

# **Myoglobin microplate assay to evaluate prevention of protein peroxidation**

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# **Myoglobin microplate assay to evaluate prevention of protein peroxidation**

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## **Abstract**

**Introduction:** The current therapeutic strategies are based on the discovery and developing of multifunctional drug candidates able to interact with various disease related targets. Drugs that have the ability to scavenge reactive oxygen species (ROS), beyond their main therapeutic action, may prevent the oxidative damage of biomolecules. Therefore, analytical approaches that monitor in a continuous mode the ability of drugs to counteract peroxidation of physiologically relevant biotargets are required.

**Objectives:** A microplate spectrophotometric assay is tested and proposed to evaluate the ability of selected cardiovascular drugs to prevent protein peroxidation.

**Methods:** The scavenging capacity of several drugs was tested and compared with two endogenous antioxidants (taurine and reduced glutathione). The studied drugs included an angiotensin-converting enzyme inhibitor (enalapril), several  $\beta$ -blockers (atenolol, labetalol and propranolol) and two statins (pravastatin and fluvastatin). Myoglobin, which is a heme protein, and peroxy radicals generated from thermolysis of 2,2'-azo-bis(2-amidinopropane) dihydrochloride at 37 °C, pH 7.4 were selected as protein model and oxidative species, respectively. Myoglobin peroxidation was continuously monitored by the absorbance decrease at 409 nm and the ability of drugs to counteract protein oxidation was determined by the calculation of the area under the curve upon the myoglobin oxidation.

**Results and Conclusions:** Fluvastatin ( $AUC_{50} = 12.5 \pm 1.2 \mu\text{M}$ ) and enalapril ( $AUC_{50} = 15.2 \pm 1.8 \mu\text{M}$ ) showed high ability to prevent myoglobin peroxidation, providing even better efficiency than endogenous antioxidants such as reduced glutathione. Moreover, labetalol, enalapril and fluvastatin prevent the autooxidation of myoglobin, while glutathione showed a pro-oxidant effect.

**Keywords:** Myoglobin; Peroxy radical; Protein oxidation; Microplate; Statins;  $\beta$ -Blockers.

## **Resumo**

**Introdução:** As estratégias terapêuticas atuais residem na descoberta e desenvolvimento de fármacos multifuncionais que sejam capazes de intervir em diferentes alvos terapêuticos. Os fármacos que possuem a capacidade de sequestrar espécies reativas do oxigênio (ERO), para além do seu principal efeito terapêutico, podem dessa forma prevenir o dano oxidativo das biomoléculas. Neste contexto, torna-se necessário dispor de metodologias que monitorizem em modo contínuo a capacidade dos fármacos impedirem a peroxidação dos alvos biológicos.

**Objetivos:** Testar e propor um método espectrofotométrico em microplaca para avaliar a capacidade de fármacos usados na área cardiovascular para prevenirem a peroxidação proteica.

**Métodos:** A capacidade antioxidante de vários fármacos foi testada e comparada com a de dois antioxidantes endógenos (taurina e glutatona reduzida). Os fármacos estudados incluíram um inibidor da enzima de conversão da angiotensina (enalapril), vários  $\beta$ -bloqueadores (atenolol, labetalol, propanolol) e duas estatinas (pravastatina e fluvastatina). Como proteína modelo foi selecionada a mioglobina, proteína heme, enquanto que os radicais peróxido foram gerados a partir da termólise a 37°C, pH 7,4 do 2,2'-azo-bis(2-amidinopropano) dicloridrato como espécie oxidativa. A peroxidação da mioglobina foi monitorizada continuamente pelo decréscimo de absorvência a 409 nm e a capacidade dos fármacos impedirem a oxidação proteica foi determinada pelo cálculo da área sob a curva da oxidação da mioglobina.

**Resultados e Conclusões:** A fluvastatina ( $AUC_{50} = 12,5 \pm 1,2 \mu M$ ) e o enalapril ( $AUC_{50} = 15,2 \pm 1,8 \mu M$ ) foram os que demonstraram maior capacidade para impedir a peroxidação da mioglobina, que foi inclusivamente superior à de antioxidantes endógenos tais como a glutatona reduzida. O labetalol, enalapril e a fluvastatina previnem a auto-oxidação da mioglobina, enquanto a glutatona demonstrou um efeito pro-oxidante.

**Palavras-chave:** Mioglobina; Radical peróxido; Oxidação proteica; Microplaca; Estatinas;  $\beta$ -Bloqueadores.

## 1. Introduction

Redox reactions are characterized by chemical mechanisms that involve the oxidation (loss of electrons) of one substance and the reduction (acceptance of electrons) of another substance [1]. These reactions are essential for numerous biochemical pathways and cellular chemistry, biosynthesis and regulation of cell signaling [2]. In biological systems, pro-oxidant is a substance that undergoes reduction by inducing oxidative damage to various biological targets such as nucleic acids, lipids and proteins. On the other hand, an antioxidant is a substance that can efficiently counteract the activity of a pro-oxidant, reducing oxidation and inducing the concomitant formation of products having no or low toxicity. In 1995, Halliwell et al. [3] suggested that an antioxidant is “*any substance that when present at low concentrations, compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate*”. In this context, not all reductants involved in a chemical reaction are antioxidants, only those compounds which are capable of protecting the biological target (*oxidizable substrate*).

The pro-oxidants are referred as reactive oxygen species (ROS) such as alkoxy radicals ( $\text{RO}^\bullet$ ), peroxy radicals ( $\text{ROO}^\bullet$ ), hydroxyl radical ( $\text{HO}^\bullet$ ), superoxide anion radical ( $\text{O}_2^{\bullet-}$ ), singlet oxygen ( $^1\text{O}_2$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hypochlorous acid ( $\text{HOCl}$ ). Reactive nitrogen species (RNS) are also produced such as nitric oxide radical ( $\text{NO}^\bullet$ ), nitrogen dioxide radical ( $\text{NO}_2^\bullet$ ) and peroxynitrite anion ( $\text{ONOO}^-$ ) [4, 5]. These reactive species, can be easily formed from exposure of organisms to exogenous sources (UV-irradiation, drugs, pollutants, toxins, pesticides and herbicides) and much more important to endogenous sources that is a continuous process such as from mitochondrial electron-transport chain and from activity of some enzymes as nitric oxide synthases and xanthine oxidase [6]. Moreover, activated phagocytes produce a variety of reactive species that play an important role in the mechanism of defense against infectious agents [7].

Depending on the site and the concentration generated, these reactive species are well recognized for playing a dual role, as both beneficial and deleterious effects have been established [8]. To

prevent the oxidative damage mediated by these species, living cells have developed a complex defense system composed of enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants (uric acid, glutathione, bilirubin and albumin) that convert them to harmless species. Dietary antioxidants such as ascorbic acid (vitamin C),  $\alpha$ -tocopherol (vitamin E), carotenoids and polyphenolic compounds have also an important role in protecting essential biological targets against ROS/RNS action [4].

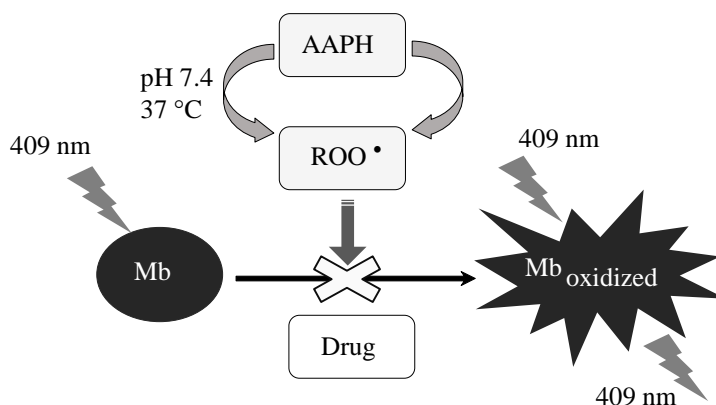
The state called ‘oxidative stress’ occurs when this balance can be shifted towards the pro-oxidant agents due to an overproduction of ROS/RNS and/or to a decrease of antioxidant protection [9]. This can be triggered by several factors such as genetic, diet, lifestyle, environmental conditions and diseases [6]. The oxidative stress has been implicated in the pathogenesis and development of several human diseases, including cardiovascular diseases [10], cancer [11], diabetes mellitus [12], inflammatory diseases [13], ischemia/reperfusion injury [14-16] and neurodegenerative disorders [17].

In this context, the interest in antioxidant research has become a topic of increasing attention in the last two decades, especially within biological, medical and nutritional fields. In the case of biological samples (e.g. plasma, serum, urine), measurement of antioxidant status may be helpful for diagnostic and treatment monitoring, especially during supplementation trials for boosting plasmatic antioxidant levels [18]. However, a validated in vitro assay that can reliably measure the total antioxidant capacity of biological samples is not yet available. This situation is due to the fact that the term total antioxidant capacity is a broader definition that covers: *i*) inhibition of generation and scavenging capacity against ROS/RNS; *ii*) reducing capacity; *iii*) metal chelating capacity; *iv*) activity of antioxidative enzymes; *v*) and inhibition of oxidative enzymes. Moreover, different antioxidants act by different mechanisms and even the same compound can have different ways of actuation [19]. For that reason, the evaluation of overall antioxidant capacity is complex and requires multiple assays to generate an “antioxidant profile” [20].

