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The Reactions of Oxicam and Sulfoanilide Non Steroidal Anti-Inflammatory Drugs with Hypochlorous Acid: Determination of the Rate Constants with an Assay Based on the Competition with Para-aminobenzoic Acid Chlorination and Identification of Some Oxidation Products

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Hypochlorous acid (HOCl) is an oxygen-derived species involved in physiological processes related to the defence of the organism that may cause adverse effects when its production is insufficiently controlled. In order to examine its reactivity with potential scavenging molecules from the non steroidal anti-inflammatory drugs (NSAIDs) family, a competition assay based on para-aminobenzoic acid (PABA) chlorination was developed. The original optimised *in vitro* fluorimetric procedure offered the possibility to determine rate constants (k_s) for the reaction with HOCl in physiologically relevant conditions. The specificity of the system was improved by a liquid chromatography (LC) which allows the separation of the drugs and their oxidation products. After determination of the rate constant for PABA chlorination by HOCl (mean ± SD in $M^{-1}\,s^{-1}$: $4.3 \pm 0.3 \times 10^3$), the applied mathematical model for a chemical competition permits to obtain linear curves from competition studies between several NSAIDs and PABA. Their slopes provided the following rate constants for the different studied drugs: tenoxicam: $4.0 \pm 0.7 \times 10^3$, piroxicam: $3.6 \pm 0.7 \times 10^3$, lornoxicam: $4.3 \pm 0.7 \times 10^3$, meloxicam: $1.7 \pm 0.3 \times 10^4$, nimesulide: $2.3 \pm 0.6 \times 10^2$. Meloxicam therefore reacted significantly faster than the other oxicams and nimesulide, which is the weakest scavenger of the studied series. The identification of some of the oxidation products by NMR or MS permitted to explore the reaction mechanism and to examine some aspects of the structure/activity relationships for the molecules of the same chemical family.

Keywords: Hypochlorous acid; Para-aminobenzoic acid; Rate constant; NSAID; Oxicam; Sulfoanilide

INTRODUCTION

Hypochlorous acid (HOCl) is a key derivative essentially produced by the myeloperoxidase/halide system during the respiratory burst of phagocytic cells and deeply involved in the antimicrobial activity of neutrophils and monocytes.[1] Such as other reactive oxygen-derived species (ROS), it may cause many adverse effects in the organism and seems mostly involved in the pathogenesis of inflammatory diseases.[2] A possible implication in atherosclerosis by oxidation of the low-density-lipoproteins contained in the artery wall was also reported.[3] Furthermore, HOCl could be responsible for many cell damages at the bronchial epithelium in respiratory diseases.[4] All these conditions are the consequence of a powerful attack by myeloperoxidase-derived HOCl of several molecular species such as the sulfhydryl groups of peptides and proteins including glutathione or the amino group of amino acids.[5] This relationship incited many authors to examine the reaction of selected ROS

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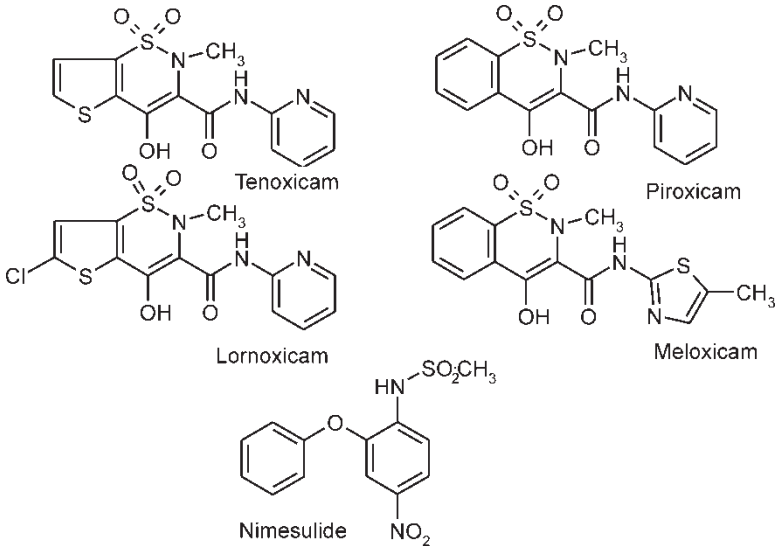

