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In Vitro Study of Antioxidant Activity of Serum and Plasma Samples As Well As Glutathione Exposed to Various Exogenous Stress Factors

Małgorzata Olszowy-Tomczyk (malgorzata.olszowy@poczta.umcs.lublin.pl)
Uniwersytet Marii Curie-Sklodowskiej w Lublinie Wydzial Chemii https://orcid.org/0000-0002-8344-8534

Łukasz Paprotny
Research and Development Centre, ALAB Laboratories

Agnieszka Celejewska
Research and Development Centre, ALAB Laboratories

Dorota Szewczak
Research and Development Centre, ALAB Laboratories

Dorota Wianowska
Uniwersytet Marii Curie-Sklodowskiej w Lublinie Wydzial Chemii

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In vitro study of antioxidant activity of serum and plasma samples as well as glutathione exposed to various exogenous stress factors

Małgorzata Olszowy-Tomczyk[1]*, Łukasz Paprotny[2], Agnieszka Celejewska[2], Dorota Szewczak[2], Dorota Wianowska[1]

[1]Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Skłodowska University in Lublin, Pl. Maria Curie-Skłodowska 3, 20-031 Lublin, Poland; Tel: +48 81 537 56 58; Fax: +48 81 533 33 48

[2]Research and Development Centre, ALAB Laboratories, ul. Ceramiczna 1, 20-150 Lublin, Poland

*Corresponding author

E-mail: malgorzata.olszowy@poczta.umcs.lublin.pl

Abstract

The imbalance between the production of Reactive Oxygen Species (ROS) and their sequestration promotes the formation of so-called oxidative stress conditions which are considered crucial in the aging process and development of many human diseases. Glutathione plays an essential role in the antioxidative barricade against ROS. Its role in the detoxification process of xenobiotics and carcinogen is also known. However, there are no comparative studies on the antioxidant properties of both biological samples and glutathione as well as the change in these properties as a result of exposure to various stress factors.

This paper fills this gap comparing the antioxidant activity of serum and plasma samples of the known glutathione content with the activity of glutathione itself assessed by the different methods. In addition, it reveals a significant role of environmental xenobiotics in oxidative stress and differentiates the stress induced by different groups of drugs, among which the greatest one has been demonstrated for antiarrhythmic drugs and cytostatics. More importantly, it proves that human plasma is more resistant to stress factors and N-acetylcysteine clearly promotes the extension of antioxidant properties of both the plasma and serum samples. The latter conclusion is consistent with the implied preventive and/or supportive action of this drug against SARS-CoV-2.

Keywords: ROS, GSH, ACC, oxidative stress, oxidative factors

Introduction

Reactive oxygen species (ROS) are an integral part of our life and as such arise from endogenous and exogenous causes as products of cellular metabolism and, for example, a consequence of environmental pollutants, X-rays and gamma rays radiation or our lifestyle and habits [1,2]. Their presence can have a positive or negative impact on a human, depending whether there is a balance between their production and sequestration by the antioxidant defense system of an organism [3,4]. When this equilibrium is disturbed, due to the ROS overproduction and/or reduced efficiency of the defense system, a condition of the so-called oxidative stress arises which is believed to be crucial in the aging process and the development of many diseases such as for example the cardiovascular system disease, diseases of the central nervous system or cancer [5,6].

During the evolution, our organism develops several mechanisms to protect against the negative action of ROS. Each of them is based on the use of the so-called antioxidants, i.e. substances that counterbalance the noxious effect of ROS [7] by preventing the formation of reactive forms (1), neutralizing (scavenging) free radicals (2), creating chelate complexes with pro-oxidative metals (3) and removing or repairing damage caused by reactive compounds [8]. The most important endogenous antioxidants are small thiol-containing molecules of peptides and proteins, such as glutathione and thio-, gluta-, and peroxi-redoxin. Of them, glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) presents in all eukaryotic cells plays a key role in antioxidative barricade against electrophiles and ROS [9,10,11].

The role of glutathione in physiological and pathological states and its impact on the redox and detoxification process of xenobiotics and carcinogen is described in the literature [12]. Similarly, there are several papers on the antioxidant properties of selected body fluids [13,14]. However, to our knowledge, there are no comparative studies on the antioxidant properties of both biological samples and glutathione as well as the change in these properties as a result of exposure to various stress factors. To fill this gap, the aim of the research described herein is to compare the

antioxidant activity of serum and plasma samples of a known glutathione content, with the activity of glutathione itself whose content was chromatographically assessed and examine to what extent the presence of stress factors will change the activity of the investigated samples. The research used various approaches to the assessment of antioxidant activity, namely the ABTS, ORAC, FRAP and CUPRAC methods. Moreover, the influence of various factors inducing oxidative stress was taken into account. As the exemplary factors of environmental stress, benzene and copper ions were used at the concentration observed in the environment. In addition, some pharmaceuticals were applied bearing in mind that this group of xenobiotics is an important but so far neglected in the research source of human oxidative stress. In assessing the impact of this group of substances, the representatives of various drug groups were used in the range of therapeutic concentrations characteristic of each of them. The following were selected for the study: atenolol and propanolol as the representatives of antiarrhythmic drugs; carbamazepine, ethosuximide, levetiracetam and primidone from the group of antiepileptic drugs, and 5-fluorouracil from cytostatics. The study also included acethylcysteine currently administered to the patients suffering from COVID-19 and, in the light of the data [15], exhibiting preventive and/or adjuvant effects against SARS-CoV-2.

Materials and Methods

Materials

$CuCl_2$, $Fe_2(SO_4)_3 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, HCl, CH_3COONH_4, benzene, ethanol, methanol, chloroform, CH_3COONa, CH_3COOH, $NaH_2PO_4 \cdot H_2O$,$Na_2HPO_4 \cdot 7H_2O$ were purchased from the Polish Chemical Plant POCh (Gliwice, Poland). 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2'-azobis(2-amidinopropane) dichloride) (AAPH), 2,2′-diphenylpicrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), di-potassium peroxdisulfate, fluorescein, linoleic acid, neocuproine (2,9-dimethyl-1,10-phenanthroline, Nc), Trolox, Tween 20, β-carotene were purchased

from Sigma-Aldrich (Poznań, Poland). Milli-Q system (Millipore, Bedford, MA, USA) was applied for water purification.