Drugs that scavenge reactive oxygen species (ROS) and/or inhibit the protein and lipid peroxidation have beneficial clinical effects in the prevention and/or treatment of oxidative-stress related diseases, usually named as pleotropic effects [21]. For instance, statins, namely 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibitors, are the first line therapy for lowering cholesterol levels but various cholesterol-independent beneficial properties, such as reduction of endothelial dysfunction, stabilization of atherosclerotic plaques and decrease of oxidative stress have been indicated, that may potentiate their clinical effects [22]. Actually, current therapeutic strategies are based on the design of multifunctional drug candidates that are able to interact with multiple disease related targets, with the advantages of reduced molecularity, absence of drug-drug interactions and improved pharmacokinetics and pharmacodynamics [23].

The analytical methods that have been applied to measure the scavenging capacity of drugs are not adequate because the assays are based on the use of synthetic radical, such as 2,2-diphenyl-1-picrylhydrazyl radical (DPPH<sup>•</sup>) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS<sup>•+</sup>) and/or on competitive assays that uses target molecules (e.g., fluorescein, luminol and pyrogallol in Oxygen Radical Absorbance Capacity, named as ORAC assay) that do not represent any reactive species and biotarget, respectively, found in living organisms [24].

The research work presented in this thesis was directed to the development of a novel analytical approach to determine the scavenging capacity of cardiovascular drugs using reactive species and oxidizable substrates found at biological milieu. Among the ROS formed in pathophysiological conditions, peroxy radicals were selected as oxidizing species because they are responsible for the propagation step of lipid and protein peroxidation [8]. Hence, the peroxy radicals generated from thermo-decomposition of 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) and an endogenous protein (myoglobin) were used as models of reactive species and biotarget, respectively (Figure 1).



**Figure 1.** Schematic representation of myoglobin (Mb) oxidation mediated by peroxy radicals (ROO<sup>•</sup>) generated from thermolysis of 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and its prevention by drugs.

The myoglobin oxidation induced by peroxy radicals was monitored by the absorbance decrease at 409 nm under reaction conditions (pH, temperature) similar to those found in vivo (Fig. 1). The analytical protocol was implemented in a microplate format in order to obtain a high-throughput procedure. The capacity of cardiovascular drugs and endogenous antioxidants to prevent protein oxidation was determined by the increase of the area under the curve (AUC) and the antioxidant effectiveness of drugs were characterized by their IC<sub>50</sub> values, which represents the drug concentration that causes 50% inhibition of myoglobin oxidation. Finally, the scavenging capacity of several drugs, such as  $\beta$ -blockers (atenolol, labetalol, propranolol), statins (pravastatin, fluvastatin) and angiotensin-converting enzyme (ACE) inhibitors (enalapril) was compared to that obtained for endogenous antioxidants (reduced glutathione and taurine).

## 2. Objectives

The main aims of the present work were:

- i) to develop a high-throughput microplate approach to determine the ROS scavenging capacity of drugs applying relevant biological reactive species (peroxyl radicals) and targets (myoglobin);
- ii) to monitor in a continuous mode the myoglobin peroxidation by the absorbance decrease at 409 nm under reaction conditions (pH, temperature) similar to those found *in vivo*;
- iii) to determine the role of ethanol and DMSO used as cosolvents upon the oxidation kinetics of myoglobin;
- iv) to determine the prevention of autoxidation of myoglobin by cardiovascular drugs;
- v) to assess the capacity of drugs to prevent protein peroxidation by the increase of the area under the curve (AUC);
- vi) to compare the scavenging capacity of several drugs used for cardiovascular pathologies, such as several ACE inhibitors,  $\beta$ -blockers and statins with endogenous antioxidants.

Finally, at the end of this work it is expected that the results obtained may elucidate the relevance of the antioxidant activity of some cardiovascular drugs as an additional pleiotropic effect.

### **3. Original paper\***

#### **Myoglobin microplate assay to evaluate prevention of protein peroxidation**

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## Myoglobin microplate assay to evaluate prevention of protein peroxidation



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### ABSTRACT

The current therapeutic strategies are based on the design of multifunctional drug candidates able to interact with various disease related targets. Drugs that have the ability to scavenge reactive oxygen species (ROS), beyond their main therapeutic action, may prevent the oxidative damage of biomolecules. Therefore, analytical approaches that monitor in a continuous mode the ability of drugs to counteract peroxidation of physiologically relevant biotargets are required.

In the present work, a microplate spectrophotometric assay is proposed to evaluate the ability of selected cardiovascular drugs, including angiotensin-converting enzyme (ACE) inhibitors, β-blockers and statins to prevent protein peroxidation. Myoglobin, which is a heme protein, and peroxy radicals generated from thermolysis of 2,2'-azo-bis(2-amidinopropane) dihydrochloride at 37 °C, pH 7.4 were selected as protein model and oxidative species, respectively. Myoglobin peroxidation was continuously monitored by the absorbance decrease at 409 nm and the ability of drugs to counteract protein oxidation was determined by the calculation of the area under the curve upon the myoglobin oxidation. Fluvastatin ( $AUC_{50} = 12.5 \pm 1.2 \mu\text{M}$ ) and enalapril ( $AUC_{50} = 15.2 \pm 1.8 \mu\text{M}$ ) showed high ability to prevent myoglobin peroxidation, providing even better efficiency than endogenous antioxidants such as reduced glutathione. Moreover, labetalol, enalapril and fluvastatin prevent the autoxidation of myoglobin, while glutathione showed a pro-oxidant effect.

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### 1. Introduction

The multi-target drug design concept is based on the integration of multiple pharmacophores into a single drug molecule in order to make it active within several biological mechanisms, with the advantages of reduced molecular weight, absence of drug–drug interactions and improved pharmacokinetics and pharmacodynamics. This research field has been applied to neurodegenerative and cardiovascular diseases where multi-target strategies are a promising alternative to the classical “one target-one drug” approach [1]. Drugs that have the ability to scavenge reactive oxygen species (ROS), which were implicated in the development of several

oxidative-stress related human diseases, have demonstrated beneficial clinical effects, usually named as pleiotropic effects [2]. For this reason, the assessment of antioxidant capacity of drugs and drug candidates is a relevant analytical step to establish their therapeutic profile [1].

Several methodologies for assessment of antioxidant properties have been applied to pharmaceutical compounds, including direct detection of free radical scavenging by electron spin resonance, reduction of synthetic radicals and of metal species, as well as the determination of biomarkers of oxidative stress [3]. Among these methods, the oxygen radical absorbance capacity (ORAC) has become a popular antioxidant assay because peroxy radicals are employed as reactive species and they represent the main lipid peroxidation propagators [4]. ORAC assay was originally proposed using 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH) as a thermal radical initiator to provide a steady flux of peroxy radicals to oxidize the model protein β-phycoerythrin which is a fluorescent probe [5]. Drugs with antioxidant properties compete with the probe for the radicals, preventing or retarding probe oxidation.

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However,  $\beta$ -phycoerythrin demonstrated several disadvantages, including the interactions of antioxidants with this protein, its reduced photostability, variations between lot preparations and relatively high cost [4]. During the last decade, other oxidation-sensitive probes with fluorescent (fluorescein [6], *p*-aminobenzoic acid [7], long-wavelength fluorophore Nile blue [8]) or absorbance properties (pyrogallol red [9], alizarin red [10]) have been proposed for reaction monitoring. Nevertheless, as the ORAC values are strongly dependent of the selected probe [10], the physiological meaning of ORAC values are limited because these probes do not represent any biological target susceptible to oxidative damage. In fact, a useful technique for the reliable evaluation of antioxidant properties should work at conditions that are as physiologically relevant and as close as possible to the biological system where they will exert the antioxidant effect.

Low density lipoproteins (LDL) have been used as biotarget of peroxidation assays. However, the inter-individual variability and the complexity of LDLs production ruled out this methodology for routine/screening assays [4]. Antioxidant assays based on the inhibition of protein oxidation mediated by peroxy radicals have been developed using human and bovine serum albumin [11], catalase [12], alkaline phosphatase [13] and citrate synthase [14]. Human serum albumin has also been used as biological target for other reactive oxygen species as hypochlorite [15]. The protein oxidation is determined after a fixed reaction time by the content of carbonyl and hydroperoxide groups formation [11,12], protein size by the band pattern of SDS-PAGE [13], enzymatic activity [12,13], dityrosine [14] and protein chloramines [15] formation. For continuous monitoring of protein oxidation, loss of intrinsic fluorescence of the aromatic residues of serum albumin has also been applied, despite the fact that several drugs can interfere in the fluorescence measurements [16].