FIGURE 1 Structure of the studied NSAIDs.

with physiological antioxidants and exogenous compounds including therapeutical drugs. In this context, thiol containing-molecules such as N-acetylcysteine are often studied as a crucial target of ROS.[6–8] This is also the case for non-steroidal anti-inflammatory drugs (NSAID)[9–12] that are widely used to treat chronic inflammatory diseases characterised by an uncontrolled production of ROS and also for drugs of the cardio-vascular system, particularly in consideration of the high sensitivity of low-density-lipoproteins to oxidation.[13–16]

Methods available in the literature for studying the *in vitro* scavenging activity of molecules towards HOCl use measurement systems such as UV-Visible spectrophotometry or chemiluminescence.[7,10,15,17] In the first technique, a potential scavenger is put in competition with a "detector" molecule for HOCl and the reaction is quantified by measurement of the absorbance of the competitor at a specified wavelength. However, the results are most frequently expressed as concentrations inhibiting 50% of total oxidation (IC_{50} values) or as relative rate constants.[6,10] Moreover, the method may suffer from serious interferences as several scavengers absorb in the same range than the detector molecule. In the second system, the amount of light produced by the oxidation of luminol (essentially) is measured and reflects the quantity of HOCl that has not reacted with the scavenging molecule. It offers a better sensitivity, but as no linear relationship exists between the concentration of the scavenging molecule and the amount of light, results have also to be expressed as IC_{50} values.[15,17] In the present state of the art, there is a real interest to dispose of a sensitive assay for measuring absolute rate constants for the reaction with HOCl, which may serve to compare the results obtained with different scavengers. Moreover, such data are essential to properly investigate a possible direct interaction of drugs with the myeloperoxidase enzymatic system.[10]

In the present paper, a procedure was developed to assess the reactivity of HOCl with several therapeutic molecules belonging to the class of NSAIDs Fig. 1. The principle of a competitive assay system set up by Halliwell *et al.*[18] for determination of the rate constants for reaction with hydroxyl radicals was adapted to the case of HOCl using para-aminobenzoic acid (PABA) as a detector molecule for the presently studied free radical species. After optimisation of the experimental conditions for PABA chlorination, the competition model was worked out using fluorimetry to quantify the decrease in PABA concentration.[19,20] The presence of interferences during the analytical process could be solved by the introduction of a liquid chromatographic (LC) separation. The rate constants for reaction of the molecules of interest with HOCl were determined. Moreover, the nature of the oxidation products for the different drugs was investigated by nuclear magnetic resonance (NMR) and mass spectrum after isolation by thin layer chromatography (TLC).

MATERIALS AND METHODS

Chemicals and Solutions

Tenoxicam (Roche, Basle, Switzerland), lornoxicam (Nycomed, Linz, Austria), piroxicam (Pfizer, Brussels, Belgium), meloxicam (Boehringer Ingelheim, Biberach, Germany) and nimesulide (Helsinn, Biasca, Switzerland) were the NSAIDs. PABA (Acros Organics, New Jersey, USA), NaOCl (Aldrich, Steimheim, Germany) and KI (VWR, Leuven, Belgium) were the reagents. A PBS buffer (pH 7.4) was prepared at a final concentration of 10 mM

phosphate ions (KH_2PO_4/KOH) and 140 mM NaCl (all from VWR, Leuven, Belgium). These chemicals were of pro-analysis quality. Ammonium acetate, acetic acid (VWR International, Leuven, Belgium) and acetonitrile (VWR International, Fontenay Sous Bois, France) were LC analytical-grade reagents, used to prepare the mobile phase of the LC system. The acetate buffer (pH 3.0) contained 5% v/v acetic acid and 0.20% m/v ammonium acetate. De-oxygenated milliQ water served for the preparation of all solutions. The NMR solvents used were $CDCl_3$, DMSO d_6 and CD_3OD with 0.03% (v/v) of TMS from Aldrich (Steimheim, Germany).

