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INTERACTION OF MYELOPEROXIDASE WITH DICLOFENAC

INHIBITION OF THE CHLORINATING ACTIVITY OF MYELOPEROXIDASE BY DICLOFENAC AND OXIDATION OF DICLOFENAC TO DIHYDROXYAZOBENZENE BY MYELOPEROXIDASE

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Abstract—The chlorinating activity of myeloperoxidase, isolated from human polymorphonuclear neutrophils, was inhibited by the non-steroidal anti-inflammatory drug diclofenac (Voltaren®). The concentration of diclofenac needed for 50% inhibition was 20 μM, a value comparable with IC_{50} values found for other drugs. Diclofenac did not react with HOCl nor with H_2O_2 but was oxidized in the presence of myeloperoxidase and H_2O_2 to an orange-coloured unstable product. The rate of oxidation was proportional to the enzyme concentration and to the concentration of diclofenac, but independent of the H_2O_2 concentration. Presumably both Compound I and Compound II, two intermediates formed during the reaction cycle of myeloperoxidase with H_2O_2, are able to oxidize diclofenac. In these redox reactions, the active short-living Compound I is reduced to Compound II, thereby inhibiting the chlorinating activity of the enzyme. Analysis by Fast Atom Bombardment mass spectrometry showed that in the presence of H_2O_2 myeloperoxidase oxidizes diclofenac to dihydroxyazobenzene.

Diclofenac (Voltaren®) is a non-steroidal anti-inflammatory drug (NSAID) with antipyretic and analgesic properties [1–4]. It is widely used in the treatment of rheumatoid arthritis and other inflammatory diseases [4–6]. The mechanism of action of diclofenac is thought to be the inhibition of the prostaglandin synthesis [7, 8]. In man, diclofenac is extensively metabolized, the major product being 4′-hydroxydiclofenac [9, 10]. Although adverse effects are infrequent and generally mild, hepatotoxic effects and renal changes associated with treatment with diclofenac have also been reported [11–13].

Myeloperoxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is present in the granules of polymorphonuclear neutrophils and participates in the microbicidal activity of these cells [14]. In the presence of H_2O_2, myeloperoxidase catalyses the oxidation of chloride ions to the highly reactive HOCl [15]. There are strong indications that in rheumatoid arthritis polymorphonuclear neutrophils are activated by tissue-bound stimuli, resulting in release into the synovial fluid of both myeloperoxidase and H_2O_2, the latter formed as part of the oxidative burst [16, 17]. This implies that HOCl can be formed extracellularly and might contribute to the tissue damage observed in rheumatoid arthritis patients [18]. We have proposed that D-penicillamine acts by means of inhibiting myeloperoxidase and scavenging of HOCl [19, 20]. We found that many other drugs have effects on myeloperoxidase and HOCl, and in this context we studied the interaction of myeloperoxidase with diclofenac. Indeed, diclofenac inhibited the chlorination of monochlorodimedone catalysed by myeloperoxidase, but it did not scavenge HOCl. In the presence of myeloperoxidase and H_2O_2, diclofenac was oxidized to an orange-coloured product. We characterized this reaction and we elucidated the structure of the oxidation product.

MATERIALS AND METHODS

Myeloperoxidase was purified from human leukocytes as previously described [21]. The concentration was calculated using an absorbance coefficient of 89 mM^{-1} cm^{-1} per heme at 428 nm. The chlorinating activity of myeloperoxidase was determined by following the conversion of monochlorodimedone (absorbance coefficient 20.2 mM^{-1} cm^{-1} at 290 nm) into dichlorodimedone (absorbance coefficient 0.2 mM^{-1} cm^{-1} at 290 nm). The reaction was started by addition of 60 μM of H_2O_2 to the sample, which contained 25 nM myeloperoxidase, 100 mM KCl, 50 μM monochlorodimedone, 10 μM 5-aminosalicylic acid and 100 mM potassium phosphate (pH 7.1). The addition of 5-aminosalicylic acid to avoid premature inactivation of myeloperoxidase during turnover has been documented in Ref. 22. It should be noted that 5-aminosalicylic acid was present only in the assay mixture in which the activity of myeloperoxidase was measured (Fig. 1) and was absent in all other described experiments. Potential scavenging of HOCl by diclofenac was tested by incubation of various concentrations of the drug with

