SCAVENGING ACTIVITY OF DICLOFENAC. INTERACTION WITH ABTS RADICAL CATION AND PEROXYL RADICALS

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ABSTRACT

The scavenging activity of diclofenac was estimated by its capability to bleach ABTS radical cation, and ORAC (oxygen radical absorbance capacity) methodology. In addition, GC-MS technique was used to establish if the lactamic derivative of diclofenac was formed as final product in the interaction with peroxy radicals. Diclofenac bleached ABTS radical cation in a concentration-dependent way. In ORAC assays, diclofenac inhibited the consumption of fluorescein and c-phycoyanin mediated by peroxy radicals. An ORAC-fluorescein, and ORAC-c-phycoyanin value of 0.3, and 0.004 was estimated, respectively. From the downcurvature observed in Stern-Volmer-like plots, we postulated that the low protection of c-phycoyanin is related to secondary damage of c-phycoyanin by the secondary radical of diclofenac. By GC-MS studies only a 2,6-dichloroaniline derivative was detected as product after the reaction with peroxy radicals.

Key Words: Diclofenac, fluorescein, c-phycoyanin, ORAC, scavenging activity.

INTRODUCTION

Diclofenac (2-[(2,6-dichlorophenyl)amino]benzenacetic acid) is a synthetic non steroidal anti-inflammatory drug, widely employed in the treatment of different pathologies associated to inflammation processes, such as chronic arthritis, rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis.^{1,2} The mechanism of action is related to the inhibition of the arachinodate metabolites synthesis through cyclooxygenase inhibition.^{1,2}

Besides of the well known anti-inflammatory effect of diclofenac, some scavenging properties have been also reported for this drug. The inhibition of both, phosphatidylcholine liposomes oxidation³ and linoleic acid peroxidation has been informed.⁴ In addition, a reduction in the chemiluminescence associated with phosphatidylcholine hydroperoxide production in rats plasma subjected to ischemia-reperfusion process was reported.⁵ Furthermore, You-Zhi et al.⁶ have reported that diclofenac protects human erythrocytes against hemolysis process induced by peroxy radicals. All these reports suggest -at least *in vitro*- some scavenging activity of diclofenac, which could effectively provide protection against lipid peroxidation caused either by hydroxyl radicals,³ Fe²⁺/ascorbate system,⁴ ischemia-reperfusion⁵ or peroxy radicals damage.⁶ Nevertheless, diclofenac was not able to protect LDL (low density lipoproteins) against oxidation induced by copper.⁷

In spite of that some studies have suggested scavenging activity of diclofenac,³⁻⁶ there is only a few studies reporting the diclofenac-free radicals interactions. Gaudiano et al.⁸ suggested the formation of hydroxylated metabolites as oxidation/degradation products of diclofenac in presence of hydroxyl radical. Recently, Fodor et al.⁹ reported that the protection of crocin afforded by diclofenac was similar to acetaminophen, but considerably lower than 3,4-dihydroxybenzoic acid.⁹

To the best of our knowledge, studies on the scavenging activity of diclofenac using a stable free radical or using an ORAC methodology have not been developed. Then, the aim of this study was to investigate the antioxidant capacity of diclofenac using both, a stable free radical as ABTS radical cation (ABTS^{•+}), and the ORAC methodology. In ORAC assays, fluorescein and c-phycoyanin were used as target molecules. In addition, GC-MS studies were performed in order to establish if the lactamic derivative of diclofenac is generated during the reaction with peroxy radicals.

EXPERIMENTAL

Chemicals

2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), fluorescein, Trolox -a water soluble vitamin E analog used as reference antioxidant- (see Figure 1), c-phycoyanin (c-Pc), and manganese dioxide (MnO₂) were purchased by Sigma-Aldrich (St. Louis, MO, USA). Diclofenac sodium salt (Figure 1) was purchased by Instituto Sanitas laboratory (Chile). All compounds were employed as received. The c-Pc concentration was estimated by considering a Mw of ca. 13,000 per each bilin group.

Figure 1: Chemical structure of diclofenac sodium salt, AAPH, Trolox, ABTS, and fluorescein.