A stock solution of NaOCl (4% w/w) was prepared and kept at 4°C. Before use, a working solution was prepared by dilution (80 µl/50 ml water) and HOCl concentration was determined at pH 6.2 by iodometry with a 20 mM KI solution. Iodine formed was measured at 350 nm and the concentration determined using an extinction coefficient of 22,900 $M^{-1}\,cm^{-1}$.[8]

Apparatus

A UV-160 Shimadzu spectrophotometer (Antwerp, Belgium) was used to perform UV-Visible absorbance measurements for the determination of HOCl concentration. A LS50B Luminescence Spectrometer from Perkin-Elmer (Bucks, UK) with the FLWinlab software was used for fluorescence measurements of chlorinated PABA. The instrument was set at 280 nm (excitation) and 340 nm (emission) with a slit width of 10 nm. A Gilson 307 pump (Gilson, Villiers le Bel, France) with a Beckman (Fullerton, California, USA) gold system 166 UV-detector and a 1100 series Agilent LC system with an autosampler and a fluorimetric detector (Palo Alto, California, USA) were used for the chromatographic separations. The column and guard-column were provided by Alltech (Alltech, Deerfield, IL). A Bruker Avance 300 NMR spectrometer (Wissembourg, France) and an Electron Ionisation (EI) mass spectrometer (40 eV, Waters ZQ Single Quad 2000 Da, Brussels, Belgium) were used for the identification of the structures of the reaction products.

Rate Constant for the Reaction of PABA with Hypochlorite

Data to determine the rate constants were obtained by fluorimetry. The time drive mode of the FLwinlab was set for duration of 30 s with time intervals of 0.5 s. A quartz cell containing a mixture of 2475 µl of PBS buffer (pH 7.4), 25 µl of PABA (0.05 µM) and 250 µl of milliQ water were pre-incubated in the cell holder under automatic stirring during 20 min at 37°C. At zero time (t_0), 250 µl of a solution containing one of the four different concentrations of HOCl (5–50 µM) were injected in the dark. The decrease in the intensity of fluorescence (λ excitation: 280 nm; λ emission: 340 nm) was plotted versus time. The results were expressed as mean ± SD for three different determinations with five measurements for each examined concentration.

Conditions for the Competition System

In a final volume of 2.0 ml, the reaction mixture contained the following reagents at the final concentrations indicated between brackets and taken from the literature:[6,8,9,21–23] PBS buffer pH 7.4 (10 mM/140 mM), PABA (30 µM) in PBS buffer and a gradient of the examined drugs (50–200 µM for the oxicams and 80–400 µM for nimesulide). After 20 min incubation at 37°C, HOCl (29 µM) was added. The mixture was kept at the same temperature during 5 min and 40 µl were finally injected in the LC system equipped with the fluorimetric detector, such as previously described. The area under the PABA peak of fluorescence (Int) was then measured for the different NSAID concentrations. The results were expressed as mean ± SD for at least three different determinations for each examined drug.

LC Separation of the Competitors and their Products and Study of Interferences

The conditions of the LC system were adapted from a procedure previously developed for separation of PABA and its chlorination product.[20] The following solutions were prepared in PBS buffer pH 7.4 at 37°C: PABA (60 µM); the different NSAIDs (60 µM); PABA treated by HOCl (50 µM); the different NSAIDs treated by HOCl; PABA and the different NSAIDs treated by HOCl. A 50 µl of these solutions were injected in the LC system where the Gilson pump was operated in isocratic mode. The mobile phase consisted of a mixture of the pH 3.0 acetate buffer and acetonitrile. The proportions of the two solvents had to be varied between 80/20 and 60/40 for the different studied drugs in order to obtain the most satisfactory separations at a flow rate of 1.0 ml/min. The column was an Alltima C18 15 cm × 4.6 mm, five microns with a guard column, 5 × 4.6 mm, five microns and the detection was performed by monitoring the absorbance at 254 nm with the Beckman UV-detector.

A second detection system was tested and finally adopted. It is based on the Agilent system equipped with a fluorimetric detector set at the previously described excitation and emission wavelengths. The other conditions were the same as described above. The following solutions were prepared in PBS pH 7.4 buffer at 37°C: PABA (60 µM); PABA treated by 10, 20, 30, 40, 50, 60 or 70 µM HOCl; the different NSAIDs (200 µM) treated by 200 µM HOCl. To 500 µl

of each PABA solution (treated by 0, 10, 20, 30, 40, 50, 60 and 70 μM HOCl), 500 μl of PBS buffer or 500 μl of the different NSAIDs solutions treated by HOCl were added in order to construct two different calibration graphs. A 20 μl of each of these solutions were injected in the LC system. The experiments were done in triplicate and both slopes and intercepts of the graphs were compared, using the ANOVA test.

