

## IN VITRO MODEL FOR TOTAL ANTIOXIDANT ACTIVITY EVALUATION WITH APPLICATIONS ON BIOLOGICAL PRODUCTS

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### Abstract

In the present paper we are proposing an *in vitro* model for assessing the overall antioxidant activity by using the radical cation chromophore of chlorpromazine (CP<sup>+</sup>) which presents a maximum absorption at 525 nm. A decrease in the radical colour is caused by the presence of antioxidants, proportional to the concentration of antioxidant. For evaluating the antioxidant action, the percentage inhibition of absorbance is being calculated for two established time moments (10 and 20 min), then the kinetic graph is drawn (percentage inhibition as a function of the time), and the area under curve (AUC) is calculated. From the calibration curve, found under similar conditions, (the AUC as a function of the standard antioxidant concentration - gallic acid) one may determine the total antioxidant activity expressed in gallic acid equivalents (GAE). The calibration curve was plotted on two concentrations intervals: 10-50 μM/L and 50-100 μM/L of gallic acid. The method we are proposing involves both the concentration of the antioxidant as well as the time of reaction, constituting a thorough measure of the antioxidant's ability to scavenge free radicals, related to a standard antioxidant, at selected time points of the reaction. The model may be applied on determining the antioxidant action of complex composition biological products.

### Rezumat

În lucrare se propune un model *in vitro* pentru determinarea activității antioxidante totale folosind radicalul cation cromofor al clorpromazinei (CP<sup>+</sup>), care prezintă absorbanta maximă la  $\lambda = 525$  nm. În prezența antioxidantilor se produce o diminuare a culorii radicalului proporțională cu concentrația antioxidantului. Pentru evaluarea activității antioxidante se calculează procentul de inhibiție a absorbanței la două intervale de timp stabilite, se trasează curba cinetică (procent de inhibiție funcție de timp) și se calculează aria de sub curbă (AUC). Din curba de calibrare obținută în condiții similare (AUC funcție de concentrația antioxidantului standard - acid galic) se determină activitatea antioxidantă exprimată în echivalenți micromolari de acid galic (GAE). Curba de calibrare a fost trasată pentru două intervale de concentrații: 10-50 μM/L și respectiv 50-100 μM/L acid galic. Metoda propusă implică atât concentrația antioxidantului cât și timpul de reacție, fiind o măsură completă a abilității antioxidantului de a capta radicalii liberi, raportată la un antioxidant standard, într-un interval de timp fixat. Modelul se poate aplica la determinarea activității antioxidante a produșilor biologici cu compoziție complexă.

**Keywords:** antioxidant activity, chlorpromazine, radical cation

### Introduction

The study of reactive oxygen species (ROS), and reactive nitrogen species (RNS), as well as that of their involvement in the starting or aggravating of some oxidative processes, is, at the moment, an important aim of research in several fields: medicine, pharmacy, cosmetics, food industry and petrochemical industry.

In living organisms, these species act as mediators of some physiological processes, playing a double role (“*two faced*”): that of redox adjusting common physiological functions and, respectively, its involvement in some pathological processes (human diseases and ageing). Over production of ROS and RNS causes multiple cellular and molecular modifications, named generically *oxidative stress*.

An important role in maintaining the cellular redox balance, also called *redox homeostasis*, is played by antioxidant compounds, substances capable of either neutralizing or stabilizing free radicals [8, 16].

Due to the obvious effect that antioxidants play in preventing the onset of some diseases, or in the reduction of the latter's effects, studies related to the action mechanism of antioxidants, especially that of natural antioxidants, as well as those related to the methods of assessing the antioxidant action are very numerous [2, 6, 7, 12, 14].

The antioxidants action mechanisms are manifold, and are in accordance to their nature. The radicals scavenging as well as the antioxidant action may be achieved in several ways: Single Electron Transfer (SET) from antioxidant to radical, Hydrogen Atom Transfer (HAT), by both mechanisms or by the

binding of the transitional metals ions that catalyse the oxidative processes [3].

As part of the methods based on Single Electron Transfer (SET), is measured the capacity of an antioxidant (analysed sample), of reducing an oxidant by one electron transfer. These methods employ as oxidants either coloured stable radicals, generated through an initial reaction of oxidation or complex combinations of transitional metals situated at higher levels of oxidation, that change their absorbance through reduction [5, 9, 11].