Terashima et al. [17] proposed the protein myoglobin as a spectrophotometric probe to evaluate antioxidant capacity. Myoglobin denaturation due to peroxidation is reflected in a decrease of absorbance at 409 nm (Soret band) owing to the exposure of the heme group, buried in a hydrophobic pocket within the protein's interior, to the polar aqueous solvent [18]. This probe was further applied toward different reactive species [19] as well as to determine antioxidant properties of flavonoids [20], leafy vegetables and beans [21] and commercially available miso [22]. However, for peroxy radical scavenging capacity, samples were pre-incubated at 47 °C and the myoglobin protective ratios were calculated from the data after 30 min of reaction, not considering the kinetic information provided by the area under the curve. Myoglobin and hydrogen peroxide have also been used as an oxidation system of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and the antioxidant capacity was assessed by the lag time required to the formation of ABTS radical cation species [23]. This assay was adapted to microplate format for measurement of total antioxidant capacity of human plasma from healthy donors. However, the probe used for antioxidant assessment is ABTS, which does not represent a biological target.

In the present work, a high-throughput microplate assay based on the structural change of myoglobin due to the reaction with peroxy radicals under physiological conditions (37 °C, pH 7.4) was developed to evaluate the ability of selected cardiovascular drugs to prevent protein peroxidation, including angiotensin-converting enzyme (ACE) inhibitors,  $\beta$ -blockers and statins. The analytical conditions, including the effect of radical generator, drugs and oxidized products in the absorbance measurement were determined. The role of ethanol and DMSO as cosolvents upon the oxidation kinetics of myoglobin and the prevention of autooxidation of myoglobin by cardiovascular drugs was also studied. The ability of drugs to counteract myoglobin peroxidation was compared to the endogenous antioxidants such as reduced glutathione and taurine.

## 2. Materials and methods

### 2.1. Reagents

All chemicals used were of analytical reagent grade with no further purification. 2,2'-Azobis-(2-methylpropionamide) dihydrochloride (AAPH), atenolol, enalapril maleate salt, L-glutathione reduced, labetalol hydrochloride, myoglobin from equine skeletal muscle (ref. M0630), potassium phosphate monobasic, pravastatin sodium salt hydrate, ( $\pm$ )-propranolol hydrochloride and taurine were obtained from Sigma-Aldrich (St. Louis, MO). Fluvastatin sodium salt, lovastatin and simvastatin were obtained from Cayman Chemical Company (Michigan, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was obtained from Fluka (Buchs, Switzerland). Water from arium water purification system (resistivity > 18 M  $\Omega$  cm, Sartorius, Goettingen, Germany) was used for preparation of all solutions. Ethanol absolute pro analysis from Panreac (Barcelona, Spain) and DMSO from Sigma-Aldrich (St. Louis, MO) were used as cosolvents.

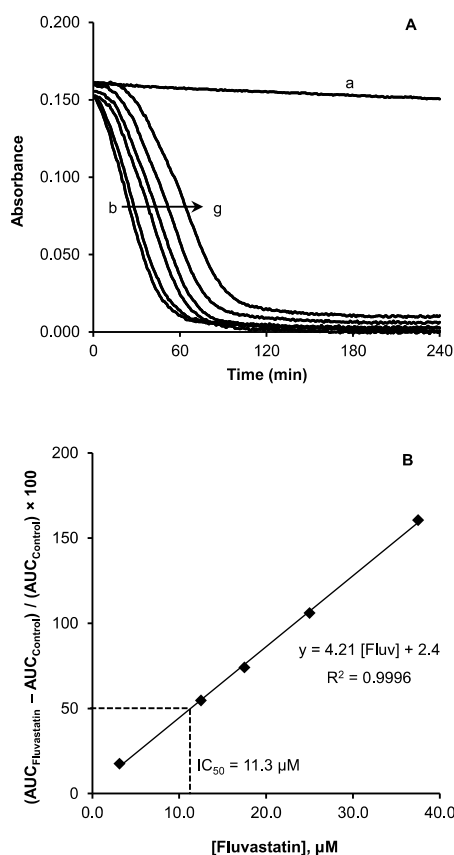
### 2.2. Myoglobin stock solution

Myoglobin stock solution (c.a. 500  $\mu$ g/mL) was prepared by dissolving the protein in phosphate buffer solution (pH 7.4; 50 mM). Before analysis, dilutions of the myoglobin stock solution (between 30 and 300  $\mu$ g/mL) were prepared in phosphate buffer solution and a linear relationship between the protein concentration and absorbance at 409 nm was established. The calibration procedure was performed every day to assure the integrity of the protein and to provide similar protein concentration for all experiments. Hence, a myoglobin solution (75  $\mu$ g/mL) that provided an absorbance value of  $0.200 \pm 0.010$  after dilution in the microplate well was prepared (25  $\mu$ g/mL, concentration in the microplate well). Standard solutions from tested drugs were prepared by dissolving the corresponding solid in phosphate buffer solution.

### 2.3. Microplate protocol for monitoring myoglobin oxidation

The capacity of drugs to prevent the peroxidation of myoglobin (Mb) was evaluated by high-throughput 96-well microplate using spectrophotometric detection at 409 nm, with wavelength selection by a monochromator (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA). Peroxy radicals (ROO $\cdot$ ) were generated by thermo-decomposition of AAPH at 37 °C and at pH 7.4. The microplate layout was tailored to provide results from 6 concentrations of drug, each analyzed in quadruplicate (Supplementary file, Fig. S1). The intrinsic absorption of AAPH at 409 nm, myoglobin (Mb) and of the highest concentration of tested drug was determined in A1–H1, A2–H2 and A3–H3 wells, respectively. The intrinsic absorbance of mixtures (AAPH + drug or myoglobin + drug) was also measured in A4–H4 and A5–H5 wells, respectively, in order to assess the potential interference of drug and/or its oxidation products in the analytical signal of myoglobin during the measurement of its oxidation.

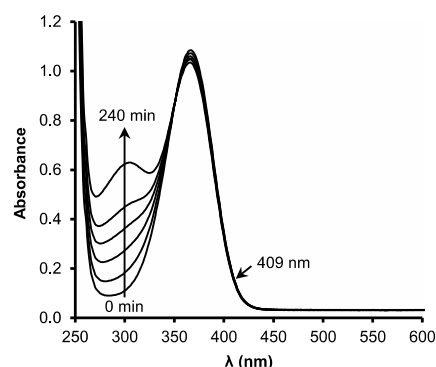
Hence, the microplate myoglobin method was carried out in phosphate buffer (pH 7.4; 50 mM) at 37 °C, where 100  $\mu$ L of Trolox standard solutions (5.0–100  $\mu$ M) or drug solution and 100  $\mu$ L of myoglobin (75  $\mu$ g/mL) were mixed in each well. After that, microplate was pre-incubated at 37 °C for 30 min. Then, 100  $\mu$ L of freshly prepared AAPH solution (180 mM) was added and the absorbance at 409 nm was recorded every minute during 240 min, beginning after the first minute of reaction. The control experiment ( $C_t$ , Fig. S1), where no protection of myoglobin oxidation is expected, was performed by replacing the drug solution with 100  $\mu$ L of buffer.



**Fig. 1.** (A) Decay of absorbance of myoglobin (25 µg/mL) mediated by peroxy radical oxidation (AAPH, 60 mM) in the presence of different concentrations of fluvastatin: (a) myoglobin (25 µg/mL) in the absence of oxidant species; (b–g) increasing concentrations of fluvastatin (0, 3.0, 12.5, 17.5, 25.0 and 37.5 µM). The intrinsic absorbance value of AAPH at 409 nm was subtracted from the absorbance decay of myoglobin. (B) Relationship between antioxidant capacity expressed as the increase of area under the curve (AUC) and the concentration of fluvastatin obtained for one experiment. The  $AUC_{50}$  correspond to the fluvastatin concentration that provided an absorbance increase of 50% of the AUC of the control.

#### 2.4. Calculation of antioxidant capacity values of drugs expressed as $IC_{50}$ and TEAC

The protein oxidation induced by  $ROO^{\bullet}$  species was indirectly monitored by the absorbance decrease at 409 nm. The kinetic profile of myoglobin oxidation mediated by AAPH-derived peroxy radicals in the absence (control) and in the presence of increasing concentrations of cardiovascular drugs is presented in Fig. 1A, using fluvastatin as example. Similar kinetic profiles were obtained for the other tested drugs, excluding Trolox (classical standard compound) that prevented the immediate decay of absorbance and provided a plateau (as myoglobin oxidation started when there was no more antioxidant left to scavenge peroxy radicals). The antioxidant curves (absorbance vs time) were normalized (relative absorbance, %) by dividing the absorbance value at a given time ( $Abs_{x_{min}}$ ) by the absorbance value attained at first minute ( $Abs_{1min}$ ). The area under the curve (AUC) was calculated by integrating the relative absorbance curve as a function of the reaction time using Microcal Origin 6.1<sup>TM</sup> software. The antioxidant capacity



**Fig. 2.** UV-vis spectra of AAPH (2,2'-azobis-(2-methylpropanamide) dihydrochloride) solution (60 mM, pH 7.4, 37 °C) at different reaction times (0, 30, 60, 120, 180 and 240 min). The absorbance signal at determination wavelength (409 nm) was constant along reaction monitoring.