The ACC optima containing 600 mg of N-acetylcysteine per 1 tablet was purchased in a local pharmacy. The analytical standards of atenolol, carbamazepine, ethosuximide, 5-fluorouracil, levetiracetam, primidone, and propranolol were purchased from Sigma-Aldrich (Poznań, Poland). Working solutions of standards were prepared in water from individual stock standard solutions prepared in methanol, except the fact that an N-acetylcysteine tablet was immediately dissolved in water. They were kept under stable conditions at -20°C.

The lyophilized whole blood controls with two levels of GSH concentration were obtained from Chromsystems Instruments & Chemicals GmbH (Gräfelfing, Germany). They were reconstituted in distilled water according to the manufacturer's instructions and stored deep-frozen until needed.

The biological samples for testing were collected from a healthy volunteer during a period of one week. In each case, a small amount of blood was collected by the qualified staff in accordance with the local and national ethical regulations. Blood was obtained by venipuncture into commercially available red topped tubes and heparinized tubes for serum and plasma preparation, respectively. For serum preparation, the collected blood was allowed to clot at room temperature. After 20 min, the clot was removed by centrifugation at 2000 x g for 10 min. The resulting supernatant was transferred into polypropylene tube using a Pasteur pipette and immediately frozen at -20°C until testing. To prepare plasma, the procedure was analogous to that described above, except the fact that the cells were centrifuged immediately (without leaving for 20 min) and the resulting supernatant was separated and frozen. For the statistical purposes, all studies were performed with the pooled aliquots of serum or plasma.

Antioxidant activity assess methods

The evaluation of the antioxidant activity was made using various approaches of spectroscopic methods. The spectrophotometric absorbance was measured by UV Probe - 2500 Spectrophotometer (Shimadzu, Kyoto, Japan) (unless stated otherwise).

ABTS method

The antioxidant activities of the examined samples (serum or plasma or serum without protein or gluthathione solutions) using the ABTS method were determined by registering of ABTS cation radical absorbance changes at 744 nm. The activity was measured after the formation of ABTS cation radical according to the procedure described by Nenadis et al. [16]. After mixing 5 mL of 7 mM aqueous ABTS solution with 88 µL of 140 mM potassium persulfate ($K_2S_2O_8$) the mixture was incubated in the dark for 16 h and diluted with water to the absorbance value equal 0.7±0.05 at 744 nm. 2900 µL of aqueous solution of ABTS cation radical was mixed in a 4 mL test tube with 10 µL of plasma or serum or glutathione solutions. The whole was supplemented with water (90 µL) up to 3000 µL. In the case of serum without protein, 50µL of deproteinized sample was mixed with 2900 µL of aqueous solution of ABTS cation radical and 50µL of water. The deproteinization process was as follows: 100 µL of serum was added to 400 µL of methanol. After mixing the whole was centrifuged at 14000 rpm for 5 min. In all cases water was used to zero the spectrophotometer.

The antioxidant properties expressed as the $ABTS^{\bullet+}$ inhibition percent were calculated according to the following equation:

$$I(\%) = \left(1 - \frac{A_{60}}{A_0}\right) \cdot 100\%$$

where: A_0 and A_{60} are the values of $ABTS^{\bullet+}$ absorbance at 0 and 60 min of the radical neutralization reaction, respectively.

FRAP method

The FRAP assay was performed using the method of Benzie and Strain [17]. To prepare the FRAP reagent: the solution of $FeCl_3 \cdot 6H_2O$ in distilled water (concentration of Fe(III) was 20 mM) was mixed with the solution of TPTZ in 40 mM HCl (concentration of TPTZ was 10 mM), and with 0.3 M CH_3COOH/CH_3COONa buffer solution at pH = 3.6. The FRAP reagent was generated daily by mixing: acetic acid buffer, TPTZ solution, and Fe (III) solution in the follow volume ratio10:1:1, respectively. In order to estimate the antioxidant activity of the examined samples, 2900 µL of FRAP reagent was mixed in a 4 mL test tube with 10 µL of plasma or serum or glutathione solutions. The whole was supplemented with water (90 µL) up to 3000 µL. In the case of serum without protein, 50µL of deproteinized sample was mixed with 2900 µL of FRAP reagent and with 50µL of water. The mixture was shaken for 30 s and left in the dark at 37 °C for 60 min. Then the measurements of the increase of absorbance at 593 nm were performed. The mixture of FRAP reagent and water without antioxidants was used to zero the spectrophotometer.

Aqueous solutions of Fe(II) in the concentration range 0.1–2.5 mM/L were used for calibration. A calibration curve was constructed mixing the above mentioned Fe(II) solutions (100 µL) with 2900 µL of FRAP reagent (R^2= 0.9934). The absorbance was read after 60 min at 593 nm. The antioxidant activities, expressed as the Fe(II) equivalents, were calculated.

CUPRAC method

Antioxidant activity of the examined sample in the CUPRAC method was established in terms of the reduction of the cupric - neocuproine complex (Cu(II)-Nc) to the cuprous form (Cu(I)-Nc)[18]. The CUPRAC test solution was prepared using:740 µL of $CuCl_2$(concentration of Cu(II) was 10 mM), 740 µL of neocuproine in the aqueous/ethanolic mixture (50/50 v/v) (concentration of neocuproine was 7.5 mM), 740 µL of CH_3COOH/CH_3COONH_4 buffer solution at pH = 7.0 (1.0 M), 770 µL of distilled

water (or 730µL in the case of deproteinized serum) and 10 µL of sample (or 50µL of deproteinized serum). The mixture was vigorously shaken for 30 s, left in the dark for 60 min and then the increase in absorbance at 450 nm was measured. A mixture of CUPRAC reagent and water was used to zero the spectrophotometer.