The methods based on Hydrogen Atom Transfer (HAT), employ a synthetic generator of free radicals, in a sample that contains both an oxidable molecule and an antioxidant [1].

Besides, more methods were devised that measure the capacity of an antioxidant of neutralizing (scavenging capacity) relevant biological oxidants such as: the hydroxyl radical ( $\text{OH}^\bullet$ ), the superoxide anion ( $\text{O}_2^\bullet$ ), the singlet oxygen ( $^1\text{O}_2$ ) or the peroxyntirite anion ( $\text{ONOO}^-$ ).

Commonly, these methods determine either the modification in fluorescence of some substrates, or the chemical luminescence of a substrate [10, 15].

In the present paper is proposed a method for assessing *in vitro* the total antioxidant activity by utilizing as marker the radical cation of chlorpromazine ([2-chloro-N-(3-dimethylamino-propyl)phenothiazine]) abbreviated as  $\text{CP}^{+\bullet}$ .

Chlorpromazine forms, through partial oxidation, a coloured radical cation ( $\lambda_{\text{max}} = 525 \text{ nm}$ ), fairly stable, with major implications in biological systems. Through these radicals, chlorpromazine acts as a redox mediator for electron transfer reactions. Following the interaction with various antioxidants, the absorbance of the radical decreases proportionally with the concentration of the antioxidant.

## Materials and Methods

*Reagent and apparatus.* All used chemicals were of analytical reagent grade.

Chlorpromazine maleate (Sigma)  $10^{-2} \text{ mol/L}$  in hydrochloric acid solution (2 mL HCl 1 mol/L in 50 final volume); dissolving was performed by slightly heating. Potassium persulfate (Merck)  $10^{-3} \text{ mol/L}$  aqueous solution. Gallic acid (Loba Chemie-Wien) standard solution  $10^{-2} \text{ mol/L}$  was prepared in distilled water. Standard solution ( $10^{-4} \text{ mol/L}$ ) was obtained by the stock solution dilution. Hydrochloric acid (Farmitalia) was used as 1 mol/L aqueous solution.

Spectrometric measurements were performed using a spectrophotometer UV-VIS type Hewlett Packard 8453 with multidiode detector.

*Generating the radical cation  $\text{CP}^{+\bullet}$ .* Chlorpromazine reacts with the potassium persulfate in an acid medium, creating the  $\text{CP}^{+\bullet}$  radical cation, colored purple-red, with a maximum of absorption at  $\lambda = 525 \text{ nm}$ . Our previous studies [8] established the optimal parameters for generating the radical cation of chlorpromazine: the detection wavelength, the influence of pH, the optimum molar ratio chlorpromazine/persulfate, the radical stability over time.

By optimizing these parameters, the method for preparing the radical cation was obtained: for a volume of 1 mL chlorpromazine maleate solution  $10^{-2} \text{ mol/L}$  adding 8.5 mL HCl 1 mol/L and 0.5 mL solution  $\text{K}_2\text{S}_2\text{O}_8$   $10^{-3} \text{ mol/L}$ .

In order to obtain a larger volume, these quantities are multiplied.

*Determination of inhibition percent.* 20 minutes after adding  $\text{K}_2\text{S}_2\text{O}_8$  solution, at 2 mL radical solution 1 mL gallic acid standard solution is added having concentrations between 10-100  $\mu\text{M/L}$ , final volume 3 mL.

Absorbance of solutions was measured at 525 nm, against water.

By using the absorbance values of the samples ( $A_s$ ) and of the radical ( $A_r$ ) the percent inhibition has been calculated at 10 minutes and 20 minutes respectively. Inhibition percent was calculated as follows:

$$\% \text{Inh} = (A_r - A_s) / A_r \cdot 100.$$

*Determination of the area under the curve (AUC).*

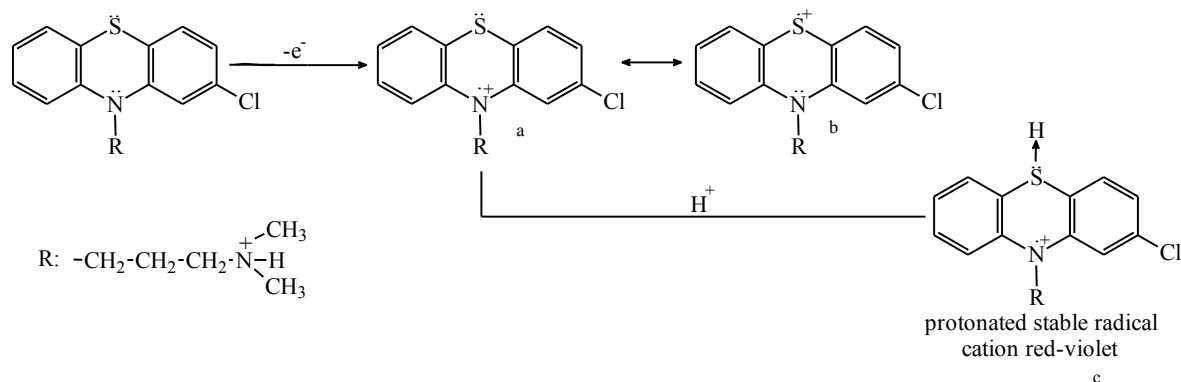
The values of the area under the curve (AUC) were calculated from the graphical representation of inhibition percent variation in a certain time slot at different gallic acid concentration.

The variation of the values of the area under the curve has been plotted as a function of the gallic acid concentration in the concentration intervals studied (10-100  $\mu\text{M/L}$ ).

With the help of calibration curves, the antioxidant activity of the samples can be determined and expressed in molar equivalents of gallic acid (GAE).

## Results and Discussion

It was proven by electrochemical studies and by Electron Spin Resonance (ESR) that the univalent oxidation product of chlorpromazine in acidic medium is a radical cation [4]. The chlorpromazine oxidation mechanism is as follows:

**Figure 1.**

The mechanism of chlorpromazine's oxidation by  $\text{K}_2\text{S}_2\text{O}_8$  in acidic medium

The radical produced through univalent oxidation displays two main resonance structures (a, b). Through protonating the sulphur atom (strong acidic medium), the radical cation is stabilized (c). The protonated radical cation coloured purple-red ( $\lambda = 525 \text{ nm}$ ) remains stable for approximately 20 minutes, at a molar ratio chlorpromazine/ persulfate of 20/1.

The excess chlorpromazine makes certain its univalent oxidation; therefore, under these circumstances, there remains no unreacted persulfate that may subsequently interfere with the radical's reaction with the antioxidants present in the sample.

Antioxidants reduce the preformed radical with an intensity and rate of reaction, dependant on the antioxidant activity, concentration and reaction time.

For this reason, the antioxidant activity may also be expressed in terms of "total contribution to antioxidant activity over the time range studied", by calculating the area under the curve, resulted from the graphical representation of inhibition percent variation in a certain time slot at different concentrations of antioxidant or by graphic representation of gradient % Inh / antioxidant concentration according to time [13].

Calibration curve is obtained by graphic representation of the area under the curve according to the concentration of the standard antioxidant.

In the case of determining the antioxidant activity in  $\text{CP}^{++}$ -gallic acid system, the values of the area under the curve have been calculated from the inhibition percent variation at 10 minutes and 20 minutes for the concentration interval studied (Table I).