(AC) of the sample was determined for each tested drug concentration using the following equation:

$$AC = \frac{AUC_{Compound} - AUC_{control}}{AUC_{control}} \times 100 \quad (1)$$

where  $AUC_{Compound}$ , corresponds to the area under the curve obtained for a given concentration of Trolox or drug, while  $AUC_{control}$  is related to the area under the curve of myoglobin oxidation in the absence of antioxidant species. A linear relationship was established between antioxidant capacity (%) and drug concentration (Fig. 1B). The concentration of drug (µM) that provided an increase of 50% of the AUC of control experiment ( $AUC_{50}$ ) was determined. Lower  $AUC_{50}$  values correspond to a higher ability of drug to prevent myoglobin oxidation. For all the drugs tested, different concentrations were evaluated and the results obtained correspond to the mean  $\pm$  standard deviation of two experiments performed in quadruplicate.

The Trolox Equivalent Antioxidant Capacity (TEAC) values were determined by the ratio between the  $AUC_{50}$  obtained for a given drug and that attained for the standard compound (Trolox). The relationship determined between antioxidant capacity (AC) and Trolox concentration was  $AC(\%) = 3.2 (\pm 0.3) \times [Trolox, (\mu\text{mol/L})] + 4.3 (\pm 2.6)$ ,  $R > 0.995$ .

### 3. Results and discussion

#### 3.1. Evaluation of analytical conditions of myoglobin microplate assay

The myoglobin method is based on the measurement of the absorbance decay at 409 nm due to oxidation of heme group of protein induced by peroxy radicals [17–19]. The UV-vis spectra of myoglobin solution at different reaction times (0, 30, 60, 90 and 120 min) in the presence of AAPH showed the decrease of absorbance peak (409 nm) of native myoglobin along reaction development (Supplementary data, Fig. S2). Moreover, myoglobin peroxidation does not generate oxidation products that absorb between 350 and 600 nm.

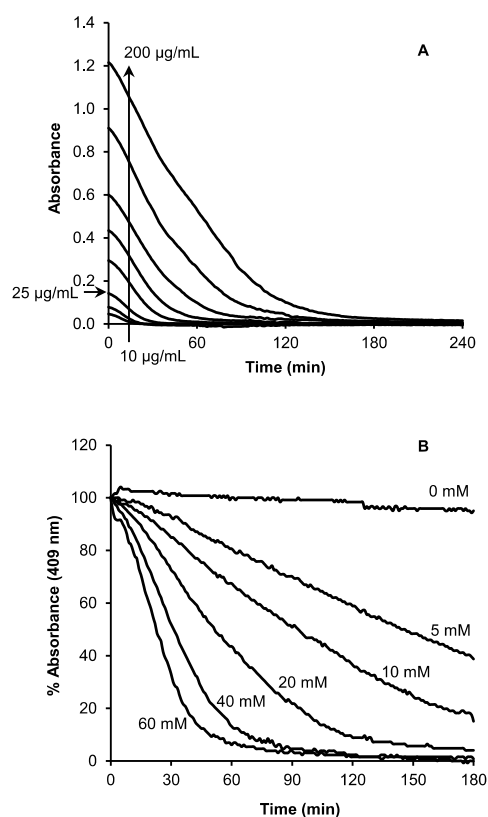
The absorbance of all components present in the reaction media (AAPH, Mb, drug) and their mixtures, at determination wavelength (409 nm), was measured in order to assess their potential interference. The absorbance spectrum of AAPH solution along reaction development was determined and it was observed that the radical generator also absorbs at 409 nm. Hence, as the analytical signal was constant throughout the reaction time (Fig. 2),

the intrinsic absorbance of AAPH at 409 nm was measured (Fig. S1, A1–H1) and the respective absorbance value was subtracted from the absorbance decay of myoglobin. This issue was not considered in the previous work published by Terashima et al. [19], which used lower concentrations of AAPH (13.9 mM) and higher reaction temperature (47 °C) in order to enhance the generation rate of peroxy radicals to oxidize protein. Moreover, as the intrinsic absorption of AAPH was not considered, a residual absorbance value was obtained at the end of the reaction [20].

For all tested drugs, intrinsic absorbance at 409 nm was not observed as well as the absorbance of myoglobin solution was not affected by the presence of drug. However, for higher concentrations of fluvastatin (>800 μM), absorbance increase was observed during reaction monitoring because its oxidation products also absorbed at the determination wavelength (data not shown). Therefore, to avoid the interference of intrinsic absorption of fluvastatin oxidation products, lower concentrations were used.

The stability of myoglobin along reaction was evaluated by monitoring the absorbance value of protein in the absence of oxidant species (Fig. S1, A2–H2). The decay of absorbance after 60, 120, 180 and 240 min was 1.8, 5.7, 10.3 and 15.0% of the initial absorbance value, respectively, indicating an autoxidation process. This issue occurs due to the direct dissociation of the neutral superoxide radical from oxymyoglobin that may oxidize amino acid residues and denature proteins [24]. Moreover, maintenance of Mb in the reduced state is not possible due to the formation of ferryl Mb species and to the dissociation of ferrityporphyrin IX from the globin, with consequent release of iron atoms that can also induce protein oxidation [25]. This autoxidation process of heme proteins plays an important role in pathophysiology of vasospasms following subarachnoid hemorrhages and renal dysfunction after rhabdomyolysis [26]. Trolox (100 μM), labetalol (100 μM), enalapril (100 μM) and fluvastatin (37.5 μM) decreased autoxidation of myoglobin to 2.5–5.0%, while taurine (500 μM), atenolol (500 μM), propranolol (100 μM) and pravastatin (100 μM) did not prevent autoxidation. On the other hand, for the major endogenous antioxidant (reduced glutathione) a pro-oxidant effect was observed since the autoxidation of myoglobin increased up to 30% after 240 min of reaction monitoring. This pro-oxidant effect was observed for higher glutathione concentrations (500 μM). In fact, the interaction of thiols with reactive radicals can generate thyl radicals which have a pro-oxidant effect, especially in iron-rich environments, which is the case of iron atoms release during Mb autoxidation [27]. However, reduced glutathione represents a powerful endogenous antioxidant as demonstrated by other antioxidant assays that applied different mechanisms of reaction such as metal reduction [28] and scavenging of multiple reactive oxygen species [29].

The influence of the probe (myoglobin) and the radical generator (AAPH) concentrations in the microplate method was studied. The concentration of myoglobin determines the initial absorbance value and the amount of radical species required to oxidize the spectrophotometric probe. The oxidation kinetics profile obtained for different concentrations of myoglobin (10–200 μg/mL) in the microplate well at similar concentration of radical generator (60 mM) is depicted in Fig. 3A. Higher myoglobin concentration (200 μg/mL) provided higher analytical signals at 409 nm and better reproducibility between assays (RSD < 1%) compared to those that applied lower myoglobin concentration (10 μg/mL), showing higher variability of initial absorbance value (RSD < 15%, data not shown). However, higher concentrations of probe required extended reaction monitoring in order to oxidize amino acid residues of myoglobin and to expose the heme group to the polar environment, that leaves the hydrophobic pocket of the protein [18]. For instance, 15 and 180 min are the time intervals required to oxidize almost all spectrophotometric probe at 10 and 200 μg/mL,



**Fig. 3.** (A) Decay of absorbance at 409 nm of myoglobin at different concentrations in microplate well (10–200 μg/mL) mediated by fixed initial peroxy radical generator concentration (AAPH, 60 mM). (B) Decay of absorbance (%) of myoglobin (25 μg/mL) mediated by different initial AAPH concentrations in the reaction media (0–60 mM). The absorbance (%) values were obtained by using the following equation:  $(Abs_{t-x} \text{ min}/Abs_{t=0 \text{ min}}) \times 100$ . For both graphs, the concentrations correspond to values present in the microplate well.

respectively (Fig. 3A). Moreover, higher concentrations of antioxidant are required to prevent the oxidation of higher concentrations of probe, which can be a problem for those drugs with low solubility in aqueous media. Therefore, 25 μg/mL of myoglobin was selected for further studies as a compromise between reproducibility and a procedure that does not require high antioxidant concentrations for protein protection. The lower reproducibility of lower protein concentrations was circumvented by a myoglobin calibration procedure performed before each analysis ( $Abs_{409 \text{ nm}} = 0.00585 [\text{Mb}, (\mu\text{g/mL})] + 0.053$ ,  $R > 0.9995$ ,  $n = 10$ ).