ORAC assay

2600 µl of fluorescein (0.04 µM) dissolved in the 0.075 M phosphate buffer (pH=7.0) was mixed with 10 µl of the examined sample (or 50 µl of serum without protein) and with 90 µl of water (or 50 µl for the determination of antioxidant properties of deproteinized serum). As the blank sample 0.04 µM fluorescein dissolved in the 0.075 M phosphate buffer (pH=7.0) (2600 µl) with the addition 100 µl of 0.075 M phosphate buffer (pH=7.0) was used. The reaction mixture was then allowed to equilibrate by incubating for a minimum of 30 minutes at 37°C. Antioxidant properties were determined examining the fluorescence decay in the reaction initiated by the addition of 200 mM AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) (300 µl) used as a peroxyl radicals generator.

Fluorescence measurements were made at 37°C using RF-551 spectrofluorometer (Shimadzu, Japan). The excitation and emission wavelengths were as follows: 485 nm and 520 nm, respectively. The fluorescence was monitored kinetically recording the data every minute for 120 min. Pure water was used to zero the spectrofluorometer. The antioxidant capacity, expressed as the AUC, was calculated as follows [19]:

$$AUC = 1 + f_1/f_0 + \ldots\ldots + f_{n+1}/f_0$$

where: *AUC* – the area under the fluorescence decay curve, f_0 - the initial fluorescence reading at 0 min, and f_n - the fluorescence reading at time *n*.

Effect of stressful factors on antioxidant properties of biological systems

There was determined the effect for the serum (with and without protein), plasma samples including the GSH concentration for both matrices, as well as for commercially available whole blood solutions of GSH (at two GSH concentration levels, GSH I and GSH II) used as the control samples in the chromatographic analysis and for three types of stressing agents such as metal ions, organic solvent and drugs. Copper ions (Cu^{2+}) and benzene were selected as the representatives of the first two factors. As for the third factor, it was represented by various kinds of commonly applied pharmaceuticals, including antiarrhythmic (atenolol, propanolol), antiepileptic (carbamazepine, ethosuximide, levetiracetam, primidone), cytostatic (5-fluorouracil) and mucolytic (N-acetylcysteine, ACC) ones.

The effect was examined by the ABTS method. Briefly, an aliquot (2900 μl) of the aqueous ABTS radical cations solution (with the initial absorbance value equal 0.7±0.05 at 744 nm) was mixed with 10 μl of a biological sample (50 μl in the case of deproteinized serum) and 50 μl of stressful factors aqueous solutions. The whole was supplemented with 40 μl of water (0 μl in the case of the deprotenized serum sample) to a volume of 3000 μl. In the case of the systems containing only serum or plasma or glutathione solutions (without a stressing agent) 90 μl of water was added to reach the same, constant volume of all measuring systems. The concentration of the aqueous solutions of metal ions and benzene was 1 μg/mL. The aqueous solutions of drugs were prepared in such a way that the concentrations in the used volume (50 μl) corresponded to the therapeutic dose of the drug in the blood (i. e. for: levetiracetam 70 μg/mL; 5-fluorouracil 0.2 μg/mL; carbamazepine 20 μg/mL; primidone 8 μg/mL; propranolol 200 ng/mL; atenolol 1.150 μg/mL; ethosuximide 90 μg/mL) [20, 21]. In the case of N-acetylcysteine, the concentration was 40 μg per 1 mL of blood.

GSH determination

GSH determinations were made using a standard diagnostic kit for the HPLC analysis (Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany) on an HPLC-FD system

(NexeraX2, Shimadzu Corporation, Kyoto, Japan). The test was performed according to the instructions provided by the kit manufacturer using a sample volume of 10 µl.

The used HPLC-FD method is a procedure intended for the routine diagnostic GSH determination using a selective separation column as well as a selective fluorescence detector (FD) operating at two wavelengths (385 nm for excitation and 515 nm for emission). Its first stage involves an appropriate sample preparation aiming at protein precipitation (precipitation reagent lot 0320), next chemical reduction of GSSG to non-oxidised glutathione (GSH) that can be determined chromatographically (reduction reagent, lot 1419), and finally derivatization using the derivatizing reagents (lot 1419 and 0320). The prepared sample is subsequently subjected to the HPLC separation followed by the FD detection of the sum of both oxidised and reduced glutathione.

During the chromatographic analysis, the individual sample components are separated based on the differences in the affinity for the stationary phase (HPLC – column, lot 4115) and the mobile phase (lot 0419), both supplied by the kit manufacturer, which causes them to migrate at different rates to a detector where they are selectively and sensitively detected. The total amount of GSH was calculated by relating the GSH chromatographic response to the GSH calibration curve obtained using the highly purified whole blood GSH calibration standard (lot 4317) and the internal standard (lot 0320), both supplied by the kit manufacturer. To verify the correctness of the calibration, the control tests at two glutathione levels (whole blood GSH control level I and whole blood GSH control Level II, lot 4317) were analyzed. The correctness of the calibration was confirmed by obtaining the results within the manufacturer's declared range of GSH concentrations in the control samples.

Statistical analysis

All the results are presented as the mean of three independent measurements. The one-way analysis of variance (ANOVA) was done to determine the significance of the main effects. Significant differences ($p < 0.05$) between the means were identified using the Fisher coefficient (F) value. If the

calculated value of F (F_{cal}) exceeds the table value F (F_{tab}), this indicates a statistically significant influence of the given parameter.

Results

Table 1 summarizes the antioxidant activity of serum, plasma and glutathione samples, estimated using the ORAC, ABTS, CUPRAC and FRAP methods. The exemplary curves of the concentration changes of the remaining ABTS radical cation, monitored for 60 minutes in the systems differing in the sample type are shown in Fig. 1 A. All data were obtained for the serum and plasma samples of the same human blood sample as well as the glutathione solutions prepared in the whole lyophilized blood at two concentration levels from the range in which this peptide is present in a healthy human. The total GSH concentration in the tested samples and the whole blood, determined chromatographically is presented in Table 2. For the sake of clarity, it should be added that the attempts to assess the antioxidant properties of the whole blood did not give unequivocal results, therefore the tests were limited to the serum and plasma samples.

