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RP-HPLC study of the degradation of diclofenac and piroxicam in the presence of hydroxyl radicals

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Abstract

The effect of hydroxyl radical attack on two non-steroidal anti-inflammatory drugs (NSAIDs) was studied in vitro. Diclofenac and piroxicam were analysed by RP-HPLC after reaction with $OH^{\bullet}$ free radicals to detect newly formed oxidation and/or degradation products. $OH^{\bullet}$ free radicals were obtained by means of ferrous sulphate and ascorbic acid mixtures. During the reaction the mixtures were exposed to irradiation by a tungsten lamp to obtain an increased and more reproducible formation of hydroxyl radicals. The chromatographic profiles showed the formation of several new peaks for both diclofenac and piroxicam due to the presence of a number of degradation/oxidation products formed in the presence of $OH^{\bullet}$ radicals.

Keywords: Degradation; Oxidation; Drugs; Hydroxyl radicals; Diclofenac; Piroxicam; RP-HPLC

1. Introduction

Reactive Oxygen Species (ROS) are constantly formed in the human body. Many of them play a useful physiological role but their overproduction can be toxic.

Overproduction of $OH^{\bullet}$ free radicals in vivo is often a consequence of pathologies or a physiological condition during ageing [1]. Hydroxyl radicals are involved in ischemia-reperfusion injury, cancer, rheumatoid arthritis, Parkinson and Alzheimer's diseases [2]. The excess of $OH^{\bullet}$ free radicals is often due to a pathological release of free iron ions that may stimulate radical reactions, as it happens in stroke, in Parkinson and Alzheimer's diseases or in cancer [3–7].

$OH^{\bullet}$ free radicals could react in vivo with several endogenous molecules, but also with xenobiotic molecules such as drugs [8–11]. Several studies regard the effect of $OH^{\bullet}$ free radicals on biological endogenous molecules, but not much work concerns the effect of hydroxyl radicals on drugs structure [8,12,13]. An example of this reaction is the hydroxyl radicals attack upon salicylic acid leading to the formation of 2,3-

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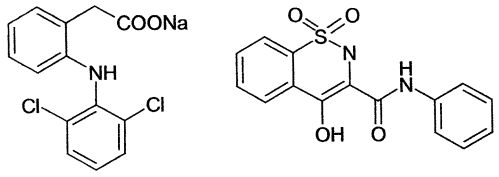
dihydroxybenzoic acid both in vitro and in vivo [14].

The starting point of the work was the hypothesis that, in the presence of $OH^{\bullet}$ free radical excess, drugs could be modified in their structure by a direct non-enzymatic reaction, giving oxidation/degradation products different or in different quantity with respect to those produced in the normal metabolic pathway. These products could be toxic and/or could induce, as secondary radicals, radicalic reactions and cellular damage [10].

In this paper the structural effects of $OH^{\bullet}$ free radicals attack on two non-steroidal anti-inflammatory drugs, diclofenac and piroxicam, were studied (Fig. 1).

Both the drugs are largely used in the treatment of rheumatoid arthritis, osteoarthritis and other inflammatory diseases [15] especially in aged patients, in whom hydroxyl radicals overproduction is a physiological condition [16]. It can be supposed that, in some cases, adverse reactions to pharmacological therapy in elders could be related to a different degradation pathway as a direct hydroxyl radicals-drug reaction. To verify this hypothesis, the formation of reaction products between the drug molecule and the $OH^{\bullet}$ radicals was investigated in vitro as a necessary first step to a successive in vivo study. To reproduce the in vivo mechanism of iron-catalysed $OH^{\bullet}$ free radicals production, hydroxyl radicals-drug reaction was performed by treating the drugs with a ferrous sulphate/ascorbic acid mixture. The evaluation of the effects of $OH^{\bullet}$ free radicals attack on diclofenac and piroxicam was performed by RP-HPLC.



(I) (II)

Fig. 1. Chemical structures of diclofenac sodium (I) and piroxicam (II).