For a fixed concentration of myoglobin (25 μg/mL), the protein oxidation rates induced by peroxy radicals were dependent of the initial AAPH concentration because it determines the amount of radical species produced within a given time interval (Fig. 3B). In fact, the absorbance decay in the first minutes of reaction (30 min) is directly related to the initial concentration of AAPH by the following equation:  $\log(\Delta Abs/\text{min}) = 0.756 \times \log[\text{AAPH}, \text{mM}] - 3.86$ ,  $R = 0.998$ ,  $n = 5$ . Lower concentrations of AAPH (5 mM) required longer time intervals to oxidize protein and to ascertain the prevention of probe oxidation by the tested drugs, originating a time-consuming procedure. The initial concentration of AAPH was selected at 60 mM, which corresponds to about 0.441 mM of ROO• radicals generated after 90 min of reaction [4], because at this time almost all protein was oxidized (Fig. 3B). Higher concentrations of

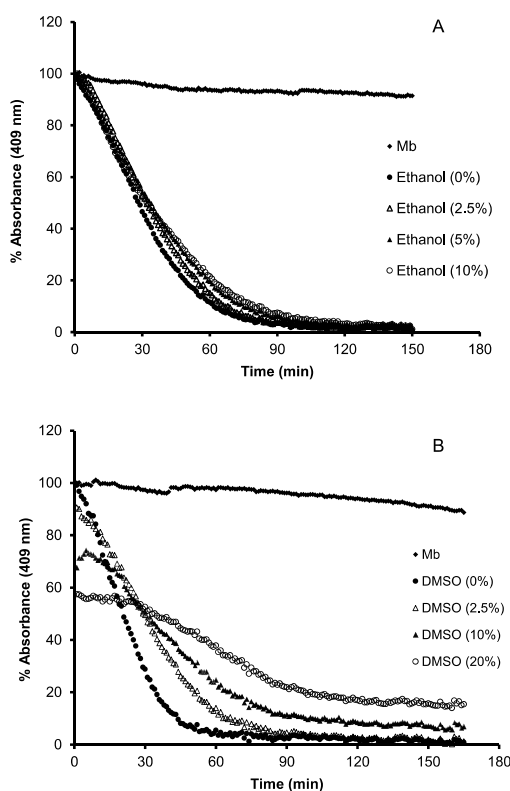


Fig. 4. Decay of absorbance (%) of myoglobin (25 µg/mL) mediated by peroxy radicals (AAPH, 60 mM) in the presence of different percentages (v/v) of ethanol (A) and DMSO (B) in the reaction media.

AAPH (>60 mM) provided shorter time intervals for probe oxidation. However, the initial kinetics of protein oxidation could not be measured as some few minutes are taken between the addition of AAPH solution and starting the absorbance measurement.

Considering that some drugs have low solubility in aqueous media, the influence of ethanol and DMSO on the oxidation kinetics of myoglobin was studied (Fig. 4). For ethanol, the initial absorbance values of myoglobin solutions were similar to those obtained in absence of ethanol. This result confirms that the structure of the heme group of myoglobin was not affected by the addition of ethanol as cosolvent. In fact, the solubility of myoglobin in pure ethanol has been described as negligible. Nevertheless, a higher solubility has been observed in c.a. 30% in volume of ethanol [30]. Ethanol concentrations (>10%, v/v) were not tested as it does not represent a biological environment. The slight increase of the AUC observed with higher concentrations of ethanol (up to 10%) could be related to the lower peroxy radical generation rate from AAPH in ethanolic medium.

In the case of DMSO, it was observed a decrease of the initial absorbance of myoglobin solution with increasing concentrations of DMSO, indicating the denaturing effect of this cosolvent toward proteins. Moreover, a delay in the protein oxidation process was also observed. Both effects were attained for lower concentrations of DMSO (2.5% v/v) and were related to DMSO concentration in the reaction media. In the literature, it has been described that sec-

**Table 1**  
Capacity of drugs to prevent myoglobin oxidation mediated by peroxy radicals.

Compound	Concentration range (µM)	AUC <sub>50</sub> <sup>a</sup> (µM)	TEAC <sup>b</sup>
Trolox	5.0–100	15.9 ± 3.8	1.0
Fluvastatin	3.0–37.5	12.5 ± 1.2	0.8
Pravastatin	5.0–100	51.5 ± 0.7	3.2
Atenolol	5.0–500	>500	n.a.
Labetalol	5.0–100	29.1 ± 3.9	1.8
Propranolol	5.0–100	21.0 ± 2.2	1.3
Enalapril	5.0–100	15.2 ± 1.8	1.0
Taurine	5.0–500	>500	n.a.
Glutathione	5.0–500	232 ± 10	14.6

<sup>a</sup> AUC<sub>50</sub> correspond to drug concentration that provided an increase of 50% of the area under the curve of the myoglobin oxidation (control experiment).

<sup>b</sup> TEAC, Trolox equivalent antioxidant capacity, calculated as the ratio of AUC<sub>50</sub> (compound)/AUC<sub>50</sub> (Trolox). n.a., not available.

ondary structure and thermal stability of myoglobin is modified, even at low concentrations of DMSO [31]. This occurs because the sulfoxide group, as a strong hydrogen acceptor, probably removes water from the hydration shell of the myoglobin [30]. Hence, drugs with low solubility in buffer media could be tested at 10% (v/v) in ethanol with minor modifications in the heme environment, while DMSO as cosolvent should be avoided. Moreover, for comparison purposes the antioxidant standard compound (Trolox) should also be tested under the same conditions.

### 3.2. Ability of cardiovascular drugs to prevent myoglobin peroxidation

To evaluate the applicability of the proposed myoglobin microplate assay to assess the ability of drugs to prevent protein peroxidation, some cardiovascular drugs such as statins (fluvastatin, pravastatin), β-blockers (atenolol, labetalol, propranolol) and ACE inhibitor (enalapril) were selected. For comparison purposes, reduced glutathione and taurine were used as endogenous antioxidants, while Trolox was applied as a classical antioxidant standard compound. The AUC<sub>50</sub> values, which correspond to the concentration of drug that provided an increase of 50% of the AUC value of control, and the Trolox equivalents antioxidant capacity (TEAC) values are given in Table 1. Simvastatin and lovastatin were also tested, however, for stock solutions of buffer/ethanol (70:30, v/v) at 200 µM, that corresponds to 10% (v/v) in ethanol in microplate well, both drugs were not soluble. Therefore, their AUC<sub>50</sub> values were not determined. Among the drugs tested, fluvastatin was the most potent scavenger protecting myoglobin from peroxidation, even more than antioxidant standard Trolox, as it can be observed for the lower AUC<sub>50</sub> value obtained (12.5 ± 1.2 µM). The antioxidant effect of the other HMG-CoA reductase inhibitor tested, pravastatin, was about 5 times lower considering the AUC<sub>50</sub> value determined was 51.5 ± 0.7 µM. The higher scavenging properties of fluvastatin are related to the electrophilic group near to the double bond and to the alcohol function that may stabilize the oxidized species formed, while pravastatin does not present this electrophilic group (Supplementary data, Fig. S3). Similar results were described by Franzoni et al. [32] which demonstrated that fluvastatin was the statin that exhibited the highest anti-peroxy radical scavenging capacity using the total oxyradical scavenging capacity (TOSC) assay. This assay evaluates the ability of compounds to prevent the peroxidation of alpha-keto-gamma-methylbutyric acid. Moreover, the ability of fluvastatin to inhibit the formation of thiobarbituric acid reactive substances in iron (II)-supported peroxidation of liposomes was much larger than that presented by pravastatin [33].

Among β-blockers, propranolol presented higher capacity to prevent myoglobin oxidation (AUC<sub>50</sub> = 21.0 ± 2.2 µM) than labetalol (AUC<sub>50</sub> = 29.1 ± 3.9 µM), whilst atenolol did not demonstrated any antioxidant properties, even at high concentrations

(500  $\mu\text{M}$ ). The peroxy radical scavenging capacity obtained for propranolol has been described for the ORAC–fluorescein assay, and labetalol and atenolol did not prevent fluorescein oxidation [34]. Propranolol was also the most potent  $\beta$ -blocker to prevent lipid peroxidation in membrane models and cellular assays, exhibiting a membrane-fluidizing effect with the capacity to disturb membrane organization [35]. This membrane effect could be related to its higher hydrophobicity and it may explain the higher protection of heme group of myoglobin present in the hydrophobic pocket. On the other hand, labetalol and atenolol have hydrophilic groups in its molecular structure that may decrease the amount of drug near to the heme group of myoglobin (Fig. S3).

Enalapril, angiotensin-converting enzyme (ACE) inhibitor, showed high ability to prevent myoglobin peroxidation ( $\text{AUC}_{50} = 15.2 \pm 1.8 \mu\text{M}$ ), similar to standard antioxidant Trolox (Table 1). This antihypertensive drug has presented, beyond its hypotensive and renoprotective effects, the capacity to reduce oxidative stress markers and enhance the activity of antioxidant enzymes in kidney of spontaneously hypertensive rat [36]. The primary and secondary prevention of cardiovascular diseases may also largely depend on its pleiotropic effects, including antioxidant, antithrombotic and profibrinolytic activities [37].