The results of research on the influence of various environmental and pharmaceutical stress factors, used alone and in a pair, on the antioxidant properties of the tested samples revealed by the ABTS method, are presented in Table 3 and Figure 2, respectively. In these experiments copper ions and toxic benzene were used as model the representatives of the environmental pollution. In turn, from the group of drugs, the research covered the representatives of the currently used antiarrhythmic, antiepileptic, cytostatic and mucolytic ones. The both groups of stress factors were used in the concentrations at which these substances either exist in the environment or are present in the human body at the so-called therapeutic level. All data are expressed as the difference (Δ) in the antioxidant activity of the biological sample without and with the addition a single xenobiotic as well as the sample with one and two xenobiotics. In the latter case, GSH I, levetiracetam, ACC or levetiracetam and ACC

were individually added to the serum and plasma samples containing primidone or benzene. The higher the difference value (Δ), the greater the influence of a given xenobiotic is. The method of calculating the difference is presented graphically in Fig. 1 B in the form of a hypothetical graph of the dependence of the unreacted radical as a function of the time measured from the addition into the measuring system of a biological sample alone (solid line), and the sample enriched with xenobiotic which slows down (dotted line) or accelerates (dashed line) the disappearance of the radical. If the added xenobiotic slows down the disappearance of the radical i.e. reduces the antioxidant activity, then the graph is above the curve observed in the absence of this substance (negative Δ). However, if the added xenobiotic accelerates the kinetics i.e. intensifies the activity, the curve lies below (positive Δ). In other words, Fig. 1 B shows the effect of the addition of a xenobiotic on the degree of suppression of the reaction kinetics (negative Δ) or its acceleration (positive Δ) in relation to the reaction kinetics observed in the biological sample alone.

For the sake of clarity, the evaluation of the antioxidant properties was also made for the solutions of xenobiotics used alone and in a pair. In all cases, the observed inhibition of the radical ABTS cation was less than 2% (data not shown). Therefore, it can be assumed that the xenothiotics used in the research do not exhibit the antioxidant activity.

Discussion

Comparative studies of the antioxidant properties of serum and plasma samples as well as glutathione using various spectrophotometric approaches

Currently, many analytical methods are used to determine the antioxidant activity of complex samples [22]. In the case of biological samples, the ABTS method is the most frequently applied [23]. Therefore, the comparative analysis of the antioxidant activity of serum and plasma samples as well as

glutathione at two concentration levels began with this method. The results obtained in this series of experiments are presented in the first row of Table 1 and Fig. 1.

The data presented in Table 1 and Fig. 1 confirm the antioxidant activity of glutathione, which increases with the increasing concentration of this peptide in the sample from 947 µmol/L for GSH I to 2186 µmol/L for GSH II (see Table 2 and compare the position of the curve obtained for GSH I with that for GSH II in Fig. 1A). Moreover, they show the influence of the type of biological matrix on the tested activity. Serum has a stronger ABTS cation radical-scavenging effect than plasma. Nevertheless, it is weaker than the action of glutathione at a higher concentration. Considering that the serum GSH content is much lower and comparable to the plasma GSH content (see Table 2), it can be concluded that other serum components exhibit antioxidant activity.

The composition of the serum differs significantly from that of the plasma. It contains, among others, soluble products of the transformation of fibrinogen into fibrin, which may be responsible for the greater antioxidant activity of the serum. To validate this reasoning, the ABTS radical cation - scavenging was determined on the protein-free serum. The obtained data are collected in the third column of Table 1, as well as in the form of a relationship marked as 5 in Fig. 1A. These data confirm the share of serum proteins in the creation of antioxidant properties and prove a very complex nature of the antioxidant activity of natural systems [24-26].

Comparing the antioxidant activity of the tested samples was revealed using the ABTS method for the activity of the same samples estimated using other methods (successive rows of Table 1), it should be stated that the change of the method differentiates the antioxidant activity even in the case of methods based on the same reaction mechanism. This makes it difficult to draw unambiguous conclusions.

Analyzing the results obtained with the ORAC method based on the mechanism of hydrogen atom transfer (HAT) [18, 26], it can be seen that the type of biological matrix does not affect the antioxidant properties. Differences in the activity of serum (with and without proteins) and plasma are statistically

insignificant (F_{cal} = 1.34, F_{tab} = 5.14). Moreover, the antioxidant properties of these three matrices are better than those obtained for the glutathione solutions. Thus, the results obtained in the ORAC assay contradict those obtained with the ABTS method. This is not unusual because the process of ABTS•+ neutralization proceeds based on the single electron transfer mechanism (SET) [19,23,27]. The worse antioxidant properties of glutathione solutions may be due to the fact that the decrease in fluorescence is often non-linear in time, which may be a problem contributing to the increase in measurement imprecision. The other possible explanation is that fluorescein is able to form complexes with the components of the sample resulting in an apparent increase in antioxidant activity of biological samples [12].