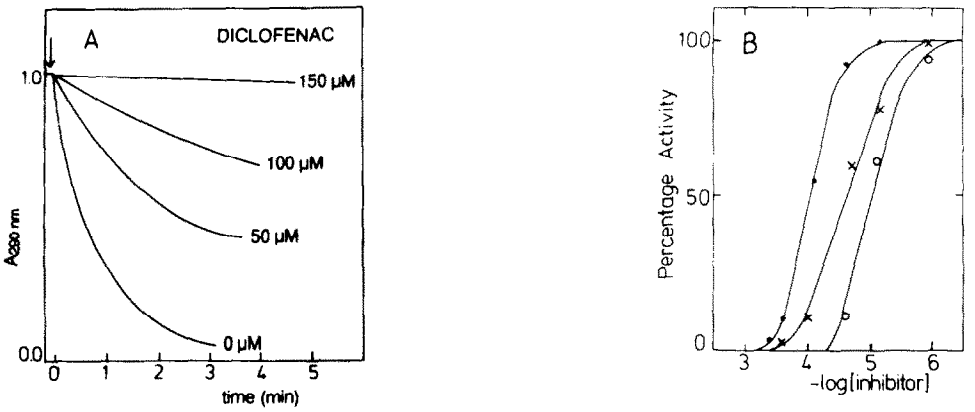

Fig. 1. (A) Inhibition of the chlorinating activity of myeloperoxidase by diclofenac. The reaction was started by the addition of H_2O_2 (arrow) to the assay mixture (see Materials and Methods), in which 0–150 μM diclofenac was present. The absorbance at 290 nm was recorded. (B) Variation in the chlorinating activity of myeloperoxidase as a function of the logarithm of the concentrations of (●) indomethacin, (×) diclofenac and (○) D-penicillamine. The monochlorodimedone chlorination assay is described in Materials and Methods.

50 μM HOCl, followed by determination of the remaining HOCl with the monochlorodimedone reagent. Since myeloperoxidase is a hemoprotein, the enzyme and its intermediates Compound I, Compound II and Compound III have characteristic absorbance spectra. Absorption spectroscopy was carried out on a Cary-17R recording spectrophotometer or on a Hewlett–Packard 8451 diode array spectrophotometer.

Diclofenac was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and from Ciba-Geigy (Basel, Switzerland). The diclofenac-containing drug Voltaren® was also a product of Ciba-Geigy. 5-Aminosalicylic acid was obtained from Merck (Rahway, NJ, U.S.A.). For each experiment appropriate H_2O_2 dilutions (absorbance coefficient 43.6 $M^{-1}cm^{-1}$ at 240 nm) were freshly prepared from a 30% stock solution (Merck). All other chemicals used were of the highest purity available.

The orange oxidation product of diclofenac was produced by the addition of 500 μM H_2O_2 to a mixture of 7.5 μM myeloperoxidase and 200 μM diclofenac. After about 1 hr an orange precipitate was separated from the reaction mixture by centrifugation. The dry-frozen orange powder was subsequently suspended in thioglycerol. Fast Atom Bombardment (FAB) mass spectrometry [23, 24] was carried out using a V.G.Micromass ZAB-2HF mass spectrometer, an instrument with reverse geometry, fitted with a high-field magnet and coupled to a V.G. 11/250 data system. The samples were loaded in a thioglycerol solution onto a stainless steel probe and bombarded with xenon atoms with an energy of 8 keV. During the high resolution FAB mass spectrometry measurements a resolving power of 15,000 (10% valley definition) was used. Mass analysed ion kinetic energy (MIKE) spectra [25] were obtained by varying the electric sector voltage, the main beam being 8 keV. FAB/MIKE spectra record the fragment ions of spontaneously decomposing $(M + H)^+$ ions, while FAB/MIKE/CA (collisional activation) mass spectra record the fragment ions of $(M + H)^+$ ions following collision with helium as the target gas. The helium pressure was such that the intensity of the main beam was reduced by 50%.