In the myoglobin method, the ability of the endogenous antioxidant reduced glutathione ( $\text{AUC}_{50} = 232 \pm 10 \mu\text{M}$ ) was lower than most of the drugs tested. This result could be related to the pro-oxidant effect observed for GSH at high concentrations due to the production of thiyl radicals that could potentiate the oxidation of myoglobin [27]. Previous studies, which sought to determine antioxidant capacity of taurine, a sulfur-containing  $\beta$ -amino acid, observed minimal free radical scavenging capacity against many oxygen-derived radicals including peroxy species [38]. Similar results were obtained in the present myoglobin method which used concentrations up to 500  $\mu\text{M}$ , that are higher than the concentrations of taurine found in plasma and extracellular fluids (10–100  $\mu\text{M}$ ). Nevertheless, when taurine concentrations were used in the range of intracellular levels that can reach up to 80 mM, depending on the type of tissue, significant scavenging potential against peroxy radical, nitric oxide and superoxide was described [39]. Therefore, to predict the ability of taurine to prevent myoglobin oxidation in intracellular environment, higher concentrations should be tested. The proposed microplate assay allowed the assessment of antioxidant capacity of two drugs in less than 240 min, with control of different reaction conditions. The estimated cost per analysis for each drug is about 0.90 €, considering disposable microplate wells.

#### 4. Conclusions

A high-throughput microplate spectrophotometric method was developed to determine the ability of drugs to prevent peroxidation of myoglobin, applied here as protein model. In contrast to other peroxy radical assays that also use proteins as oxidation probes, this microplate myoglobin method continuously monitors the time-dependent oxidation process and its inhibition by drugs. Autoxidation process of myoglobin as physiological oxidation inductor was inhibited by labetalol, enalapril and fluvastatin, while glutathione showed a pro-oxidant effect. Fluvastatin showed higher ability to prevent myoglobin peroxidation, about 20 times higher than the widely spread endogenous antioxidant (reduced glutathione), which can be physiologically relevant due to muscle toxicity induced by statins. Therefore, the proposed microplate myoglobin assay is a simple and cost-effective analytical approach suitable as front-end procedure to predict the ability of multifunctional drugs to prevent protein oxidation, enhancing the knowledge about their pleiotropic effects.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2015.06.006>

#### References

- [1] Y. Bansal, O. Silakari, Multifunctional compounds: smart molecules for multifactorial diseases, *Eur. J. Med. Chem.* 76 (2014) 31–42.
- [2] G.J. Moon, S.J. Kim, Y.H. Cho, S. Ryoo, O.Y. Bang, Antioxidant effects of statins in patients with atherosclerotic cerebrovascular disease, *J. Clin. Neurol.* 10 (2014) 140–147.
- [3] G. Beretta, R.M. Facino, Recent advances in the assessment of the antioxidant capacity of pharmaceutical drugs: from in vitro to in vivo evidence, *Anal. Bioanal. Chem.* 398 (2010) 67–75.
- [4] D.J. Huang, B.X. Ou, R.L. Prior, The chemistry behind antioxidant capacity assays, *J. Agric. Food Chem.* 53 (2005) 1841–1856.
- [5] G.H. Cao, H.M. Alessio, R.G. Cutler, Oxygen-radical absorbency capacity assay for antioxidants, *Free Radical Biol. Med.* 14 (1993) 303–311.
- [6] B.X. Ou, M. Hampsch-Woodill, R.L. Prior, Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe, *J. Agric. Food Chem.* 49 (2001) 4619–4626.
- [7] K. Guclu, G. Kibrislioglu, M. Ozyurek, R. Apak, Development of a fluorescent probe for measurement of peroxy radical scavenging activity in biological samples, *J. Agric. Food Chem.* 62 (2014) 1839–1845.
- [8] J. Godoy-Navajas, M.P.A. Caballos, A. Gomez-Hens, Long-wavelength fluorimetric determination of food antioxidant capacity using Nile Blue as reagent, *J. Agric. Food Chem.* 59 (2011) 2235–2240.
- [9] C. Lopez-Alarcon, E. Lissi, A novel and simple ORAC methodology based on the interaction of Pyrogallol Red with peroxy radicals, *Free Radical Res.* 40 (2006) 979–985.
- [10] I. Martin, A. Aspee, P. Torres, E. Lissi, C. Lopez-Alarcon, Influence of the target molecule on the Oxygen Radical Absorbance Capacity index: a comparison between alizarin red- and fluorescein-based methodologies, *J. Med. Food* 12 (2009) 1386–1392.
- [11] M. Anraku, M. Kabashima, H. Namura, T. Maruyama, M. Otagiri, J.M. Gebicki, N. Furutani, H. Tomida, Antioxidant protection of human serum albumin by chitosan, *Int. J. Biol. Macromol.* 43 (2008) 159–164.
- [12] J.C. Mayo, D.X. Tan, R.M. Sainz, S. Lopez-Burillo, R.J. Reiter, Oxidative damage to catalase induced by peroxy radicals: functional protection by melatonin and other antioxidants, *Free Radical Res.* 37 (2003) 543–553.
- [13] F. Bertolini, L. Novaroli, P.A. Carrupt, M. Reist, Novel screening assay for antioxidant protection against peroxy radical-induced loss of protein function, *J. Pharm. Sci.* 96 (2007) 2931–2944.
- [14] N.L. Chepelev, J.D. Bennett, J.S. Wright, J.C. Smith, W.G. Willmore, Oxidative modification of citrate synthase by peroxy radicals and protection with novel antioxidants, *J. Enzyme Inhib. Med. Chem.* 24 (2009) 1319–1331.
- [15] O. Firuzi, L. Giansanti, R. Vento, C. Seibert, R. Petrucci, G. Marrosu, R. Agostino, L. Saso, Hypochlorite scavenging activity of hydroxycinnamic acids evaluated by a rapid microplate method based on the measurement of chloramines, *J. Pharm. Pharmacol.* 55 (2003) 1021–1027.
- [16] D.E. Epps, T.J. Raub, V. Caiolfa, A. Chiari, M. Zamai, Determination of the affinity of drugs toward serum albumin by measurement of the quenching of the intrinsic tryptophan fluorescence of the protein, *J. Pharm. Pharmacol.* 51 (1999) 41–48.
- [17] M. Terashima, I. Nakatani, A. Harima, S. Nakamura, M. Shiiba, New method to evaluate water-soluble antioxidant activity based on protein structural change, *J. Agric. Food Chem.* 55 (2007) 165–169.
- [18] P.A. Sykes, H.C. Shiu, J.R. Walker, R.C. Bateman, Determination of myoglobin stability by visible spectroscopy, *J. Chem. Educ.* 76 (1999) 1283–1284.
- [19] M. Terashima, R. Watanabe, M. Ueki, S. Matsumura, Comprehensive evaluation of antioxidant activity for various substances with 5-axe cobweb chart, *Food Chem.* 120 (2010) 150–155.
- [20] M. Terashima, Y. Kakuno, N. Kitano, C. Matsuoka, M. Murase, N. Togo, R. Watanabe, S. Matsumura, Antioxidant activity of flavonoids evaluated with myoglobin method, *Plant Cell Rep.* 31 (2012) 291–298.
- [21] M. Terashima, A. Fukukita, R. Kodama, H. Miki, M. Suzuki, M. Ikegami, N. Tamura, A. Yasuda, M. Morikawa, S. Matsumura, Evaluation of antioxidant activity of leafy vegetables and beans with myoglobin method, *Plant Cell Rep.* 32 (2013) 349–357.