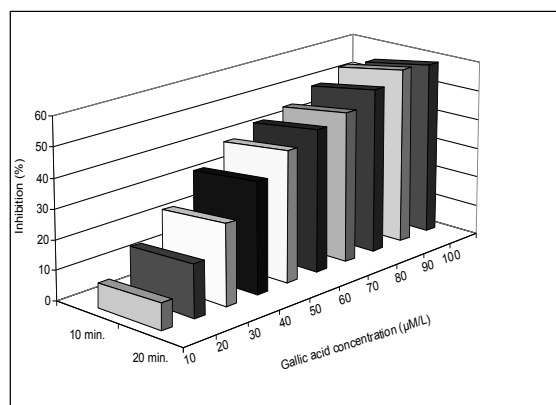
**Table I**

AUC values calculated in  $\text{CP}^{++}$  - gallic acid system

| AG<br>( $\mu\text{M/L}$ ) | Absorbance |         | Inhibition (%) |          | AUC      |
|---------------------------|------------|---------|----------------|----------|----------|
|                           | 10 min.    | 20 min. | 10 min.        | 20 min.  |          |
| 0                         | 0.470300   | 0.43847 | —              | —        | —        |
| 10                        | 0.431170   | 0.39934 | 8.320221       | 8.924214 | 86.22217 |
| 20                        | 0.392947   | 0.36112 | 16.44759       | 17.64157 | 170.4458 |
| 30                        | 0.352120   | 0.32029 | 25.12864       | 26.95281 | 260.4073 |
| 40                        | 0.307730   | 0.27590 | 34.56730       | 37.07665 | 358.2198 |
| 50                        | 0.276823   | 0.24499 | 41.13906       | 44.12548 | 426.3227 |
| 60                        | 0.260783   | 0.22895 | 44.54965       | 47.78366 | 461.6665 |
| 70                        | 0.248500   | 0.21667 | 47.16139       | 50.58499 | 488.7319 |
| 80                        | 0.227863   | 0.19603 | 51.54944       | 55.29158 | 534.2051 |
| 90                        | 0.210290   | 0.17846 | 55.28599       | 59.29938 | 572.9268 |
| 100                       | 0.214457   | 0.18263 | 54.39996       | 58.34903 | 563.7449 |

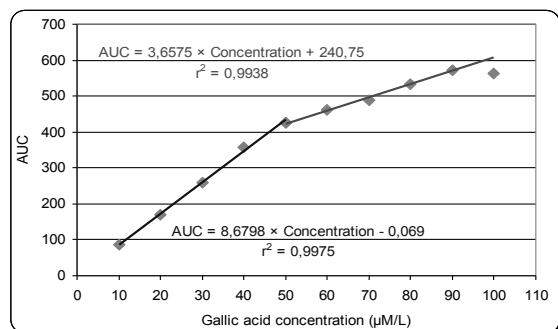
AG = gallic acid concentration; AUC = area under the curve

The profile of the area under the curve has been represented at different concentrations of gallic acid (Figure 2).

**Figure 2.**

Area under the curve (expressed as inhibition of gallic acid concentration) calculated for 10-100  $\mu\text{M/L}$  domain

The variation of the values of area under the curve has been plotted as a function of the gallic acid concentration (Figure 3).



**Figure 3.**

Area under the curve (AUC) according to gallic acid concentration

According to the calibration curves it has been noticed that the considered domain of concentration (10-100 µM/L gallic acid) can be divided in two subdomains of linearity: 10-50 µM/L gallic acid and 50-100 µM/L respectively.

The equations of the regression straight lines for the two concentration domains are:

$$\text{AUC} = 8.6798 \times \text{Concentration} - 0.069; r^2 = 0.9975 \quad (10\text{-}50 \mu\text{M/L});$$

$$\text{AUC} = 3.6575 \times \text{Concentration} + 240.75; r^2 = 0.9938 \quad (50\text{-}100 \mu\text{M/L}).$$

With the help of calibration curves and using the two equations of the regression function antioxidant activity of the samples can be determined and expressed in molar equivalents of gallic acid (GAE).

In order to achieve that, the absorbance of the sample and of the radical have been determined at two specific time moments, same time moments as in the case of the standard (fixed time) and inhibition percent was calculated; inhibition percent variation was graphically represented in the fixed time range and area under the curve was also calculated. From the calibration curves it was determined the total antioxidant activity of the sample.

It has been proven that the reaction of quenching radicals and therefore their discoloration speed are in accordance with the time chosen for performing the measurement. The higher reduction strength, the antioxidants shall react more rapidly with the radicals, whereas in the case of low reduction strength antioxidants the reaction speed would be lower, and the time required for initiating the reaction, greater (lag phases) [3].

The advantage presented by the proposed model consists in the fact that it may be applied at both evaluating antioxidants that present a lag phase, and of those that have no lag phases (with initial rate).

We believe that the method may be used on complex composition biological products: food, vegetable and fruit extracts, plasma, etc.

## Conclusions

It is proposed an *in vitro* model for assessing the total antioxidant activity, based on modifying the preformed radical cation chromophore of chlorpromazine absorbance in acid environment, in the presence of antioxidants. The method is based on measuring the radical colour inhibition percentage, at two selected moments in time, on the graphic representation of the inhibition percentage variation over time (kinetic curve), and determining the area under the curve. Based on the calibration curve obtained in the same way, at different standard antioxidant concentrations (gallic acid) one may evaluate the overall antioxidant activity of the analysed sample.