2. Experimental

2.1. In vitro $OH^{\bullet}$ free radical production and drugs oxidant treatment

$OH^{\bullet}$ free radicals were produced by means of ferrous sulphate and ascorbic acid mixtures. Stock solutions of ferrous sulphate (ACS reagent, Sigma, St. Louis, MO, USA) and L-ascorbic acid (minimum 99.0%, Sigma) were freshly prepared. The proper volumes of ferrous sulphate and ascorbic acid stock solutions were then mixed and diluted to obtain the following final concentrations:

Oxidant Mixture 1 (MIX 1)

$= 5.0 \times 10^{-5}$ M $FeSO_4 + 1.0 \times 10^{-4}$ M ascorbic acid

Oxidant Mixture 2 (MIX 2)

$= 1.0 \times 10^{-4}$ M $FeSO_4 + 2.0 \times 10^{-4}$ M ascorbic acid

These mixtures produce $OH^{\bullet}$ free radicals in the presence of dissolved oxygen [17].

Diclofenac and piroxicam stock solutions were prepared at a concentration of 5.0×10^{-3} and 1.1×10^{-3} M, respectively. Reference solutions were obtained by diluting stock solutions to a final concentration of 1.0×10^{-4} and 2.2×10^{-5} M, respectively. Diclofenac sodium salt and piroxicam were purchased from Sigma.

Reaction solutions were obtained mixing proper volumes of stock solutions and then bringing to volume to obtain a final concentration in the oxidant mixtures of 1.0×10^{-4} and 2.2×10^{-5} M for diclofenac and piroxicam, respectively.

All solutions were prepared in 0.05 M sodium phosphate buffer, pH 7.40 ± 0.02 and filtered through 0.45 μm Minisart 17597 filters (Sartorius AG, Goettingen, Germany) before analysis.

As previously reported, light accelerates the $OH^{\bullet}$ free radicals formation in the oxidant mixture [18,19]. This phenomenon makes the

hydroxyl production non-reproducible because of the light exposition variability during the reaction. To overcome this problem and to have a reproducible and rapid $OH^{\bullet}$ free radicals formation, an irradiation with a 60 W tungsten lamp was applied. Reaction solutions as well as oxidant mixtures and reference solutions were placed into a closed box 30-cm below the lamp immediately after their preparation. A 2-h oxidation time was fixed on the ground of the results related to the $OH^{\bullet}$ free radicals production (see Section 3).

LC control measurements were carried out on reference solutions before and after light exposure to insure that the light treatment itself did not damage the drug molecules.

LC control measurements were carried out also on irradiated MIX 1 and MIX 2 to verify that no peaks had origin from ascorbic acid or its degradation products.

2.2. $OH^{\bullet}$ free radical quantitation

To quantitate $OH^{\bullet}$ free radicals production from irradiated and non-irradiated oxidant mixtures, the coumarin method was employed [20].

An appropriate volume of coumarin-3-carboxylic acid (CCA) (99%, Aldrich-Chemical Co., Gillingham, UK) stock solution (5.0×10^{-4} M in 0.05 M phosphate buffer) was added to the oxidant mixtures to reach a final concentration of 2.0×10^{-4} M. CCA reacts with $OH^{\bullet}$ free radicals produced by the oxidant mixtures to give the fluorescent compound 7-hydroxy-coumarin-3-carboxylic acid (7OHCCA).

By excitation at 400 nm, 7OHCCA emits a fluorescence band centred at 450 nm. Fluorescence emission is proportional to 7OHCCA concentration and, consequently, to $OH^{\bullet}$ free radicals concentration. A calibration curve of 7OHCCA (99%, Aldrich-Chemical Co.) standard solutions was employed to quantitate hydroxyl radicals. Fluorescence spectra were recorded on a FluoroMax spectrofluorimeter (Instrument S.A., Inc., JOBIN YVON/SPEX Division, Edison, NJ, USA).

2.3. HPLC apparatus and chromatographic conditions

LC analyses were performed using a liquid chromatograph series 1100 (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) equipped with an automatic injector (series G1329A, Agilent) and a diode array detector (series G1315B, Agilent).

To detect diclofenac oxidation/degradation products, two different LC methods were employed. The first one (Method I) was developed slightly modifying the European Pharmacopoeia LC method for related substances [21].

Two Kromasil C8KR100, 5 μm, 150 × 4.6 mm I.D. columns connected in series (Eka Chemicals AB, Bohus, Sweden) were used. The mobile phase consisted of a mixture (58:42 v/v) of methanol and a mixture of phosphoric acid (1.6 g/l) and sodium dihydrogen phosphate monohydrate (1.4 g/l) in equal volumes, whose pH was adjusted to 2.50 with phosphoric acid. Column temperature was set at 30 °C. Mobile phase was delivered at a flow rate of 1 ml/min and the injection volume was 100 μl. In accordance with literature [22], chromatograms were recorded at 236, 254, 275 and 282 nm to better detect all possible drug oxidation products.