The FRAP method similar to the ABTS ones, based on the mechanism of a single electron transfer (SET) [27], reveals the similarity of the properties of the serum sample and the plasma as well as the lower activity of the protein-free serum sample. The antioxidant properties assessed with this method for the glutathione solutions are significantly greater than for serum and plasma. However, they do not correlate with the increase in glutathione concentration, as for GSH II a smaller and statistically significant difference in the tested activity was found (F_{cal} = 8.93, F_{tab} = 7.71). At this point it should be recalled that the FRAP method measures the reduction of ferric to ferrous ions in the presence of antioxidants in a non-physiological environment as it requires an acidic pH to maintain iron solubility. Moreover, this method does not detect antioxidants with the thiol group, which may explain the observed data. On the other hand, the CUPRAC method, based on the SET mechanism too [26], reveals a statistically significant influence of both the matrix and glutathione concentration on the antioxidant properties. These results are consistent with these obtained by ABTS. However, while in the case of the latter method the properties increased in the order: protein-free serum < GSH I < plasma < serum < GSH II, in the case of the CUPRAC method these properties increased in the following order: serum without proteins < serum < plasma < GSH I < GSH II. The reasons for the change of order can be seen, for example, in the fact that the CUPRAC assay measures the thiol-group antioxidants as well as other

blood antioxidants such as ascorbic acid, α-tocopherol, β-carotene, uric acid, albumin, and bilirubin. Nevertheless, although the CUPRAC method is an indirect method of assessing antioxidant capacity, the results obtained using it correlate best with those obtained by the ABTS method and the total GSH content.

Summarizing, the analysis of the data collected in Table 1 shows differences in the antioxidant properties of serum and plasma as well as glutathione solutions tested with different methods. Nevertheless, each of the methods confirms the antioxidant activity of biological matrices, and the visible differences may result from methodological differences in the approaches and/or the composition of the tested systems. The last remark applies especially to the glutathione solutions prepared in the reconstituted whole blood lyophilisate, the composition of which may be different from that of the blood from which the serum and plasma were obtained in the tests.

The influence of various stress factors on the antioxidant activity of biological samples

The analysis of the data collected in Table 3 reveals a very diverse influence of individual xenobiotics on the antioxidant properties of the tested samples. Overall, the changes in the antioxidant capacity caused by environmental pollutants far outweigh the effects of drugs. With regard to drugs, a reduction in the antioxidant effect (negative Δ) is observed for most of them, the effect being the strongest for antiarrhythmic drugs and cytostatics. Only in the case of levetiracetam and ACC, positive Δ values were observed regardless of the sample type. Bearing in mind the preventive and/or supportive action against SARS-CoV-2 of the ACC drug, it is worth stressing of the studied stress factors this drug gives the highest delta values for the serum and plasma samples, which proves that it evidently promotes the increase of antioxidant properties. On the other hand, in the case of carbamazepine and primidone, the induced effect depends on the sample type, as in the case of the former drug, a decrease in antioxidant properties was observed for the serum and plasma samples, and in the latter - only for the serum sample

Generally, the smallest delta values were obtained for the serum, which may prove that this biological matrix is more susceptible to the influence of xenobiotic. The effect also depends on the concentration of glutathione and it decreases with the increase in the GSH concentration (compare the results for GSH I with those for GSH II). However, this conclusion does not apply to ethosuximide, levetiracetam and 5-fluorouracil because in the case of these xenobiotics, a decrease in the antioxidant properties is observed with an increase in the GSH concentration. The reasons for this phenomenon are difficult to explain clearly, taking into account the extremely complex nature of biological samples and the fact that many factors can affect the antioxidant properties.

From a comparison of the delta values obtained for the serum and plasma samples subjected to the action of one stress factor with those revealed after the introduction of the other one (see Table 3 and Fig. 2), it can be seen that the addition of a second xenobiotic significantly changes the kinetics of the neutralization of ABTS cation radicals. Greater variation in the delta value is observed for the serum. For this matrix also the resultant effect of the action of two xenobiotics in all cases exceeds the effect that would be after summing the effects of single xenobiotics (compare the delta values in Fig. 2 with those collected in Table 3, e.g. the delta value obtained for the system: serum + primidone + ACC with the values of serum + primidone and serum + ACC). In the case of plasma, the resultant effect of two xenobiotics is not so unequivocal. While in the system: plasma + primidone + levetiracetam the effect is comparable to the sum of the effects observed for the plasma + primidone and plasma + levetiracetam systems, in the plasma + primidone + ACC system it is lower than the sum of the respective effects. Since plasma is distinguished from serum by the presence of clotting factors, and these are known to be responsible for maintaining homeostasis, perhaps they are what differentiates the plasma response from the serum response to oxidative stress caused by the same factors. Another series of experiments is needed to address the relationship ambiguity and to explain why plasma is less prone the stressing agents. The task, however, is very complex, given how very complex mixtures are

biological samples, and that the effect may depend on our diet, habits, medications and even the proportions of the individual compounds.

Conclusions

The article compares the antioxidant activity of human serum and plasma samples of the known glutathione content with the activity of glutathione alone, assessed by the ABTS, ORAC, FRAP and CUPRAC methods, and demonstrates greater resistance to oxidative stress of plasma samples. Moreover, the important role of environmental xenobiotics in the oxidative stress as well as the diversified influence of pharmaceutical ones were revealed. Of the all examined pharmaceutical xenobiotics, including the representatives of antiarrhythmic, antiepileptic, cytostatic and mucolytic drugs, the greatest stress was demonstrated for the antiarrhythmic drugs and cytostatics. More importantly, it was proved that N-acetylcysteine evidently promotes the increase of antioxidant properties of both plasma and serum samples. The latter conclusion is consistent with the implied preventive and/or supportive action of this drug against SARS-CoV-2.

Nevertheless, further series of experiments are needed to address the ambiguity of the observed relationships and to explain why human plasma is more resistant to the effects of various stressing agents. However, this task is very difficult considering the complexity of the mixtures that are biological samples, and the stress resistance effect may depend on our diet, habits and medications.

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References:

[1] Yanai, N., Shiotani, S., Hagiwara, S., Nabetani, H., Nakijma, M. (2008) Antioxidant combination inhibits reactive oxygen species mediated damage. Biosci. Biotechnol. Biochem. 72, 3100–3106. https://doi.org/10.1271/bbb.80159.

[2] Sen, S., Chakraborty, R., Sridhar, C., Reddy, Y.S., De, R.B. (2010) Free radicals, antioxidants, diseases and phytomedicines: current status and future prospect. Int. J. Pharm. Sci. Rev. Res. 3, 91–100.