Characterization of the Products of the Reaction between HOCl and the Scavengers

A 500 mg of each investigated drug were dissolved in 250 ml PBS buffer pH 7.4 and incubated during 20 min at 37°C. A 30 ml of HOCl (~0.4 M) at neutral pH were progressively added to the stirred solution maintained at 37°C. For some studied drugs, a precipitate appeared which was then separated by filtration, washed with milli-Q water and dried before analysis. The aqueous solution was acidified to pH 3 with diluted HCl (2 M) and then extracted with ethyl acetate. This organic solution was deposited on a silicagel TLC plate and developed with adapted solvent systems. The detected spots were extracted by an appropriate solvent, dried and identified by ^{1}H and ^{13}C NMR and eventually EI mass spectrometry. After identification, the products were submitted to the LC system equipped with the UV detector as previously described in order to measure the retention time with the respective pre-determined mobile phases.

RESULTS

Experimental Conditions for PABA Chlorination and Determination of the Rate Constant of the Reaction

PABA is chlorinated by hypochlorite ions to give a non-fluorescent derivative called 3-chloro-4-aminobenzoic acid and abbreviated PABA–Cl.[20] The rate constant for this reaction can be measured by a pseudo-order 1 kinetics as shown in Fig. 2a. Figure 2b shows the graph obtained by plotting the reaction rates determined in the previous graph versus HOCl concentrations. The slope of the rate as a function of HOCl concentration represents the rate constant for the reaction (k_{PABA}). A rate constant of $4.3 \pm 0.3 \times 10^3\ M^{-1}\ s^{-1}$ was experimentally obtained and used for the calculation of the rate constants for the various tested molecules in the competition assay.

Calculation of the Rate Constant for Reaction of Scavengers with HOCl in the Developed Competition Model

The principle of the calculation was derived from the one of the deoxyribose assay set up by Halliwell *et al.*

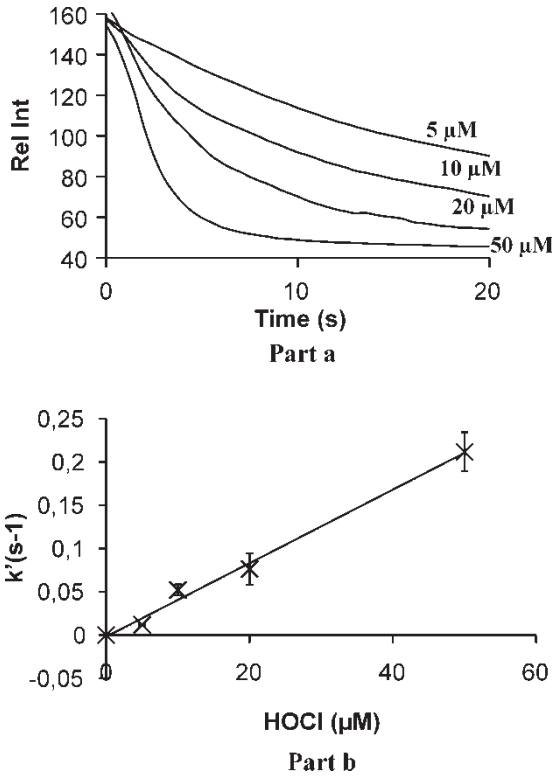

FIGURE 2 Part a: Effects of increasing amounts of HOCl (5–50 μM) on the intensity of fluorescence of PABA (0.05 μM) in PBS buffer pH 7.4 and at 37°C. Part b: Effects of increasing amounts of HOCl (5–50 μM) on the value of the rate constant of the reaction with PABA ($n = 5$).

and based on the expression of a chemical competition.[18,24] Considering the same assumptions, (1) and (2) are the kinetic equations for the reaction of each competing molecule with HOCl:

$$R_{PABA} = k_{PABA}[PABA]\,[HClO] \quad (1)$$

$$R_s = k_s[S]\,[HOCl], \quad (2)$$

where R_s and R_{PABA} are the rates of the reactions of the scavenger (S) and PABA with HOCl, respectively, k_s and k_{PABA} are the rate constants and [S] and [PABA] are the concentration of the scavenging molecule and of the competitor, respectively, at time 0.