The second LC method (Method II) was developed by Bort et al. in the study of hepatic metabolites of diclofenac [23]. A Kromasil C18, 5 μm, 250 × 4.6 mm I.D. column (Restek Corporation, Bellefonte, PA, USA) was employed with a mobile phase consisting of triethanolamine (0.02%) in phosphate buffer (0.02 M, pH 7.40) and acetonitrile (75:25 v/v). The flow rate was 1 ml/min and the injection volume was 100 μl. Effluents were monitored at 282 nm, as indicated in the original R. Bort work, and at 254 nm to better compare the results from the two methods. Column temperature was 30 °C.

Piroxicam samples were analysed by the European Pharmacopoeia LC method for related substances [24] opportunely modified. The chromatographic column was a Hypersil BDS C18, 5 μm, 250 × 4.6 mm I.D. (Alltech, IL, USA). The mobile phase was acetonitrile and potassium dihydrogen phosphate solution (6.81 g/l, pH 3.0)

(28:72 v/v) delivered at a constant flow rate of 1.0 ml/min. Column temperature was 40 °C and the injection volume was 50 μl. The chromatograms were recorded at 230 nm.

HPLC-grade methanol and acetonitrile were from Carlo Erba (Milan, Italy). All other reagents were of analytical grade.

3. Results and discussion

3.1. Effect of light on $OH^{\bullet}$ free radical production

Table 1 shows the fluorescence intensity and the $OH^{\bullet}$ free radical concentration in the oxidant mixtures (MIX 1 and MIX 2) with and without lamp irradiation.

The data showed an increase of $OH^{\bullet}$ free radical concentration in the irradiated oxidant mixtures. Moreover, the twofold concentrated MIX 2 produced a higher $OH^{\bullet}$ concentration in comparison with MIX 1. After a 2-h reaction time, non-irradiated MIX 1 and MIX 2 produced nearly the same radical quantity. The $OH^{\bullet}$ production was time-dependent, but after 2 h of light exposure the process was complete. Longer irradiation times produced no increase of $OH^{\bullet}$ concentration whereas shorter irradiation times caused a lower hydroxyl radicals production (data not shown).

Table 1
Fluorescence intensity and hydroxyl free radical concentration in MIX 1 and MIX 2 obtained by coumarin method

Sample	Fluorescence intensity (a.u.)	$[OH^{\bullet}]$ ($\times 10^{-5}$ M)[b]
Irradiated MIX 1[a]	0.73	0.57
Irradiated MIX 2[a]	1.2	0.81
Non-irradiated MIX 1	0.28	0.35
Non-irradiated MIX 2	0.33	0.37

[a] Solutions were irradiated as described in the text.
[b] $OH^{\bullet}$ free radical molar concentration calculated from the calibration curve of fluorescence intensity as a function of 7OHCCA concentration.

3.2. $OH^{\bullet}$ free radical effect on diclofenac

The chromatographic profile of diclofenac reference solution obtained by method I is shown in Fig. 2A. Diclofenac showed a retention time (r.t.) of 60.2 min, while the peak at r.t. 36.5 min was due to 1-(2,6-dichlorophenyl)indolin-2-one, the lactamic form of the molecule, as reported in the European Pharmacopoeia monograph [21]. The HPLC analysis of the same sample after a 2-h irradiation gave the same chromatographic profile, indicating that the drug structure was not affected by light exposure if ferrous sulphate and ascorbic acid had not been added (data not shown).

The chromatograms of diclofenac submitted to the oxidant treatment are reported in Fig. 2B and C. Several new peaks could be detected using either MIX 1 or MIX 2. In the absence of the drug, irradiated MIX 1 and MIX 2 showed no peaks in the 10–70 min time range, proving that none of the peaks detected in the chromatograms of diclofenac after the oxidant treatment had origin from ascorbic acid or its degradation products (data not shown). Chromatograms reported in Fig. 2A–C were recorded at 254 nm.

In MIX 1 (Fig. 2B) the sum of the areas of the newly formed peaks was 4.3% estimated with respect to the main peak area of diclofenac reference solution. The simultaneous decrease of diclofenac peak area was 6.0%. This discrepancy is probably due to differences between response factors of diclofenac and its oxidation/degradation products at 254 nm. Diclofenac in MIX 2 showed the same trend: the new product area sum was 7.1%, whereas the decrease of diclofenac peak was 10.7%. The increase of the oxidation/degradation products total area in MIX 2 was proportional to the increase of $OH^{\bullet}$ free radical concentration. This observation suggested that the newly formed products were originated from the reaction between diclofenac and hydroxyl radicals.