RESULTS

The chlorinating activity of myeloperoxidase was inhibited by diclofenac as shown in Fig. 1A. It can be calculated that the initial activity in the presence of 50 μM diclofenac was only 20% of that in its absence. From a plot of the initial activity in the presence of inhibitors against the logarithm of the inhibitor concentration (Fig. 1B) it could be derived that under the conditions of our assay 20 μM diclofenac caused 50% inhibition of the myeloperoxidase activity. This IC_{50} value lies in between those measured for indomethacin and D-penicillamine. We have previously found that D-penicillamine not only inhibits the myeloperoxidase activity, but also scavenges the reaction product HOCl [19, 20]. It was found that diclofenac did not scavenge HOCl (not shown in figures).

For D-penicillamine we have previously found that the drug inactivates myeloperoxidase by the formation of Compound II and Compound III [19, 20]. These compounds are not in the pathway leading to the formation of HOCl. Therefore, the effect of diclofenac on the formation of these compounds of myeloperoxidase was investigated.

Addition of diclofenac to native myeloperoxidase did not cause spectral changes. Reaction of native myeloperoxidase with its substrate H_2O_2 converts the enzyme into Compound I which, in the absence of chloride ions, is reduced to Compound II by a second molecule of H_2O_2 [15, 26–28]. Upon addition of H_2O_2 to a mixture of myeloperoxidase and diclofenac in the absence of chloride ions, within 5 sec the absorbance maximum of myeloperoxidase at 428 nm

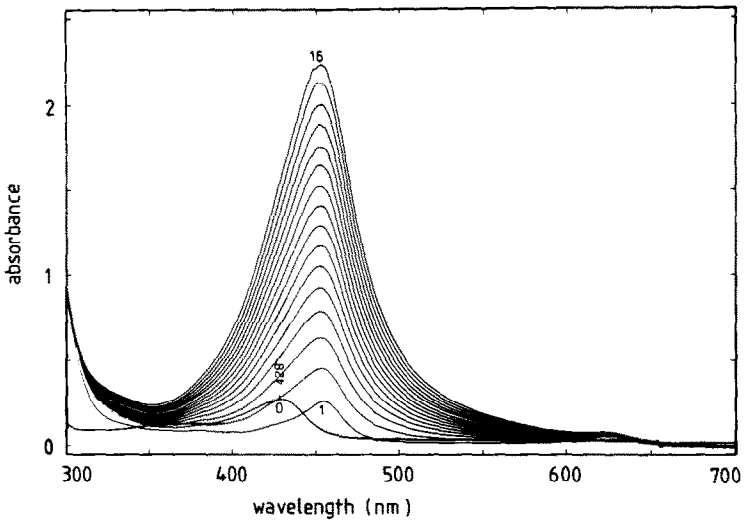

Fig. 2. Formation of the orange-coloured product of diclofenac catalysed by myeloperoxidase in the presence of H_2O_2. To a mixture of 3 μM myeloperoxidase and 200 μM diclofenac in 50 mM potassium phosphate buffer (pH 7.2) (spectrum 0) 150 μM H_2O_2 was added; 5 sec after mixing spectrum 1 was recorded. Every minute a spectrum was taken until maximal absorbance at 452 nm was reached (spectrum 16).

shifted to 454 nm, indicating the formation of Compound II as shown in Fig. 2. However, after 30 sec the absorbance peak shifted to 452 nm and the absorbance increased dramatically, far above the level of any myeloperoxidase compound. During the increase in absorbance the colour of the solution changed from light green (myeloperoxidase) to bright orange. The solution of the orange-coloured product was not stable but decomposed in a few hours, leaving the spectrum of myeloperoxidase only (not shown in Fig. 2).

To exclude the possibility that a contamination in the diclofenac sample was responsible for the unexpected formation of the orange-coloured product, the same experiment was performed with diclofenac samples from two companies, and with the drug Voltaren®. With all diclofenac samples similar results were obtained. Upon addition of H_2O_2 to diclofenac in the absence of myeloperoxidase only a trace of the orange product was formed. These results indicate that myeloperoxidase catalyses the oxidation of diclofenac by H_2O_2. In line with this, we observed that upon addition of sodium dithionite to the orange-coloured solution, formed by incubation of diclofenac with H_2O_2 and myeloperoxidase, the colour vanished immediately and only the spectrum of reduced myeloperoxidase could be detected, characterized by its absorbance at 472 and 642 nm.