Considering the competition system, $-\mathrm{dInt}/\mathrm{d}t$ of Eq. (1) can be replaced by Int° − Int where Int° is the area under the peak of fluorescence intensity when HOCl and S are absent and Int is this intensity measured after addition of the oxidant species [Eq. (3)] when S is present.

$$R_{PABA} = k_{PABA}[PABA]\,[HOCl] = Int° - Int \quad (3)$$

If Int′ is the area under the peak of fluorescence of PABA after addition of HOCl in the absence of any scavenging molecule, Int° − Int′ is the rate of

the reaction for PABA with HOCl when no drug is added. Equation (4) can then be derived:

$$\mathrm{Int}^\circ - \mathrm{Int}' = k_{\mathrm{PABA}}[\mathrm{PABA}][\mathrm{HOCl}] + k_s[S][\mathrm{HOCl}] \quad (4)$$

By replacing [HOCl] in Eq. (3) and dividing Eq. (4) successively by ($\mathrm{Int}^\circ - \mathrm{Int}'$) and ($\mathrm{Int}^\circ - \mathrm{Int}$), the general Eq. (5) is obtained provided that the reaction between PABA and HOCl is an elementary process:

$$1/(\mathrm{Int}^\circ - \mathrm{Int}) = 1/(\mathrm{Int}^\circ - \mathrm{Int}') + k_s[S]/k_{\mathrm{PABA}}[\mathrm{PABA}](\mathrm{Int}^\circ - \mathrm{Int}') \quad (5)$$

When plotting the concentration of S versus $1/(\mathrm{Int}^\circ - \mathrm{Int})$, a linear graph should be obtained and k_s calculated from the slope:

$$\mathrm{Slope} = k_s/k_{\mathrm{PABA}}[\mathrm{PABA}](\mathrm{Int}^\circ - \mathrm{Int}') \quad (6)$$

Efficiency of the Chromatographic Separation and Determination of the Rate Constants

Separation of the reaction products by LC revealed that the oxidation of PABA by HOCl gives rise to two peaks corresponding to the unchanged molecule and its chlorinated product, which in the mobile phase acetonitrile/acetate buffer 20/80, had retention times of 3.1 and 10.1 min, respectively. Addition of scavenging molecules caused the appearance of several other peaks (3–4 for most compounds). In accordance with literature,[25] the competition with tenoxicam for PABA chlorination gave rise to four more peaks with the following retention times: 1.9, 5.0, 14.4 (peak for tenoxicam) and 15.7 min (Fig. 3). Lornoxicam and piroxicam, which were examined in the mobile phase containing the same mixture of solvents in a ratio 30:70, gave quite similar results with the following peaks: PABA (2.5 min), PABA–Cl (5.2 min), lornoxicam (16.4 min) and piroxicam (17.9 min) and three more peaks (lornoxicam: 1.5, 3.8 and 22.2 min and piroxicam: 1.5, 3.6 and 9.4 min). Finally, meloxicam and nimesulide, examined in the mobile phase 40:60, gave retention times of 2.3 min for PABA, 3.5 min for PABA–Cl, 16.6 min for meloxicam which had also three oxidation products (2.2, 3.1 and 6.0 min) and 25.5 min for nimesulide which had no detectable oxidation product.