Interaction of diclofenac with ABTS radical cation (ABTS^{•+})

ABTS^{•+} was generated from ABTS oxidation by manganese dioxide activated solid (MnO₂).¹⁰ Oxidation of ABTS (150 μM) in phosphate buffer (75 mM at pH 7.4) was carried out at room temperature employing an excess of MnO₂, which was removed by centrifugation and filtration. The resulting blue-green solution of ABTS^{•+} was kept at 4°C before its use. The ABTS^{•+} concentration was derived from its absorbance at 734 nm ($\epsilon_{734} = 0.015 \mu\text{M}^{-1} \text{cm}^{-1}$).¹¹ The absorbance of ABTS^{•+} remained constant for several hours. The reaction was started by adding diclofenac to the ABTS^{•+} solution, and the reaction was followed by measuring the decay in absorbance at 734 nm. Measurements were recorded in a thermostated cuvette of an Agilent 8453 (Palo Alto, CA, USA) UV-visible spectrophotometer. The reaction was carried out at room temperature in air saturated solutions. In addition, the decrease in the

absorption units of ABTS $\dot{Y}^+$ at 734 nm after 6 min of the addition of diclofenac or Trolox was used to calculate TEAC (Trolox equivalent antioxidant capacity) value (Eqn 1).¹²

$$TEAC = \frac{[\Delta A_{\text{diclofenac}}]}{[\Delta A_{\text{Trolox}}]} \times \frac{[\text{Trolox}]}{[\text{Diclofenac}]} \quad (1)$$

Where:

$\Delta A_{\text{diclofenac}}$ = Difference between initial absorbance ($UA_{0 \text{ minutes}}$) and after 6 minutes of diclofenac addition ($UA_{6 \text{ minutes}}$).

ΔA_{Trolox} = Difference between initial absorbance ($UA_{0 \text{ minutes}}$) and after 6 minutes of Trolox addition ($UA_{6 \text{ minutes}}$).

[Trolox] = Trolox molar concentration.

[Diclofenac] = Diclofenac molar concentration.

Protection of c-phycoerythrin, and fluorescein

Stock solutions of diclofenac (1 mM), and Trolox (1×10^{-2} M) were prepared daily in ethanol immediately before their use. Stock solutions of AAPH (0.6 M), fluorescein (1×10^{-5} M), and c-Pc (80 μ M) were prepared daily in phosphate buffer (75 mM, pH 7.4). A reaction mixture containing AAPH (10 mM), the target molecule (fluorescein, 70 nM, or c-Pc, 0.8 μ M) with or without diclofenac or Trolox was incubated in phosphate buffer (75 mM) at pH 7.4, at 37°C in the thermostated cuvette of a Perkin Elmer LS-55 spectrofluorimeter (Beaconsfield, U.K). The consumption of fluorescein, and c-Pc were evaluated from the decrease in its fluorescence intensity. In fluorescein protection assays, 493 nm and 515 nm were used as excitation and emission wavelengths, respectively, meanwhile 620 nm and 640 nm were the respective wavelengths in c-Pc experiments.

ORAC determinations

ORAC experiments were carried out according to Ou et al. procedure.¹³ Fluorescein and c-Pc consumption were evaluated from the progressive decrease in the sample fluorescence intensity (F). Values of (F/F₀) were plotted as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that (F/F₀) reached a value of 0.2. These areas were employed to obtain ORAC values (Eqn. 2). All experiments were carried out in triplicate.

$$ORAC = \frac{[AUC - AUC^0]}{[AUC_{\text{Trolox}} - AUC^0]} \times \frac{[\text{Trolox}]}{[\text{Diclofenac}]} \quad (2)$$

Where:

AUC = Area under curve in presence of diclofenac, integrated between time zero and that corresponding to 80 % of the probe consumption.

AUC^0 = Area under curve for the control (in absence of diclofenac).

AUC_{Trolox} = Area under curve for Trolox.

[Trolox] = Trolox molar concentration.

[Diclofenac] = Diclofenac molar concentration.