The rate of oxidation of diclofenac by myeloperoxidase in the presence of H_2O_2 was proportional to the concentration of diclofenac (Fig. 3A) and to the concentration of myeloperoxidase (Fig. 3B), but independent of the concentration of H_2O_2. In order to study the effect of diclofenac on the Compound II formation we determined the rate of Compound II formation in the presence of H_2O_2 at different concentrations of diclofenac (not shown in figures). The dependence of these two parameters upon each other appeared to be linear and a rate constant of $6.0 \times 10^5\ M^{-1}\ sec^{-1}$ for the conversion of Compound I to Compound II by diclofenac could be calculated.

In the presence of chloride ions (100 mM) the maximal amount of the orange-coloured oxidation product of diclofenac was only about 20% of the value found in the absence of chloride. Probably diclofenac and chloride compete for Compound I, in which diclofenac converted Compound I to Compound II and chloride converted Compound I to native enzyme. In addition, diclofenac was able to convert Compound II to the native enzyme. The rate constant was determined in an experiment in which Compound II was produced with H_2O_2, the excess H_2O_2 eliminated by the addition of catalase, whereafter diclofenac was added. The decomposition rate of Compound II proved to be linearly dependent on the concentration of diclofenac; the calculated rate constant was $97.5\ M^{-1}\ sec^{-1}$, which is more than three orders of magnitude slower than that for the conversion of Compound I to Compound II by diclofenac.

An orange oxidation product of diclofenac was prepared for analysis by Fast Atom Bombardment (FAB) mass spectrometry as described in Materials and Methods. The FAB mass spectra obtained in the positive-ion and negative-ion mode (see Materials and Methods) are shown in Fig. 4. The (+)FAB spectrum is characterized by m/z values of 197, 214, 215, 237 and 253 (Fig. 4A). As sodium (from diclofenac sodium) and potassium (from the phosphate buffer) were present, cationized molecules could be expected. The m/z values of 237 and 253 can therefore be ascribed to $(M + Na)^+$ and $(M + K)^+$, respectively, supposing the compound has a molecular mass (M) of 214 daltons. The peak at

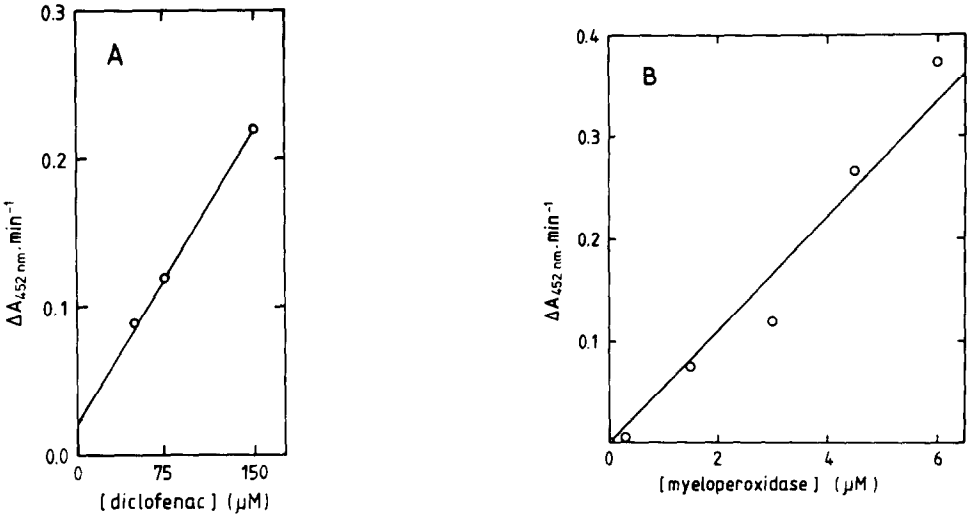

Fig. 3. (A) Velocities of the formation of the oxidation product of diclofenac catalysed by myeloperoxidase as a function of the concentration of diclofenac. H_2O_2 (50 μM) was added to a solution of 3 μM myeloperoxidase and various concentrations of diclofenac in 100 mM potassium phosphate buffer (pH 7.2). The absorbance at 452 nm was measured and expressed as absorbance change per minute. (B) Velocities of the formation of the oxidation product of diclofenac as a function of the concentration of myeloperoxidase. H_2O_2 (150 μM) was added to a solution of various concentrations of myeloperoxidase and 150 μM diclofenac in 50 mM potassium phosphate buffer (pH 7.1). The absorbance at 452 nm was measured and expressed as absorbance change per minute.

