties on the microsomes LPO model may be a quantitative characteristic of convenient test conducting for preliminary quantitative estimation of antioxidant properties of new medical substances and its preparations.

Using the models of enzymatic and ascorbate-dependent LPO intact microsomes by polagraphic method in the *in vitro* system, it is possible to determine the mechanism of substances antioxidant action, that includes antiradical activity as to lipid radicals and oxygen radicals (ROS). The proposed method allows us to investigate the peculiarities of the antioxidant action of medicinal substances and its preparations, which is determined by their nature, peculiarities of the chemical structure, concentration and interaction in the pharmaceutical form.

The peculiarities of antioxidant action of polyphenolic medicinal substances and preparations based on their enzymatic and ascorbate-dependent LPO microsomes models from rat liver have been determined by polarographic method in vitro. It has been stated that the concentration of polyphenols-antioxidants reduces significantly in composition of synergistic substances (Flacumin and Silibor granules). Altan tablets and Flacumin granules are related to the most potent antioxidants. The flacumin substance (on the altan substance level) demonstrates significant synergistic antioxidant activity in comparison with other polyphenolic substances in all preparations investigated.

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