GC-MS studies

Solutions of diclofenac (20 μ M) plus AAPH (10 mM), in phosphate buffer (pH 7.4, 75mM), before and after 3 hours incubation at 37°C, were analyzed by GC-MS. Previously to the chromatographic analysis, the solutions were extracted with ethyl acetate. Briefly, 1 mL of ethyl acetate was added to an equal volume of each sample, and the organic phase was filtered and injected immediately to the chromatograph. GC-MS analysis was performed with a Thermo Electron Corporation Trace GC Ultra double-focusing sector instrument (MAT 95 XP Thermo Electron Bremen, Germany). The GC Ultra with autosampler AS 3000, was equipped with a MDN-5S ms. capillary column (30m, 0.25 mm ID, 0.25 μ m film; SUPELCO 595, North Harrison Road, Bellefonte, USA). Chromatographic conditions: Source temperature, 260 °C; Injector temperature, 260 °C; splitless/w Surge, split Flow 12 ml/min, splitless time 1.50 min, surge pressure 45 psi; surge duration 1.50 min.

Temperature program: The oven temperature was programmed from 50 (1 min), to 180 °C (hold for 7 min) at 10 °C min⁻¹, to 250 °C (hold for 5 min) at 10 °C min⁻¹; run time was 33 minutes. Helium was used as carrier gas with an inlet pressure of 14.5 psi. The identification of the samples was based on the analyses of the mass spectra (full scan). Quantification of the GC-MS peaks was carried out using lidocaine as internal standard.

RESULTS AND DISCUSSION

Interaction of diclofenac with ABTS radical cation (ABTS $\dot{Y}^+$)

The reaction of antioxidants with stable free radicals has been widely employed as an estimation of their free radical scavenging capacities. ABTS $\dot{Y}^+$ has been frequently employed for these purposes, mainly due to their solubility in buffer solutions.¹⁴ When different diclofenac concentrations were added to an ABTS $\dot{Y}^+$ solution, a decrease of the absorbance at 734 nm (corresponding to ABTS $\dot{Y}^+$) was observed (Figure 2). From a bi-exponential adjust of the data presented in Figure 2, a stoichiometric factor (*n*, defined as the number of ABTS $\dot{Y}^+$ consumed per each molecule of diclofenac) of 2.0 ± 0.5 was estimated. In addition, the initial rate of ABTS $\dot{Y}^+$ bleaching was dependent of the diclofenac concentration (Figure 2). For example, at diclofenac 5 μ M an initial rate of 3.1 μ M/min was estimated, while at diclofenac 20 μ M, the initial rate was 20 μ M/min. These values show that diclofenac efficiently bleached ABTS $\dot{Y}^+$. In fact, nearly 80% of ABTS $\dot{Y}^+$ (initial concentration, 30 μ M) was bleached after 6 min incubation with 20 μ M diclofenac, leading to a TEAC (Trolox equivalent antioxidant capacity) value of 1.4. Nevertheless, it is important to consider that kinetic profiles of the ABTS $\dot{Y}^+$ bleaching induced by Trolox and diclofenac were different each other. While diclofenac reacted with ABTS $\dot{Y}^+$ giving the kinetic profiles of Figure 2, Trolox bleached ABTS $\dot{Y}^+$ instantaneously.

Figure 2: Kinetics profiles of ABTS radical cation (ABTS $\dot{Y}^+$) bleaching by diclofenac. ABTS $\dot{Y}^+$ (30 μ M) was incubated in phosphate buffer (75 mM, pH 7.4) in presence of different diclofenac concentrations. Control (S); diclofenac: 5 μ M (○), 10 μ M (r), 15 μ M (£), and 20 μ M (TM). ABTS $\dot{Y}^+$ consumption was monitored through the decrease in ABTS $\dot{Y}^+$ absorption intensity at 734 nm.

Protection of target molecules by diclofenac

Protection of fluorescein (ORAC- fluorescein)

The ORAC index has been widely employed in the antioxidant capacity evaluation of single antioxidants and their complex mixtures.¹³ This methodology measures the protection afforded by an antioxidant to a target molecule that is being oxidized by peroxy radicals, estimating the changes of area under curve of the kinetics profiles for the target molecule decay in fluorescence or absorbance. ORAC assay was proposed originally by Cao et al.¹⁵ using phycoerythrin as target molecule, but at present, fluorescein is the target molecule most employed.¹³ In order to evaluate the antioxidant capacity of diclofenac, protective experiments using fluorescein as target molecule were carried out. As can be seen in Figure 3, a reduced consumption rate of fluorescein was observed when diclofenac was added to a solution containing fluorescein plus AAPH. The protection of fluorescein afforded by diclofenac, would be related with the reaction between diclofenac and peroxy radicals, and can be explained by the follow set of reactions:

For simplicity, we do not consider the formation of alkoxy radicals in reaction (6), and the self-reactions of the radicals produced in step (5).