m/z = 215 would then be due to the protonated form $(M + H)^+$ and the peak at m/z = 197 could be interpreted as $(M + H\text{-}H_2O)^+$. There is no indication that chlorinated components were present, as judged from the absence of $^{35}Cl/^{37}Cl$ isotope effects. Also diclofenac itself (Fig. 4C) was not detectable in the spectrum of the oxidized product. In the (−)FAB mass spectrum (Fig. 4B) the m/z = 213, corresponding to the $(M\text{-}H)^-$ ion, is relatively abundant. Based upon the accurate mass measurement of 215.08251 for the protonated $(M + H)^+$, the elemental composition was determined to be $C_{12}H_{10}N_2O_2$.

Since the strong orange colour and the molecular composition can be expected for a dihydroxyazobenzene we compared the mass spectra with these of the commercially available *ortho,ortho'*-dihydroxyazobenzene (Figs 5A and B). As can be seen, they are very similar, taking into account that the diclofenac product contains potassium salts which give rise to additional peaks (Fig. 4A).

Conclusive evidence that the isolated oxidation product is a dihydroxyazobenzene has been provided by so-called FAB/MIKE and FAB/MIKE/CA spectra (see Materials and Methods) of the orange product (Fig. 6A and B) and the reference compound (Fig. 6C and D). Comparison of these spectra leaves no doubt that the isolated oxidation product is a dihydroxyazobenzene. However, its hydroxyl groups are not in the *ortho,ortho'*-positions.

DISCUSSION

For many years now diclofenac sodium has been successfully used as an anti-inflammatory drug, especially in the treatment of rheumatoid arthritis [6, 29, 30]. The mechanism of action proposed for the drug is inhibition of the enzyme cyclooxygenase, which catalyses the first step in the biosynthesis of prostaglandins [7, 8]. In the present study it is shown that diclofenac inhibited the catalytic activity of myeloperoxidase, thereby preventing the formation of HOCl (Fig. 1). Myeloperoxidase is present in the granules of polymorphonuclear leukocytes and macrophages, and is present in the inflamed synovia of patients suffering from rheumatoid arthritis [31]. Upon stimulation of these cells, myeloperoxidase and products of the oxidative burst, e.g. H_2O_2, are released from the cells. Furthermore, formation of HOCl from H_2O_2 and chloride ions, catalysed by myeloperoxidase, can occur. The resulting extracellular HOCl may facilitate tissue degradation [18]. The inhibition of the chlorinating activity of myeloperoxidase by diclofenac may add to the beneficial effect of diclofenac in rheumatoid arthritis. Although diclofenac is a mild reducing agent, the drug did not scavenge HOCl in contrast to several other NSAIDs [32, 33]. Surprisingly, in the presence of myeloperoxidase and H_2O_2 at neutral pH diclofenac was oxidized to an orange-coloured product (Fig. 2); without myeloperoxidase only a trace amount of this product was formed. The oxidation of diclofenac to its orange product could not be mimicked by addition of oxidants such as HOCl and potassium ferricyanide. Only with ferrous sulphate plus ammonium persulphate was it possible to oxidize a small percentage of diclofenac to the orange product (results not shown). It is known that myeloperoxidase reacts with H_2O_2 under formation of the short-living Compound I. The formation of Compound I from native myeloperoxidase and H_2O_2 was not a rate-limiting