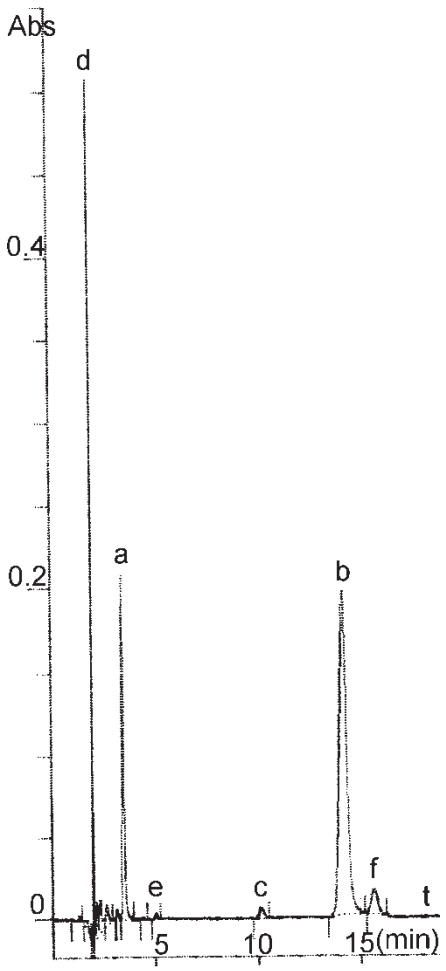

FIGURE 3 Chromatograph of a solution resulting from the competition between PABA (60 μM) and tenoxicam (60 μM) for HOCl (50 μM). The LC conditions: C18 column, mobile phase acetonitrile/acetate buffer 20/80, flow rate of 1 ml/min, UV-detection at 254 nm. The respective retention times are: PABA 3.1 min (a), tenoxicam (b) 14.4 min, PABA–Cl 10.1 min (c) oxidation product of tenoxicam 1.9, 5.0 and 15.7 min (d, e and f).

The effect of the oxidation products of the different NSAIDs on the fluorimetric signal was assessed in their respective mobile phase and a statistical comparison of the slopes and the intercepts (Student's t test for paired samples) showed no significant difference, demonstrating the absence of effect of the oxidation products. The influence of the composition of the mobile phase (different ratios of acetonitrile/acetate buffer) was also studied and it

TABLE I Determination of the rate constants for reaction of HOCl with examined molecules (ks)

	Tenoxicam	Piroxicam	Lornoxicam	Meloxicam	Nimesulide
n	4	5	5	5	3
Mean ($M^{-1}s^{-1}$)	4.0×10^3	3.6×10^3	4.3×10^3	$1.7 \times 10^{4*}$	$2.3 \times 10^{2**}$
SD ($M^{-1}s^{-1}$)	0.7×10^3	0.7×10^3	0.7×10^3	0.3×10^4	0.6×10^2
CV %	17	19	16	17	26

The values are mean ± SD for n daily experiments where the ratios of the mobile phase (acetate buffer/acetonitrile) is 20/80 for tenoxicam; 30/70 for lornoxicam and piroxicam and 40/60 for meloxicam and nimesulide. *significantly different from the other NSAID ($P < 0.05$). **significantly different from the oxicams ($P < 0.05$).

was demonstrated that it did not significantly affect the measurement of the rate constant (ANOVA test). The fluorimetric detection was then preferred to the UV-visible detection due to its better sensitivity and specificity.

The rate constants of the different studied NSAIDs were then determined according the newly developed procedure and results reported in Table I. The precision reached is quite satisfactory. Significant differences were observed for meloxicam as compared to the three other drugs of the same family ($P < 0.05$) and for nimesulide, which appeared as the weakest scavenger as compared to the other NSAIDs ($P < 0.05$).

Identification of the Products of the Reaction between HOCl and Drugs

Meloxicam was selected as representative of the oxicam family. The drug (4-hydroxyl-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide) gave ^{1}H NMR (300 MHz, DMSO) signals consistent with those reported in the literature.[26] The ethyl acetate extract of the product of the reaction between HOCl and the drug was submitted to TLC with the following solvent system chloroform/methanol/formic acid 40/5/1. Two spots could be identified: the first (Rf of 0.8) was extracted with chloroform and second (Rf of 0.45) with ethyl acetate. The respective ^{1}H-NMR (300 MHz) spectra gave the following signals: (in $CDCl_3$) 3.27 (N–CH_3, s, 3H), 7.81–7.91 (m, 2H), 7.93–7.96 (dd, J = 6.7 and 2.2 Hz, 1H), 8.05–8.08 (dd, J = 2.2 and 6.7 Hz, 1H) and (in CD_3OD): 2.57 (N–CH_3, s, 3H), 7.47–7.54 (m, 1H), 7.61–7.66 (m, 1H), 7.71–7.73 (d, J = 7.71 Hz, 1H), 7.89–7.92 (d, J = 7.5 Hz, 1H). The ^{13}C NMR (75 MHz, $CDCl_3$ or CD_3OD) of both products showed only 8 carbons (instead of 14 in meloxicam), respectively, δ: 23.4, 121.1, 125.3, 127.7, 134.4, 134.6, 137.7, 158.8 and δ: 29.7, 129.2, 129.7, 130.7, 133.7, 134.8, 136.1 and 172.0. Such features indicate the presence of N-methylbenzylsulfimide (Rf 0.8) and 2-(N-methyl-sulfamoyl)-benzoic acid (Rf 0.45)[27,28] which in the chromatographic system previously described had retention times of 6.0 and 3.2 min, respectively.