Figure 3 shows that diclofenac protects fluorescein without generating an induction time. Considering that the protection of fluorescein by antioxidants usually is through a lag time (induction time), even for antioxidants of low reactivity,¹⁶ the data presented in **figure 3**, indicate that diclofenac would have a very low reactivity towards peroxy radicals. However, when the area under curve (AUC) estimated from the kinetic profiles of **Figure 3** were compared with Trolox (**Figure 3, insert**), the ORAC-fluorescein index of diclofenac (**Eqn. (2)**) was nearly three times lower than Trolox (ORAC-fluorescein ≈ 0.3). Interestingly, the antioxidant capacity estimated from AUC (ORAC) contrasts with the evaluation of initial rates of fluorescein fluorescence decay. Thus, while diclofenac protected fluorescein without induction time (**Figure 3**), Trolox inhibited fluorescein consumption through a neat lag time.¹⁶ Therefore, the antioxidant capacity of diclofenac strongly depends on the criteria of analysis of **figure 3** data. If only AUC is considered to estimate the antioxidant capacity, diclofenac seems with a comparable scavenging capacity that Trolox. Nevertheless, if is considered the initial consumption rate as an antioxidant capacity parameter, diclofenac appears to have a very lower reactivity than Trolox.

Figure 3: Kinetic profiles of fluorescein consumption AAPH mediated in presence of diclofenac. Fluorescein (70 nM) was incubated in presence of AAPH (10 mM) with or without diclofenac in phosphate buffer (75 mM, pH 7.4) at 37°C. The reaction was followed by the decrease in fluorescein fluorescence intensity (excitation at 493 nm, emission at 515 nm). Control ($^{\text{TM}}$); diclofenac: 5 μM ($\square$), 8 μM (r), 10 μM ($\cdot$), and 16 μM (S). Insert: dependence of the area under curve after 80 % of reaction *versus* diclofenac ($^{\text{TM}}$) or Trolox ($\cdot$) concentration (μM). Trolox data were taken from ref. 16.

Protection of c-Phycocyanin (ORAC- c-Pc)

The bleaching rate of c-Pc by peroxy radicals can be modified by the presence of compounds able to react with the primary radicals. Phycobiliproteins, such as c-Pc and phycoerythrin,¹⁷ have been employed in order to evaluate the free radical scavenging capacity of several compounds. The presence of additives modifies the AUC of the kinetic profiles of c-Pc consumption (ORAC type methodologies), and the initial rate of c-Pc bleaching. We have employed

both approaches to estimate the protective capacity of diclofenac. **Figure 4** shows the effect of diclofenac on c-Pc bleaching promoted by AAPH. From the AUC of the kinetics profiles (F/F_0) of c-Pc bleaching shown in **Figure 4**, an ORAC-c-Pc value nearly to 0.004 was estimated. This value was 75 times lower than the ORAC-fluorescein index, and shows that ORAC values are extremely dependent upon the employed target molecule.¹⁸

As can be seen in **figure 4**, the addition of high diclofenac concentrations (50-500 μM) generated a decrease in the bleaching rate of c-Pc. This behaviour would be related with a low efficiency of diclofenac, and could be represented by reactions (3), (5), (6), and (7):¹⁷

The dependence of R°/R ratio (where R° is the initial rate of c-Pc bleaching in absence of diclofenac, and R the initial rate of the process in presence of diclofenac) with diclofenac concentration was studied (Stern-Volmer like plots). As is shown in the insert of **figure 4**, a clear downcurvature was observed in this plot. This result can not be explained in terms of the simple mechanism depicted by reactions (3), (5), (6), and (7), and could be related to secondary reactions of diclofenac derived radicals (diclofenac $\dot{Y}$), such as:^{19,20}

The maximum attainable R°/R value (R°/R_∞ values) can be considered as an indicator of the damaging capacity of the diclofenac $^\bullet$ radical. The R°/R_∞ value of diclofenac (estimated in the plateau of **Figure 4, insert**) was nearly 2.0. This value implies that diclofenac have a secondary damage higher than phenols such as protocatechuic acid, kaempferol, and luteolin.¹⁹ However, the secondary damage of diclofenac was similar to galangin.¹⁹ This would be compatible with the suggestion that compounds with lower reactivity towards AAPH derived peroxy radicals would have higher secondary damage.¹⁹ Interestingly, in the protection of fluorescein by diclofenac a linear relationship ($R^\circ/R = 1.042 + 0.079$, $r = 0.9944$) was observed in the R°/R *versus* diclofenac concentration plots (data not shown), indicating that in this system the secondary radical of diclofenac would not be able to damage fluorescein.