[3] Phaniendra, A., Jestadi, D.B. & Periyasamy, L. (2015) Free radicals: properties, source, targets, and their implication in various diseases. Indian J. Clin. Biochem. 30, 11-26. https://doi.org/10.1007/s12291-014-0446-0.

[4] Olszowy-Tomczyk, M. (2020) Synergistic, antagonistic and additive antioxidant effects in the binary mixtures. Phytochem. Rev. 19, 63-103. https://doi.org/10.1007/s11101-019-09658-4.

[5] Young, I.S. & Woodside, J.V. (2001) Antioxidants in health and disease. J. Clin. Pathol. 54, 176-186. https://doi.org/10.1136/jcp.54.3.176.

[6] Willcox, J.K., Ash, S.L. & Catignani, G.L. (2010) Antioxidant and prevention of chronic disease. Crit. Rev. Food Sci. Nutr. 44, 275-295. https://doi.org/10.1080/10408690490468489.

[7] Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S. & Kalayci, O. (2012) Oxidative stress and antioxidant defense, World Allergy Organ. J. 5, 9-19. https://doi.org/ 10.1097/WOX.0b013e3182439613.

[8] Olszowy, M. (2019) What is responsible for antioxidant activities of polyphenolic compounds from plants. Plant Physiol. Biochem. 144 (2019) 135-143. https://doi.org/10.1016/j.plaphy.2019.09.039.

[9] Ahmadpoor, P., Eftekhar, E., Nourooz-Zadeh, J., Servat, H., Makhdoomi, H., Ghafari, A. (2009) Glutathione, glutathione-related enzymes, and total antioxidant capacity in patients on maintenance dialysis. Iran J. Kidney Dis. 3, 22-27. https://doi.org/10.1088/2058-7058/12/7/6.

[10]Gaucher, V., Boudier, A., Bonetti, J., Clarot, I., Leroy, P. & Parent, M. (2018) Glutathione: antioxidant properties dedicated to nanotechnologies. Antioxidants. 7, 62-83. https://doi.org/10.3390/antiox7050062.

[11]Grazian, D.G., Garavaglia, M. L., Astori, E., Portinaro, N. M., Reggiani, F., Badalamenti, S., Aloisi, A.M., Santucci, A., Rossi, R., Milzani, A., Dalle-Donne, I. (2017) Assessment of glutathione/glutathione disulphide ratio andS-glutathionylated proteins in human blood, solid tissues, and cultured cells. Free Radic. Biol. Med. 112, 360-375. https://doi.org/10.1016/j.freeradbiomed.2017.08.008.

[12]Cheng, S.B., Liu, H.T., Chen, S.Y., Lin, P.T., Lai, C.Y., Huang, Y.C. (2017) Changes of oxidative stress, glutathione, and its dependent antioxidant enzyme activities in patients with hepatocellular carcinoma before and after tumor resection. PLOS ONE. 12, 1-10. https://doi.org/10.1371/journal.pone.0170016.

[13] Shahidi, F., Zhong,Y. (2015) Measurement of antioxidant activity. J. Funct. Foods. 18, 757-781. https://doi.org/0.1016/j.jff.2015.01.047.

[14] Ziobro, A., Bartosz, G. (2003) A comparison of the total antioxidant capacity of some human body fluids. Cell Mol. Biol. Lett. 8, 415-419.

[15] Jorge-Aarón, R.M. & Rosa-Ester, M.P. (2020) N-acetylcysteine as a potential treatment for COVID-19. Future Microbiol. 15 (11), 959-962, https://doi.org/10.2217/fmb-2020-0074.

[16] Nenadis, N., Wang, L.F., Tsimidou, M. & Zhang, H.Y. (2004) Estimation of scavenging activity of phenolic compounds using the $ABTS^{\bullet+}$ assay, J. Agric. Food Chem. 52, 4669-4674. https://doi.org/10.1021/jf0400056.

[17] Benzie, I.F.F., Strain, J.J. (1996) The Ferric Reducing Ability of Plasma (FRAP) as a Measure of ‘‘Antioxidant Power’’: The FRAP Assay. Anal. Biochem, 239, 70-76. https://doi.org/10.1006/abio.1996.0292.

[18] Olszowy, M., Dawidowicz, A. L. (2016) Essential oils as antioxidants: their evaluation by DPPH, ABTS, FRAP, CUPRAC, and β-carotene bleaching methods, Monatsh. Chem. 147, 2083-2091. https://doi.org/10.1007/s00706-016-1837-0.

[19] Huang, D., Ou, B., Prior, R. L. (2005) The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53(6)1841-56. https://doi.org/10.1021/jf030723c.

[20] Winek, L.C., Wahba, W.W., Winek Jr, C.L., Winek Balzer, T. (2001) Winek's Drug & Chemical Blood-Level Data.

[21] Schultz, M., Schmoldt, A., Andresen-Streichert, H., Iwersen-Bergman, S. (2020) Revisited: Therapeutic and toxic blood concentrations of more than 1100 drugs and other xenobiotics, Crit. Care. 24, 195-199. https://doi.org/10.1186/s13054-020-02915-5.

[22] Antolovich, M., Prenzler, P.D., Patsalides, E., McDonald, S., Robards, K. (2002) Methods for testing antioxidant activity. Analyst. 127, 183-198. https://doi.org/ 10.1039/B009171P.

[23] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26 (9-10), 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3.

[24] Dawidowicz, A.L., Olszowy, M. (2011) Antioxidant properties of BHT estimated by ABTS assay in systems differing in pH or metal ion or water concentration. Eur. Food Res. Technol. 232, 837-842. https://doi.org/10.1007/s00217-011-1451-7.

[25] Dawidowicz, A.L., Wianowska, D., Olszowy, M. (2012) On practical problems in estimation of antioxidant activity of compounds by DPPH method (Problems in estimation of antioxidant activity). Food Chem. 131, 1037-1043. https://doi.org/10.1016/j.foodchem.2011.09.067 .