In general, the best detection wavelength was 254 nm, but some peaks were more evident at 236 nm. In fact, the chromatogram of the same solution recorded at 236 nm (not reported) showed that the first two peaks at r.t. 11.9 and 13.3 min were more evident (about 70% area increase). Analogously, the peak at r.t. 36.5 min, corre-

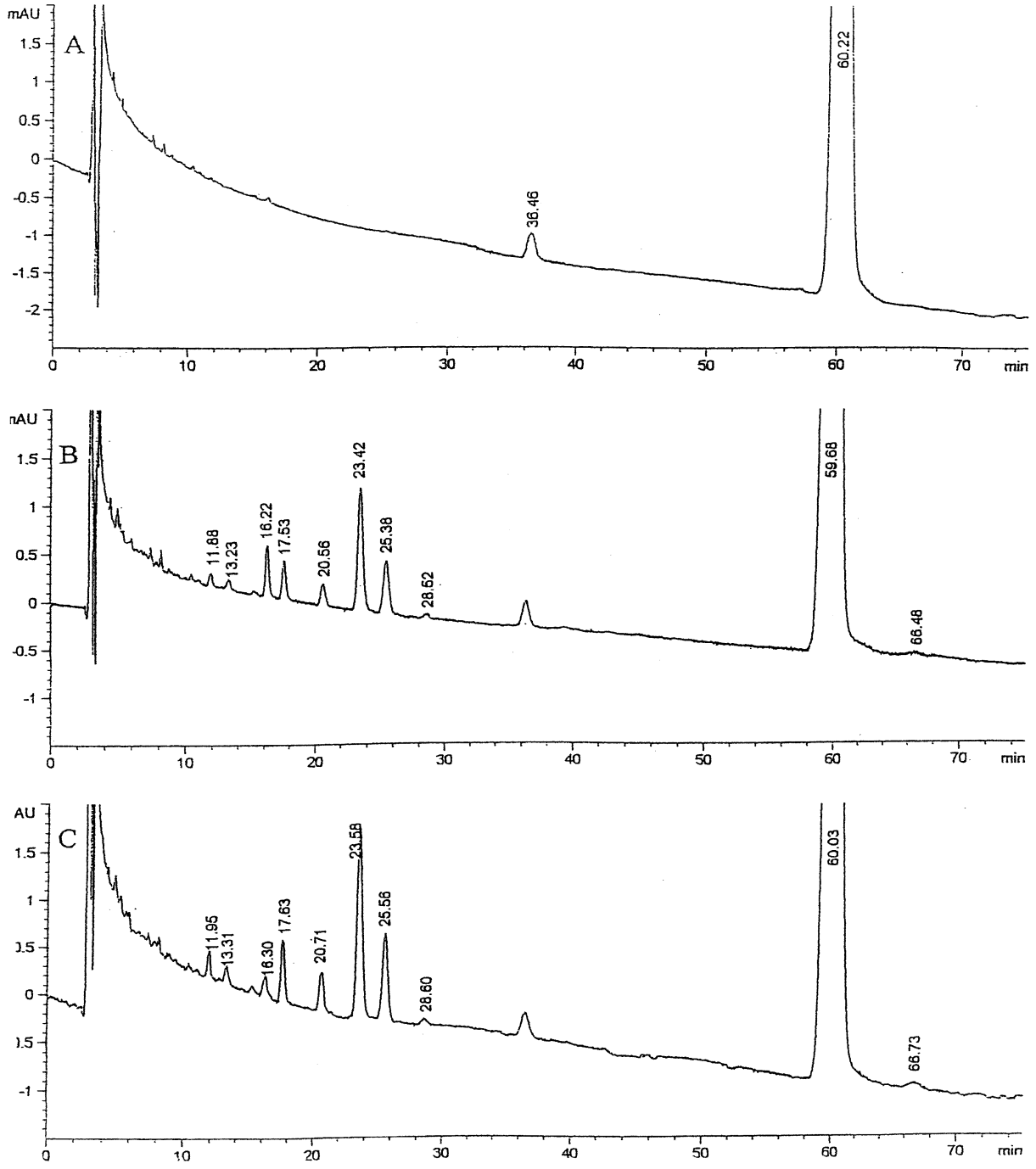


Fig. 2. Chromatographic profile of diclofenac reference solution (A), diclofenac submitted to the oxidant treatment in MIX 1 (B) and MIX 2 (C). Data refer to chromatographic method I described in Section 2.3. Detection wavelength was 254 nm.