Figure 4: Bleaching of c-Phycocyanin elicited by AAPH (10 mM) in presence of diclofenac. c-Pc (0.8 μM) was incubated in presence of AAPH (10 mM) with or without diclofenac. Control (S). Diclofenac: 50 μM ($\square$); 250 μM (r); 500 μM ($\cdot$). The reaction was followed by the decrease in c-Pc fluorescence intensity (excitation at 620 nm, emission at 640 nm) at 37°C. Insert: dependence of R°/R ratio with diclofenac concentration. R° , and R represent the initial rate of c-Pc consumption in absence, and presence of diclofenac, respectively.

GC-MS studies

In spite of the low efficiency of diclofenac as scavenging compound, the results above shown are a clear evidence that diclofenac reacted with peroxy radicals, and could explain previous reports that show some antioxidant effect of diclofenac.³⁻⁶ You-Zhi et al.⁶ reported that diclofenac inhibited human erythrocytes hemolysis mediated by peroxy radicals AAPH derived. The authors described the effect of diclofenac through a diclofenac-peroxy radicals interaction with the formation of a lactamic form of diclofenac (1-(2,6-dichlorophenyl)indolin-2-one) as final product. In order to determine if in our experimental conditions the lactamic product is formed, we used GC-MS technique. For this purpose, direct extraction of a diclofenac-AAPH solution (before and after incubation at 37°C) was carried out, and the organic phase was injected in to the chromatograph. No acidification of the solutions was used for minimizing the formation of 1-(2,6-dichlorophenyl)indolin-2-one as catalytic product.²¹ **Figure 5**, and **Figure 6** show the GC-MS chromatogram and the fragmentation pattern of a diclofenac-AAPH solution at zero time, and after three hours incubation at 37°C, respectively. As can be seen in **Figure 5A**, at zero time only a peak at 26.6 minutes was registered. The fragmentation pattern of this peak (**Figure 5B**), with a base peak (Bp) = 214, m/z (1) = 242, and an ion molecular (M^+) = 277, corresponds to the lactamic form of diclofenac.²¹ This result indicates that a fraction of lactame can be formed from diclofenac before the reaction with peroxy radicals. This agree with reports describing the lactame as a synthesis intermediate of diclofenac (impurity A of the British pharmacopoeia).^{22,23} As can be seen in **Figure 6**, after three hours incubation with AAPH, two peaks were registered. The peak at 26.6 minutes, corresponding to the lactamic form, had a similar area under curve compared with zero time (data not shown), leading that the lactamic derivative was not formed during the reaction. The second one at 10.2 minutes, had a pattern fragmentation according to 2,6-dichloroaniline (**Figure 6B**). This result implies that a fragmentation of diclofenac during the reaction with AAPH derived radicals is involved.

Figure 5: Extracted ion chromatogram (**graphic A**) and mass spectrum

(**graphic B**) of a solution containing diclofenac (20 μ M) plus AAPH (10 mM) at zero reaction time in phosphate buffer 75 mM, pH 7.4.

Figure 6: Extracted ion chromatogram (**graphic A**) and mass spectrum

(**graphic B**) of a solution containing diclofenac (20 μ M) plus AAPH (10 mM) after 3 hours incubation at 37°C in phosphate buffer 75 mM, pH 7.4. **Graphic B** represents the fragmentation pattern of the chromatographic peak at 10.2 minutes.

CONCLUSIONS

All data obtained in the present work indicate that diclofenac is able to react with a stable free radical (ABTS radical cation), and peroxy radicals. The scavenging capacity of diclofenac estimated by ORAC-like methodologies lead that diclofenac is a mild peroxy radical scavenger. However, its ability for trapping peroxy radicals should be considered in topical administration of pharmaceutical preparations containing high diclofenac concentrations.

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