[26] Dawidowicz, A.L., Olszowy, M. (2013) The importance of solvent type in estimating antioxidant properties of phenolic compounds by ABTS assay. Eur. Food Res. Technol. 236, 1099-1105.https://doi.org/10.1007/s00217-013-1982-1.

[27] Prior, R.L., Wu, X., Schaich, K. (2005) Standardized methods for the determination of antioxidant. J. Agric.Food Chem. 53, 4290-4302. https://doi.org/ 10.1021/jf0502698.

Figure captions

Figure 1. The changes of the remaining ABTS radical cation observed within 60 min in: A) the real system containing serum, glutathione I, glutathione II, plasma and serum without protein;

B) a hypothetical system explaining the way of Δ calculating: solid line -the kinetic curve in a reference system; dashed and dotted line - the kinetic curve in a system containing components accelerating and decelerating the antioxidant/ABTS•+ reaction rate, respectively.

Figure 2. Effect of adding GSH I or levetiracetam or ACC (levetiraceteam or ACC) to a primidone (benzene) containing system on altering the antioxidant activity of serum (A) and plasma (B) samples, expressed as a difference of the antioxidant activity of the matrix with one and two factors

Table 1. Antioxidant properties of serum samples (with and without protein), plasma, and whole blood glutathione at two concentration levels (GSH I and GSH II), determined by the FRAP, CUPRAC and ORAC methods

Method	Sample type				
	Serum	**Protein-free serum**	**Plasma**	**GSH I**	**GSH II**
ABTS [μM/L of Trolox]	515.58 ± 25.78	270.81 ± 13.54	420.36 ± 21.02	376.71 ± 18.84	548.69 ± 27.44
ORAC [AUC]	56.83 ± 2.84	57.34 ± 2.87	53.45 ± 2.67	43.00 ± 2.15	49.08 ± 2.45
FRAP [μM Fe^{2+}]	101.83 ± 5.10	76.28 ± 3.81	111.28 ± 5.54	246.83 ± 12.34	216.83 ± 10.84
CUPRAC [mg/mL of gallic acid]	0.14 ± 0.01	0.02 ± 0.00	0.19 ± 0.01	0.60 ± 0.03	0.71 ± 0.04

Table 2. The total GSH concentration in the tested samples and the whole blood determined chromatographically and expressed as the mean ± SD (n = 3)

GSH concentration (in µmol/L)				
Serum	**Plasma**	**Whole Blood**	**GSH I**	**GSH II**
6.14 ± 0.03	5.17 ± 0.01	1027.03 ± 2.17	947.32 ± 2.53	2186.57 ± 1.62

Table 3. The influence of the different stressful factors used alone on the antioxidant activity change of the examined matrices expressed as a difference of the antioxidant activity of the matrix with and without the factor

Stress factor	Sample type				
	Serum	Protein-free serum	Plasma	GSH I	GSH II
	Δ				
Cu^{2+}	-18.16 ± 0.91	-2.30 ± 0.11	-18.80 ± 0.94	-20.71 ± 1.04	-14.98 ± 0.75
Benzene	-26.03 ± 1.30	-3.93 ± 0.20	-9.09 ± 0.45	-40.70 ± 2.03	-13.41 ± 0.67
atenolol	-21.09 ± 1.05	-3.63 ± 0.18	-9.81 ± 0.49	-14.35 ± 0.72	-5.61 ± 0.28
propranolol	-6.77 ± 0.34	-3.64 ± 0.18	-10.16 ± 0.51	-19.39 ± 0.97	-11.06 ± 0.55
carbamazepine	-2.19 ± 0.11	-0.34 ± 0.02	-3.64 ± 0.18	0.13 ± 0.01	6.25 ± 0.31
ethosuximide	-12.46 ± 0.62	-1.62 ± 0.08	-7.00 ± 0.35	-5.60 ± 0.28	-7.89 ± 0.39
levetiracetam	5.08 ± 0.25	3.91 ± 0.19	7.46 ± 0.37	4.86 ± 0.25	3.00 ± 0.15
primidone	-20.38 ± 1.02	12.41 ± 0.62	5.64 ± 0.28	2.29 ± 0.11	12.89 ± 0.64
5-fluorouracil	-5.61 ± 0.28	-5.43 ± 0.27	-2.38 ± 0.12	-14.44 ± 0.72	-18.67 ± 0.93
ACC	9.42 ± 0.47	1.70 ± 0.08	12.14 ± 0.61	7.90 ± 0.39	8.32 ± 0.41