As a result, determining the area under the curve is an alternative way of describing the antioxidant activity and different reaction rates of antioxidant with free radicals were considered.

The area under the curve implies both antioxidant concentration and reaction time, being a complete measure of the antioxidant's ability to capture free radicals, related to a standard antioxidant within a fixed time interval. The method may be used on complex composition biological products which present an intrinsic antioxidant activity at low pH values. The proposed assay is sensitive and inexpensive.

## References

1. Badarinath A.V., Mallikarjuna K. RAO., Madhu Sudhana Chetty C., Ramkanth S., Rajan T.V.S., Guanaprakash K., A Review on *In-vitro* Antioxidant Methods: Comparisons, Correlations and Considerations. *Int. J. Pharm. Tech. Res.*, 2010; 2(2): 1276-1285.
2. Gruia V., Aramă C., Mitrea N., Arsene A., Grădinaru D., Drăgoi C., The HPLC plasmatic profile of some fat-soluble antioxidant micronutrients (all-Trans-Retinol,  $\alpha$ -tocopherol, coenzyme Q10) in diabetic and dyslipidemic patients. *Farmacia*, 2009; 57(5): 630-638.
3. Huang D., Ou B., Prior R.L., The Chemistry behind Antioxidant Capacity Assays. *J. Agric. Food Chem.*, 2005; 53(6): 1841-1856.
4. Joshi R., Ghanty T.K., Mukherjee T., Reactions and structural investigation of chlorpromazine radical cation. *J. Mol. Struct.*, 2008; 888(1-3): 401-408.
5. Magalinăes L.M., Segundo M.A., Reis S., Lima J.L., Methodological aspects about *in vitro*

- evaluation of antioxidant properties. *Anal. Chim. Acta.*, 2008; 613(1): 1-19.
6. Manea St., Mazilu E., Ristea C., Setnic S., Crețu I., Neagu M., Ivopol G., Popescu M., Comparative determination of certain antioxidants in sea buckthorn (*Hippophae Rhamnoides*) leaves, fruits, juice, oil and oil extraction residues. *Farmacia*, 2006; 54(4): 97-103.
  7. Miron A., Stănescu U., Antioxidanții naturali între medicament și aliment. Iași, Ed. Gr. T. Popa, 2003.
  8. Miftode A.M., Stefanache A., Spac A., Dorneanu V., Spectrometric determination of total antioxidant activity in chlorpromazine radical cation-ascorbic acid system. *Rev. Med. Chim. Soc. Med. Nat.*, Iasi, 2013; 117(3): 806-811.
  9. Niki E., Assessment of antioxidant capacity *in vitro* and *in vivo*. *Free Radic. Biol. Med.*, 2010; 49(4): 503-515.
  10. Papuc C., Crivineanu M., Nicorescu V., Predescu C., Rusu (Mircea) E., Scavenging Activity of Reactive Oxygen Species by Polyphenols Extracted from Different Vegetal Parts of Celandine (*Chelidonium majus*). Chemiluminiscence Screening. *Rev. Chim.*, (Bucharest) 2012; 63(2): 193-197.
  11. Prior R.L., Wu X., Schaich K., Standardized methods for the determination of antioxidant capacity and phenolic in food and dietary supplements. *J. Agric. Food Chem.*, 2005; 53(10): 4290-4302.
  12. Păduraru I., Păduraru O., Miron A., Assessment of antioxidant activity of *Basilici Herba* aqueous extract-*in vitro* studies. *Farmacia*, 2008; 56(4): 402-408.
  13. Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.*, 1999; 26(9-10): 1231-1237.
  14. Robu S., Aprotosoiaie A.C., Miron A., Cioancă O., Stănescu U., Hâncianu M., *In vitro* antioxidant activity of ethanolic extracts from some *Lavandula* species cultivated in Romania. *Farmacia*, 2012; 60(3): 394-401.
  15. Stanciu G., Chirilă E., Dobrinaș S., Negreanu-Pîrjol T., Studies Regarding the Determination of Antioxidant Properties of New Plant Extracts for Cosmetic Purposes. *Rev. Chim. București*, 2010; 61(1): 41-44.
  16. Valko M., Leibfritz D., Moncol J., Cronin M., Mazur M., Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.*, 2007; 39(1): 44-84.