- [22] M. Morikawa, Y. Ando, A. Kita, M. Sugita, S. Matsumura, M. Terashima, Comprehensive evaluation of the antioxidant activity of Miso by the myoglobin method, *Food Sci. Technol. Res.* 20 (2014) 1221–1228.
- [23] Y. Kambayashi, N.T. Binh, H.W. Asakura, Y. Hibino, Y. Hitomi, H. Nakamura, K. Ogino, Efficient assay for total antioxidant capacity in human plasma using a 96-well microplate, *J. Clin. Biochem. Nutr.* 44 (2009) 46–51.
- [24] R.E. Brantley, S.J. Smerdon, A.J. Wilkinson, E.W. Singleton, J.S. Olson, The mechanism of autooxidation of myoglobin, *J. Biol. Chem.* 268 (1993) 6995–7010.
- [25] M.P. Richards, Redox reactions of myoglobin, *Antioxid. Redox Signal.* 18 (2013) 2342–2351.
- [26] B.J. Reeder, D.A. Svistunenko, C.E. Cooper, M.T. Wilson, The radical and redox chemistry of myoglobin and hemoglobin: from in vitro studies to human pathology, *Antioxid. Redox Signal.* 6 (2004) 954–966.
- [27] M.L. Sagrista, A.F. Garcia, M.A. De Madariaga, M. Mora, Antioxidant and pro-oxidant effect of the thiolic compounds *N*-acetyl-L-cysteine and glutathione against free radical-induced lipid peroxidation, *Free Radical Res.* 36 (2002) 329–340.
- [28] S.S. Marques, L.M. Magalhaes, I.V. Toth, M.A. Segundo, Insights on antioxidant assays for biological samples based on the reduction of copper complexes—the importance of analytical conditions, *Int. J. Mol. Sci.* 15 (2014) 11387–11402.
- [29] Y. Sueishi, M. Hori, M. Ishikawa, K. Matsu-Ura, E. Kamogawa, Y. Honda, M. Kita, K. Ohara, Scavenging rate constants of hydrophilic antioxidants against multiple reactive oxygen species, *J. Clin. Biochem. Nutr.* 54 (2014) 67–74.
- [30] K.C.S. Figueiredo, H.C. Ferraz, C.P. Borges, T.L.M. Alves, Structural stability of myoglobin in organic media, *Protein J.* 28 (2009) 224–232.
- [31] Q.C. Li, P.A. Mabrouk, Spectroscopic and electrochemical studies of horse myoglobin in dimethyl sulfoxide, *J. Biol. Inorg. Chem.* 8 (2003) 83–94.
- [32] F. Franzoni, A. Quinones-Galvan, F. Regoli, E. Ferrannini, F. Galetta, A comparative study of the in vitro antioxidant activity of statins, *Int. J. Cardiol.* 90 (2003) 317–321.
- [33] A. Yamamoto, K. Ichihara, K. Hoshi, Antioxidative effect of fluvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, on peroxidation of phospholipid liposomes, *J. Pharm. Pharmacol.* 53 (2001) 227–232.
- [34] A. Gomes, D. Costa, J. Lima, E. Fernandes, Antioxidant activity of beta-blockers: an effect mediated by scavenging reactive oxygen and nitrogen species? *Bioorg. Med. Chem.* 14 (2006) 4568–4577.
- [35] C. Pereira-Leite, C. Carneiro, J.X. Soares, C. Afonso, C. Nunes, M. Lucio, S. Reis, Biophysical characterization of the drug-membrane interactions: the case of propranolol and acebutolol, *Eur. J. Pharm. Biopharm.* 84 (2013) 183–191.
- [36] G. Chandran, K.N.S. Sirajudeen, N.S.N. Yusoff, M. Swamy, M.S. Samarendra, Effect of the antihypertensive drug enalapril on oxidative stress markers and antioxidant enzymes in kidney of spontaneously hypertensive rat, *Oxidative Med. Cell. Longev.*, Vol. 2014, 10, Article ID 608512, <http://dx.doi.org/10.1155/2014/608512>
- [37] R. Krysiak, B. Okopien, Pleiotropic effects of angiotensin-converting enzyme inhibitors in normotensive patients with coronary artery disease, *Pharmacol. Rep.* 60 (2008) 514–523.
- [38] O.I. Aruoma, B. Halliwell, B.M. Hoey, J. Butler, The antioxidant action of taurine, hypotaurine and their metabolic precursors, *Biochem. J.* 256 (1988) 251–255.
- [39] M.W.S. Oliveira, J.B. Minotto, M.R. de Oliveira, A. Zanotto, G.A. Behr, R.F. Rocha, J.C.F. Moreira, F. Klamt, Scavenging and antioxidant potential of physiological taurine concentrations against different reactive oxygen/nitrogen species, *Pharmacol. Rep.* 62 (2010) 185–193.

#### **4. General conclusions**

The analytical approach developed using biomimetic system represents a step forward for physiologically relevant antioxidant assays and for the assessment of ability of drugs to protect biotargets from oxidative damage. The microplate protocol allows monitoring in a continuous mode the myoglobin peroxidation providing kinetic information about the ability of drugs to prevent protein oxidation. Drugs with low solubility in buffer media could be tested at 10% (v/v) in ethanol with minor modifications in the heme environment of myoglobin, while dimethyl sulfoxide (DMSO) as cosolvent should be avoided. Moreover, for comparison purposes the antioxidant standard compound (Trolox) should also be tested under the same conditions.

The autoxidation process of myoglobin, which is an important role in pathophysiology of vasospasms following subarachnoid hemorrhages and renal dysfunction after rhabdomyolysis, was inhibited to 2.5–5.0% by Trolox (100  $\mu\text{M}$ ), labetalol (100  $\mu\text{M}$ ), enalapril (100  $\mu\text{M}$ ) and fluvastatin (37.5  $\mu\text{M}$ ), while taurine (500  $\mu\text{M}$ ), atenolol (500  $\mu\text{M}$ ), propranolol (100  $\mu\text{M}$ ) and pravastatin (100  $\mu\text{M}$ ) did not prevent autoxidation. On the other hand, for reduced glutathione a pro-oxidant effect was observed since the autoxidation of myoglobin increased up to 30% after 4h of reaction monitoring. This took place because the interaction of thiols with reactive radicals can generate thiyl radicals which have a pro-oxidant effect, especially in iron-rich environments, which is the case of iron atoms release during Mb autoxidation.

Among the drugs tested, fluvastatin ( $12.5 \pm 1.2 \mu\text{M}$ ), enalapril ( $15.2 \pm 1.8 \mu\text{M}$ ) and propranolol ( $21.0 \pm 2.2 \mu\text{M}$ ) were those that presented higher ability to prevent myoglobin oxidative damage, even higher than that determined for reduced glutathione ( $232 \pm 10 \mu\text{M}$ ). These results indicate that these drugs may have pleiotropic effects by preventing oxidative damage of biomolecules, such as myoglobin. Finally, the proposed microplate protocol allowed the assessment of antioxidant capacity of two drugs in less than 4h, with control of different reaction conditions and

the estimated cost per analysis for each drug is about 0.90 €. And this is particularly important since health care costs is one of the most important challenges of our time.

## **5. References**

- [1] K. Jomova, M. Valko, Advances in metal-induced oxidative stress and human disease, *Toxicology* 283 (2011) 65-87.
- [2] H.J. Forman, M. Maiorino, F. Ursini, Signaling functions of reactive oxygen species, *Biochemistry* 49 (2010) 835-842.
- [3] B. Halliwell, M.A. Murcia, S. Chirico, O.I. Aruoma, Free radicals and antioxidants in food and in vivo: what they do and how they work. *Crit. Rev. Food Sci. Nutrition* 35 (1995) 7–20.
- [4] L.M. Magalhães, Development of automatic methods based on flow techniques for evaluation of antioxidant capacity in pharmaceutical and food products, PhD Thesis of Faculty of Pharmacy of University of Porto (2007).
- [5] B. Kalyanaraman, Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. *Redox Biology* 1 (2013) 244-257.
- [6] R. Kohen, A. Nyska, Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol. Pathol.* 30 (2002) 620–650.
- [7] A. Tlili, S. Dupre-Crochet, M. Erard, O. Nusse, Kinetic analysis of phagosomal production of reactive oxygen species. *Free Radic. Biol. and Med.* 50 (2011) 438-447.
- [8] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease. *Intern. J. Biochem. Cell Biol.* 39 (2007) 44–84.
- [9] A.M. Pisoschi, A. Pop, The role of antioxidants in the chemistry of oxidative stress: A review. *Eur. J. Med. Chem.* 97 (2015) 55-74.
- [10] K. Sugamura, J.F.Jr. Keaney, Reactive oxygen species in cardiovascular disease, *Free Radic. Biol. and Med.* 51 (2011) 978-992.
- [11] V. Nogueira, N. Hay, Molecular Pathways: Reactive Oxygen Species Homeostasis in Cancer Cells and Implications for Cancer Therapy. *Clin. Cancer Res.* 19 (2013) 4309-4314.