sponding to the lactamic form of diclofenac, showed an area increase at 236 nm of about 70% with respect to 254 nm, in accordance with Stierling et al. who reported for the lactamic form of 4′-hydroxy-diclofenac a maximum in the absorption spectrum at 236 nm [22].

On the contrary, diclofenac peak showed only a 40% area increase and the major oxidation product at r.t. 23.5 min a 9% increase.

The experimental observations, supported by the above cited literature information, allowed to suppose that the two peaks at r.t. 11.9 and 13.3 min (Fig. 2B and C) could be assigned to the lactamic forms of some hydroxylated products.

The UV–DAD absorption spectra of diclofenac and its main oxidation product at r.t. 23.5 min were quite similar (spectra not shown). The latter showed only a slight red-shift of the maximum at 200 nm and a 6–8 nm blue-shift of the maximum at 276 nm indicating that 4′-hydroxy-diclofenac could be the major oxidation product [22] (spectra not shown). Unfortunately, the low absorption intensity of the other peaks did not allow any evaluation of their spectroscopic characteristics.

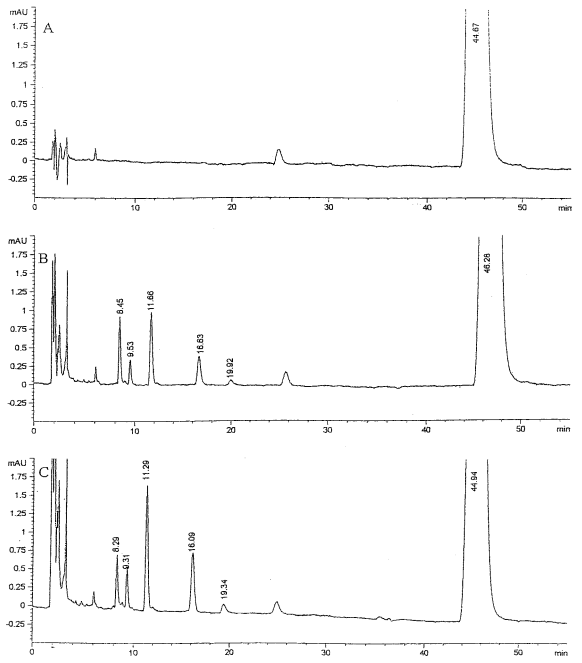


Fig. 3. Chromatographic profile of diclofenac reference solution (A), diclofenac submitted to the oxidant treatment in MIX 1 (B) and MIX 2 (C). Data refer to chromatographic method II described in Section 2.3. Detection wavelength was 282 nm.

The chromatographic profiles of diclofenac reference solution and diclofenac after the oxidation treatment obtained at 282 nm by method II are shown in Fig. 3. Five main new peaks in the chromatographic profile of diclofenac both in MIX 1 and MIX 2 were detected. A good agreement between the percent area data obtained by chromatographic methods I and II was found both at 254 and 282 nm.

All the LC analyses have been performed immediately after the end of the oxidant treatment and then changes have been followed through 24 h. Only negligible differences were observed in chromatographic profiles showing that in 2 h the reaction was terminated.

It is known that in vivo hepatic metabolism of diclofenac induces the formation of hydroxylated derivatives responsible for hepatic toxicity [25]. Some isoenzymes of cytochrome P450 and, in particular, CYP2C9, are responsible in vivo for 4′-hydroxy-, 5′-hydroxy- and 3′-hydroxy-diclofenac derivatives formation. Although the enzymatic reaction is the main transformation route of this xenobiotic molecule, the results obtained suggested that other oxidation products can be formed by a direct non-enzymatic reaction with hydroxyl radicals. This possible different reaction pathway could be influenced by intracellular $OH^{\bullet}$ free radicals concentration and it is more probable in pathological states characterised by oxidative stress or during ageing.