The sulfoanilide nimesulide (N-(4-Nitro-2-phenoxyphenyl) methanesulfonamide) was also first characterised by ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$). Addition of HOCl to a solution of the drug at pH 7.4 gave a precipitate that was isolated, washed with water and dried. The remaining solution was then acidified, extracted by ethyl acetate and dried. The two solutions gave products with similar signals; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.36 (SO_2–CH_3, s, 3H), 6.78 (SO_2–NH, s, 1H), 7.15–7.18 (m, 2H), 7.30–7.35 (m, 1H), 7.45–7.52 (m, 2H), 7.52 (d, J = 2.5 Hz, 1H) 8.04–8.05 (d, J = 2.5 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) gave the δ: 43.5, 110.8, 118.9, 120.5 (2C), 126.5, 130.7, 130.8 (2C), 131.3, 145.6, 153.5, 153.9. The shift effect on the CH_3–SO_2–R and the coupling constant demonstrated the presence of the N-(6-chloro-4-Nitro-2-phenoxyphenyl)methanesulfonamide. The chlorine atom in this derivative was confirmed by EI mass spectrum where two peaks were detected at 343 and 345 m/z in an approximate ratio 2/3:1/3 for the molecular ion peak ($C_{13}H_{11}N_2O_5SCl$). This derivative had a retention time of 25.5 min in the previously described chromatographic system.

DISCUSSION

Overproduction of ROS is frequently observed in various conditions with inflammation leading to oxidative damages that are poorly controlled by the physiological antioxidant system. Among these species, HOCl, which is mostly produced by the myeloperoxidase/H_2O_2/Cl^- system of the neutrophils, may be highly relevant as a causative agent of damages to several biomolecules or cell structures.[2] Anti-inflammatory drugs such as NSAIDs of the oxicam and sulfoanilide groups are nowadays largely used in therapeutics due to their powerful inhibiting capacity of the enzyme cyclo-oxygenase (COX) and, for some of them, of its two-isoform which is highly expressed in inflammatory diseases.[29] From a pharmacological point of view, it could be of interest to employ NSAIDs that possess a significant activity as scavengers of ROS or have a possible interaction with myeloperoxidase. Some of us previously investigated the scavenging effects of classical NSAIDs towards a large panel of ROS in various experimental conditions but this kind of activity is poorly documented in COX-2 preferential or selective drugs.[10,17,30,31]

The application of existing methodology for studying the interactions of drugs with HOCl was rapidly hindered by the appearance of several interferences due to the tested molecules themselves. Methods involving UV-Visible spectrophotometry such as the taurine chlorination assay[7,10] were indeed impossible to use due to the strong absorbance of the studied molecules in the range 200–420 nm. A procedure was therefore developed on the basis of a competition assay system for calculation of the absolute rate constants. To this purpose, PABA, a compound frequently encountered as the active component of UV-protecting creams, and the scavenging molecule were put in competition for the reactive oxygen species and fluorimetry was used to quantify the inhibition of PABA chlorination by increasing amounts of the molecule under assay. Indeed, PABA gives rise to a chlorinated derivative (3-chloro-4-aminobenzoic acid or PABA–Cl) in presence of HOCl generated from NaOCl at