Figures

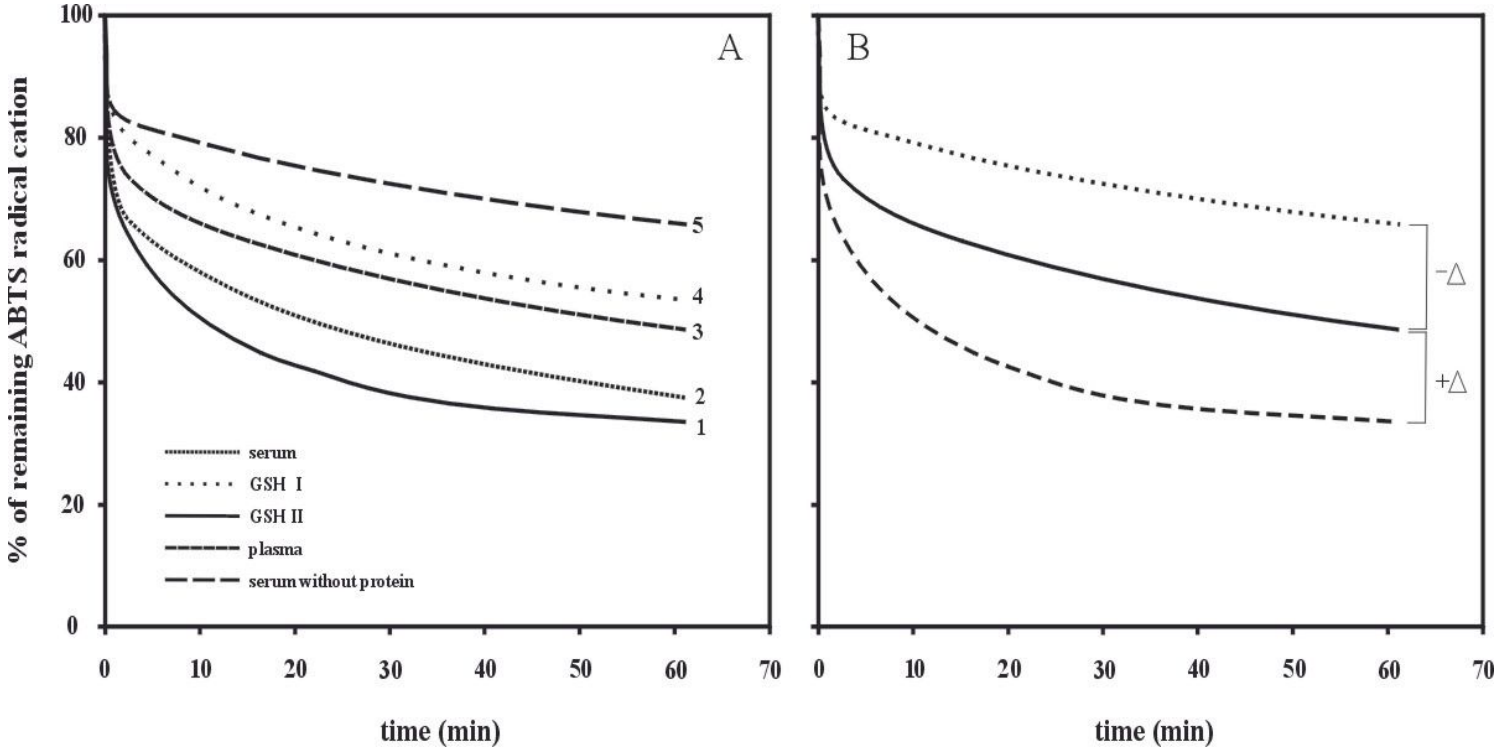

Figure 1

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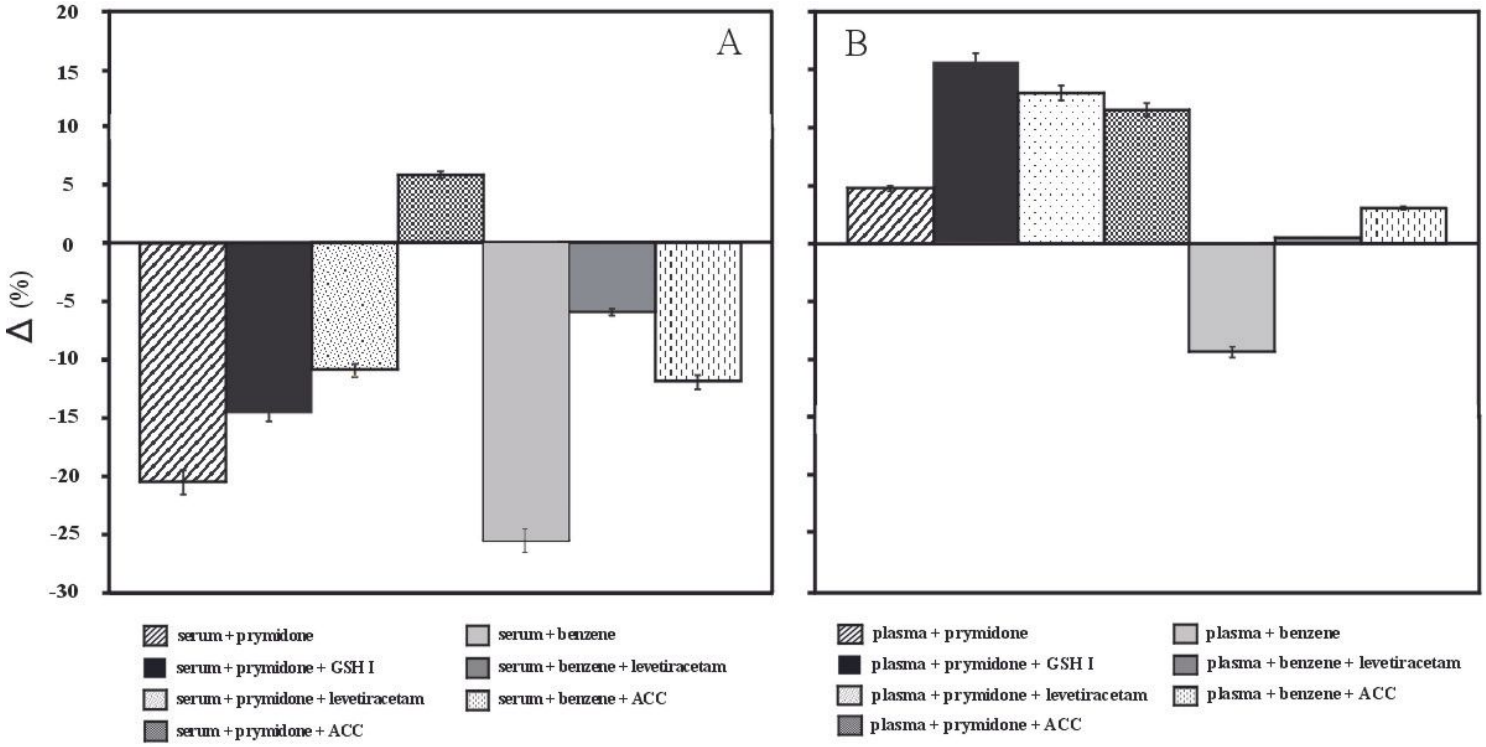

Figure 2

Effect of adding GSH I or levetiracetam or ACC (levetiraceteam or ACC) to a primidone (benzene) containing system on altering the antioxidant activity of serum (A) and plasma (B) samples, expressed as a difference of the antioxidant activity of the matrix with one and two factors

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