3.3. $OH^{\bullet}$ free radical effect on piroxicam

The chromatographic profiles of piroxicam reference solution and piroxicam after the oxidant treatment are shown in Fig. 4. The piroxicam peak at 15.2 min and a secondary peak at about 6.5 min can be seen in chromatogram A.

As already observed for diclofenac, the HPLC analysis of piroxicam reference solution after a 2-h irradiation gave the same chromatographic profile and irradiated MIX 1 and MIX 2 showed no peaks in the 3–18 min time range. On the contrary, the chromatograms of samples submitted to the oxidant treatment showed at least 13 new secondary

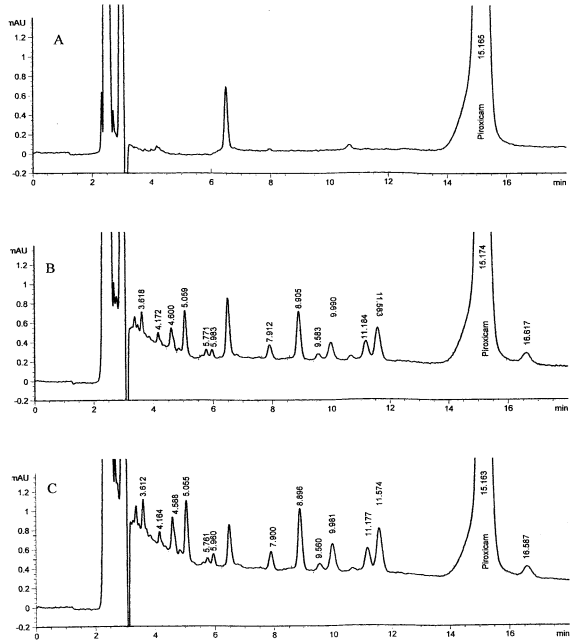


Fig. 4. Chromatographic profile of piroxicam reference solution (A), piroxicam submitted to the oxidant treatment in MIX 1 (B) and MIX 2 (C). Data refer to chromatographic method described in Section 2.3. Detection wavelength was 230 nm.

peaks (Fig. 4B and C). The chromatographic profiles obtained for the samples in the two oxidant mixtures were similar; however, the sum of the areas of degradation/oxidation products was higher for the sample in the more concentrated mixture (MIX 2). This observation again suggested that the quantity of degradation/oxidation products is proportional to the quantity of $OH^{\bullet}$ free radicals produced in the oxidant mixtures.

The total area percent values of the newly formed peaks, estimated with respect to the main peak area of piroxicam reference solution, and the decrease of the piroxicam peak area percent in MIX 1 and MIX 2 showed that more than 15% of the drug reacts with the hydroxyl radicals giving rise to the formation of new degradation/oxidation products.

The formation in vivo of the metabolic oxidation product 5-hydroxypiroxicam, that was found in human plasma as product of the enzymatic transformation catalysed by CYP2C [26], is known. The results obtained suggested that several other sub-products can be obtained by direct reaction with hydroxyl radicals. A study performed on tenoxicam [4-hydroxy-*N*-(2′-pyridyl)-2-methyl L-2*H*-thieno-(2,3e)-1,2-thiazine-3-carboxamide-1,1-dioxide] showed that in the presence of peroxidases and H_2O_2 four novel metabolites are formed by a one-electron oxidation reaction at the central carbon atom of the β-diketone [27]. The same mechanism in the reaction of piroxicam with hydroxyl radicals can be supposed.

4. Conclusion

The results reported in this paper represent a first attempt of an in vitro study aimed to understand if non-enzyme catalysed hydroxyl radical attack can modify NSAIDs structures.

The spin-trapping action of many NSAIDs could be, in some cases, the cause of the formation of radicalic and non-radicalic more toxic products. Moreover, it has been previously shown that radicals resulting from the attack of $OH^{\bullet}$ on some drugs (e.g. peroxy radicals or thiyl radicals from attack of $OH^{\bullet}$ on penicillamine) might cause biological damage [9,28,29].

In this work the formation of a number of oxidation/degradation products for both diclofenac and piroxicam in the presence of hydroxyl radicals was evidenced.

Further studies are in progress to elucidate the structure of the products originated in the reaction with $OH^{\bullet}$ radicals.

This study should be considered the first result of a more wide research that would evaluate if, in oxidative stress conditions, drugs and in particular NSAIDs can be modified by direct reaction with $OH^{\bullet}$ radicals in vivo to give sub-products that may cause drug adverse reaction, toxicity and therapeutic inefficacy.

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