physiological pH and the reaction is accompanied by a decrease in the intensity of fluorescence of PABA.[19,20] The better sensitivity of this detection system as compared to UV-Visible spectrometry allowed to test rather low concentrations of the NSAIDs, in a range compatible to the therapeutic use. However, as interference from the drugs under assay also appear with this system, a further step to the method was added consisting in a chromatographic separation of the interfering analyses by LC. Indeed, most of examined molecules and their reaction products with HOCl significantly interfered with the measurement of the signal by fluorimetry. The experimental conditions for PABA chlorination were then optimised, the competition model developed and validated and finally the rate constant for reaction with HOCl determined with good analytical performances by the use of simple linear regression calculations. No significant difference for the rate constants of the reactions with HOCl could be observed for three of the four members of the oxicam family (tenoxicam, lornoxicam and piroxicam) while meloxicam more quickly reacted with the oxidant species. Nimesulid, the sole representative of the sulfoanilide family, had the slowest reactivity as compared to the other NSAIDs. The presently determined rate constants for oxicams (in the range 10^3–$10^4\,M^{-1}\,s^{-1}$) are somewhat lower than those already published in the literature for HOCl scavengers. Peskins *et al.* (2001) mentioned a possible rate constant higher than $10^7\,M^{-1}\,s^{-1}$ for glutathione and thiol-containing molecules whereas values of 2×10^4 and $2\times10^5\,M^{-1}\,s^{-1}$ were reported for molecules such as methionine and cysteine at pH 12.[7] In the meantime, ascorbic acid has a rate constant of about $6\times10^6\,M^{-1}\,s^{-1}$ at pH 7.4 whereas taurine (primary aliphatic amine) gives a chloramine at pH 7 with a rate constant of $4.8\times10^5\,M^{-1}\,s^{-1}$.[32] Even if the rate constants obtained with NSAIDs are lower than those of physiological antioxidants (glutathione, ascorbic acid), they reflect quite well the reactivity of these molecules with HOCl showing some differences in a same chemical family and between two different families. Moreover, such data are useful to further study their interaction with the enzyme myeloperoxidase.[10]

The characterisation of the oxidation products by ^{1}H-NMR and ^{13}C-NMR associated with mass spectroscopy allowed to draw some conclusions on the structure/scavenging activity relationships. Such as already described in the literature for tenoxicam,[25] the reaction with HOCl was accompanied by an oxidation of the C-3 carbon of the enolic function (Fig. 4). Similar mechanisms were observed for lornoxicam and piroxicam. The observation that the rate constants for reaction are not significantly different for these three derivatives indicated that the chemical modification concerning the thiophene ring of tenoxicam does not affect the scavenging activity towards HOCl of the oxicams (see Fig. 1). The replacement of the pyridine ring of piroxicam by a methyl-thiazole ring in meloxicam (see Fig. 1) was accompanied by an increase in the rate constant for reaction with HOCl, demonstrating that this ring has a clear influence on the scavenging activity of the drug towards this oxygen-derived species. Moreover, a reaction on the C-3 carbon of the enolic function was also observed for meloxicam suggesting that oxidation of this function is also involved in the scavenging activity of this drug. As far as nimesulide is concerned, few characteristics of its structure should be the target of the examined oxygen-derived species. Nevertheless, only a limited number of papers described its interaction with HOCl, which could be the consequence of the reaction of the NH group on the sulfonamide function (Fig. 1).[17,33] Even if the determined rate constant is rather low, we demonstrated thanks to the NMR and MS spectrum, the presence of a chlorinated derivative resulting from an electrophilic substitution on the C-6 carbon by a chlorine atom. Incidentally, the retention times of nimesulide and its chlorinated derivative were

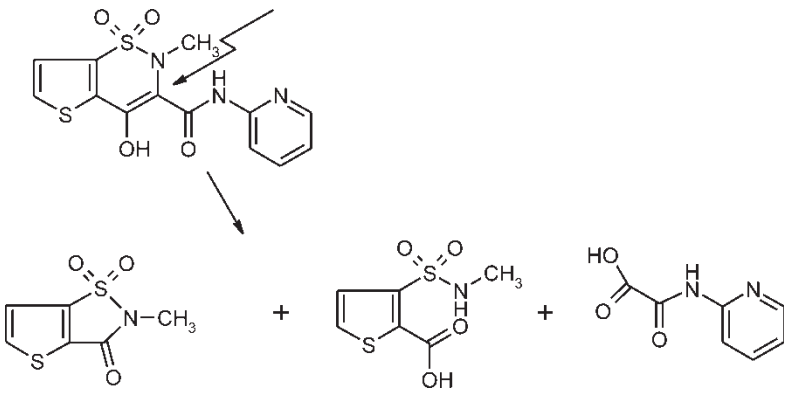

FIGURE 4 Oxidation of tenoxicam by hypochlorite according to Ichihara *et al.*[24]

similar, explaining that only one peak is observed during the LC process.

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