- [12] L. Rochette, M. Zeller, Y. Cottin, C. Vergely, Diabetes, oxidative stress and therapeutic strategies. *Biochim. Biophys. Acta-Gen. Subj.* 1840 (2014) 2709-2729.
- [13] C.M. Steyers, F.J. Miller, Endothelial Dysfunction in Chronic Inflammatory Diseases, *Int. J. Mol. Sci.* 15 (2014) 11324-11349.
- [14] N.G. Frangogiannis, The inflammatory response in myocardial injury, repair, and remodeling. *Nat. Rev. Cardiol.* 11 (2014) 255-265.
- [15] I. Fonseca, H. Reguengo, M. Almeida, L. Dias, L.S. Martins, S. Pedroso, J. Santos, L. Lobato, A.C. Henriques, D. Mendonça, Oxidative stress in kidney transplantation: malondialdehyde is an early predictive marker of graft dysfunction. *Transplantation* 97 (2014) 1058-1065.
- [16] I. Fonseca, Malondialdehyde as a Biomarker in Kidney Transplantation In Patel, Vinood B., Preedy, Victor R. (Eds.), *Biomarkers in Disease: Methods, Discoveries and Applications. Biomarkers in Kidney Disease.* Springer Netherlands. ISBN 978-94-007-7699-9 (2016).
- [17] F. Di Domenico, E. Barone, M. Perluigi, D.A. Butterfield, Strategy to reduce free radical species in Alzheimer's disease: an update of selected antioxidants. *Expert Rev. Neurother.* 15 (2015) 19-40.
- [18] L.G. Wood, P.G. Gibson, M.L. Garg, A review of the methodology for assessing in vivo antioxidant capacity. *J. Sci. Food Agric.* 86 (2006) 2057–2066.
- [19] L.M. Magalhães, M.A. Segundo, S. Reis, J.L.F.C. Lima, Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta*, 613 (2008) 1-19.
- [20] C. Lopez-Alarcon, A. Denicola, Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. *Analytica Chimica Acta*, 763 (2013) 1-10.
- [21] G.J. Moon, S.J. Kim, Y.H. Cho, S. Ryoo, O.Y. Bang, Antioxidant effects of statins in patients with atherosclerotic cerebrovascular disease, *J. Clin. Neurol.* 10 (2014) 140-147.
- [22] V.G. Athyros, A.I. Kakafika, K. Tziomalos, A Karagiannis, D.P. Mikhailidis, Pleiotropic Effects of Statins - Clinical Evidence. *Curr. Pharm. Design* 15 (2009) 479-489.
- [23] Y. Bansal, O. Silakari, Multifunctional compounds: Smart molecules for multifactorial diseases, *Eur. J. Med. Chem.* 76 (2014) 31-42.
- [24] G. Beretta, R.M. Facino, Recent advances in the assessment of the antioxidant capacity of pharmaceutical drugs: from in vitro to in vivo evidence. *Anal. Bioanal. Chem.* 398 (2010) 67-75.

## Supplementary Data

### Myoglobin microplate assay to evaluate prevention of protein peroxidation Journal of Pharmaceutical and Biomedical Analysis\*

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Salette Reis, Marcela A. Segundo

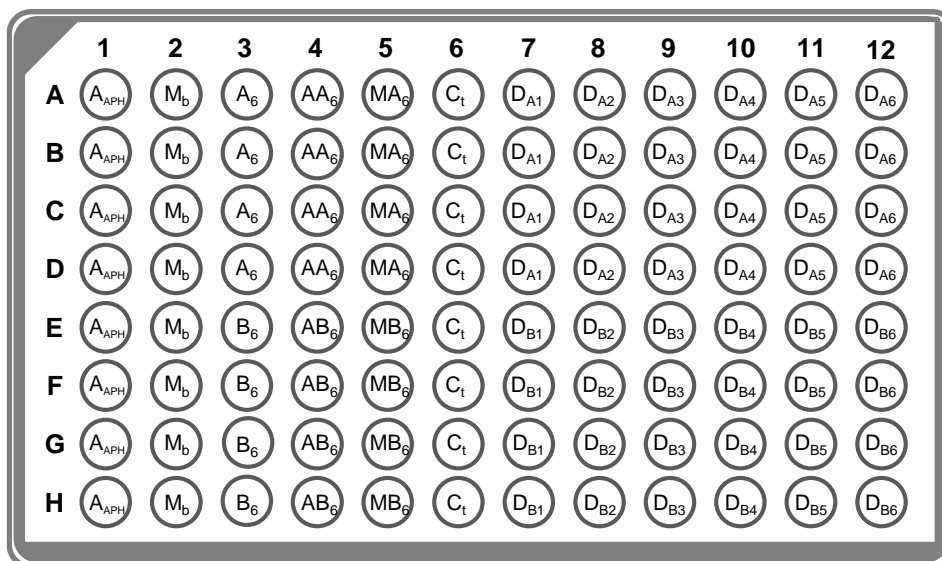
UCIBIO, REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, 228, Porto 4050-313, Portugal

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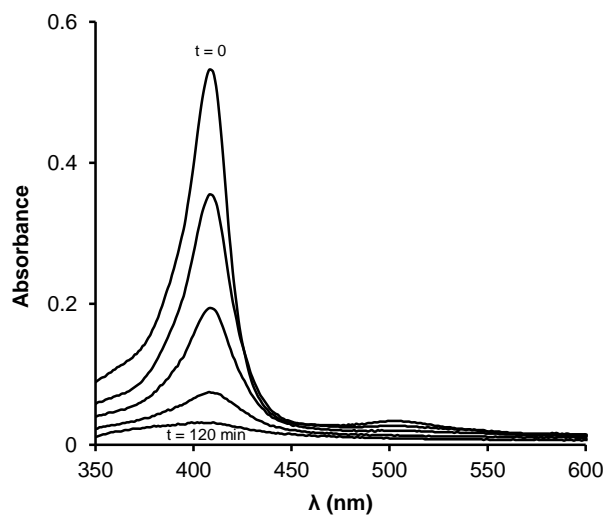
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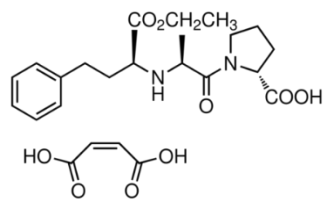
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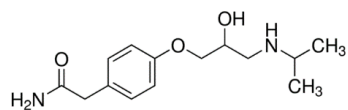
**Figure S1.** Representation of the 96-well microplate scheme applied for monitoring of myoglobin oxidation from peroxy radicals and its protection by drugs: **AAPH**, 100  $\mu$ l of AAPH (180 mM) + 200  $\mu$ l phosphate buffer (pH 7.4, 50 mM); **Mb**, 100  $\mu$ l of myoglobin (75  $\mu$ g/mL) + 200  $\mu$ l of phosphate buffer; **A6** or **B6**, 100  $\mu$ l of the highest concentration of drug A or B + 200  $\mu$ l phosphate buffer; **AA6** or **AB6**, 100  $\mu$ l of AAPH (180 mM) + 100  $\mu$ l of the highest concentration of drug A or B + 100  $\mu$ l phosphate buffer; **MA6** or **MB6**, 100  $\mu$ l of myoglobin (75  $\mu$ g/mL) + 100  $\mu$ l of the highest concentration of drug A or B + 100  $\mu$ l phosphate buffer; **Ct**, 100  $\mu$ l of myoglobin (75  $\mu$ g/mL) + 100  $\mu$ l of AAPH (180 mM) + 100  $\mu$ l phosphate buffer; **DAi**, 100  $\mu$ l of myoglobin (75  $\mu$ g/ml) + 100  $\mu$ l of AAPH (180 mM) + 100  $\mu$ l of drug A with increasing concentrations; **DBi**, 100  $\mu$ l of myoglobin (75  $\mu$ g/ml) + 100  $\mu$ l of AAPH (180 mM) + 100  $\mu$ l of drug B with increasing concentrations. Each experiment was performed in quadruplicate and in three different days.



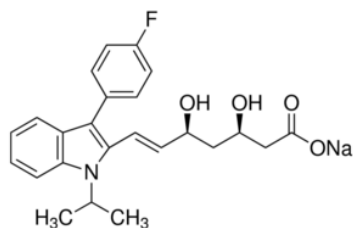
**Figure S2.** UV-Vis spectra of myoglobin solution (90 μg/mL) in the presence of AAPH solution (60 mM) obtained at different reaction times (0, 30, 60, 90 and 120 min). The absorbance peak (409 nm) of native myoglobin decrease throughout reaction time due to protein oxidation induced by peroxy radicals. Intrinsic absorbance of AAPH was subtracted from the absorbance decay of myoglobin.



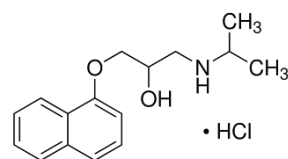
Enalapril maleate salt



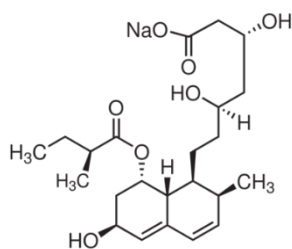
Atenolol



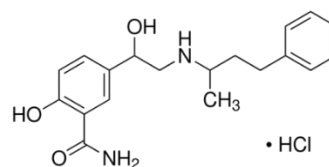
Fluvastatin sodium salt



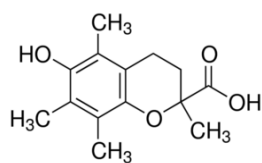
(±)-Propranolol hydrochloride



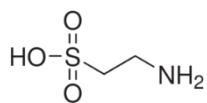
Pravastatin sodium salt



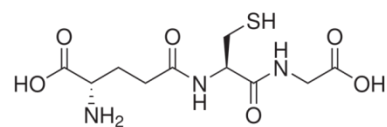
Labetalol hydrochloride



Trolox



Taurine



Reduced glutathione

**Figure S3.** Molecular structures of cardiovascular drugs, endogenous antioxidants (glutathione and taurine) and standard antioxidant (Trolox) tested.