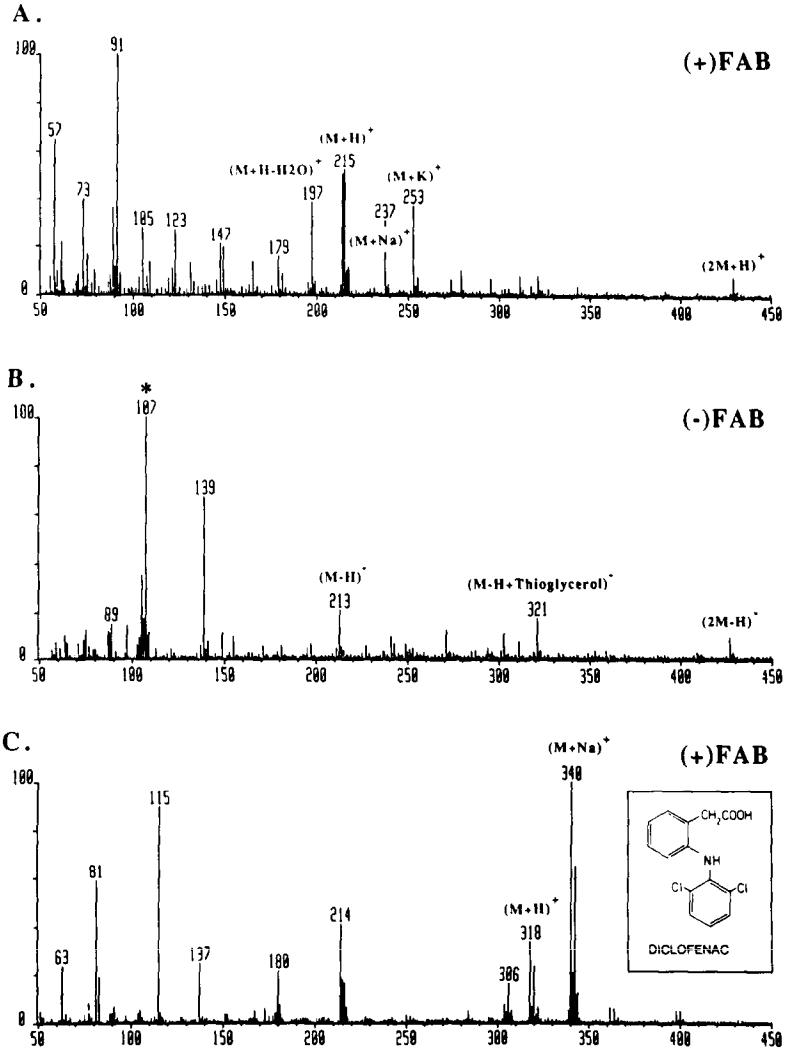

Fig. 4. (+) and (−)FAB mass spectra of the orange-coloured oxidation product of diclofenac (A and B) and (+)FAB mass spectrum of diclofenac (C) dissolved in thioglycerol as matrix. Peaks denoted by an asterisk are due to ions originating from the matrix used.

step in the oxidation of diclofenac by myeloperoxidase because the rate of this oxidation was independent of the concentration of H_2O_2. Compound I can either be reduced to Compound II or react with a proton and a chloride ion to produce the bactericidal HOCl and regenerate native myeloperoxidase [26, 27]. Compound II, in turn, can also be reduced to native myeloperoxidase by an electron-donating agent [22, 34]. A scheme of the above-mentioned reactions of myeloperoxidase is shown in Fig. 7.

Results in this paper show that diclofenac can reduce both Compound I and Compound II, with rate constants of respectively 6.0×10^5 M^{-1} sec^{-1} and 97.5 M^{-1} sec^{-1}. The nett accumulation of Compound II would explain the inhibition of the chlorinating activity of myeloperoxidase, since less Compound I is available for chloride ions to form HOCl. Presumably a series of reactions takes place, culminating in the formation of an orange-coloured product. The initial water soluble product has a strong absorbance peak at 452 nm. We determined the structure of the final orange precipitate which is no longer soluble in water.

The analysis of the molecular structure of this orange-coloured oxidation product has been carried out by Fast Atom Bombardment (FAB) mass spectrometry [23, 24]. From these data (Fig. 4) it is proposed that the oxidation product is dihydroxyazobenzene, with a molecular weight of 214 daltons. Further support has been obtained from the very similar (+) and (−)FAB spectra of the isolated product (Fig. 4A and B) compared to those of the commercially available *ortho,ortho'*-dihydroxyazobenzene (Fig. 5A and B). From the so-called FAB/MIKE and FAB/MIKE/CA spectra (Fig. 6) conclusive evidence has been provided that the isolated oxidation product is a dihydroxyazobenzene. However, its hydroxyl groups are not in the *ortho,ortho'*-positions, as can be concluded from the

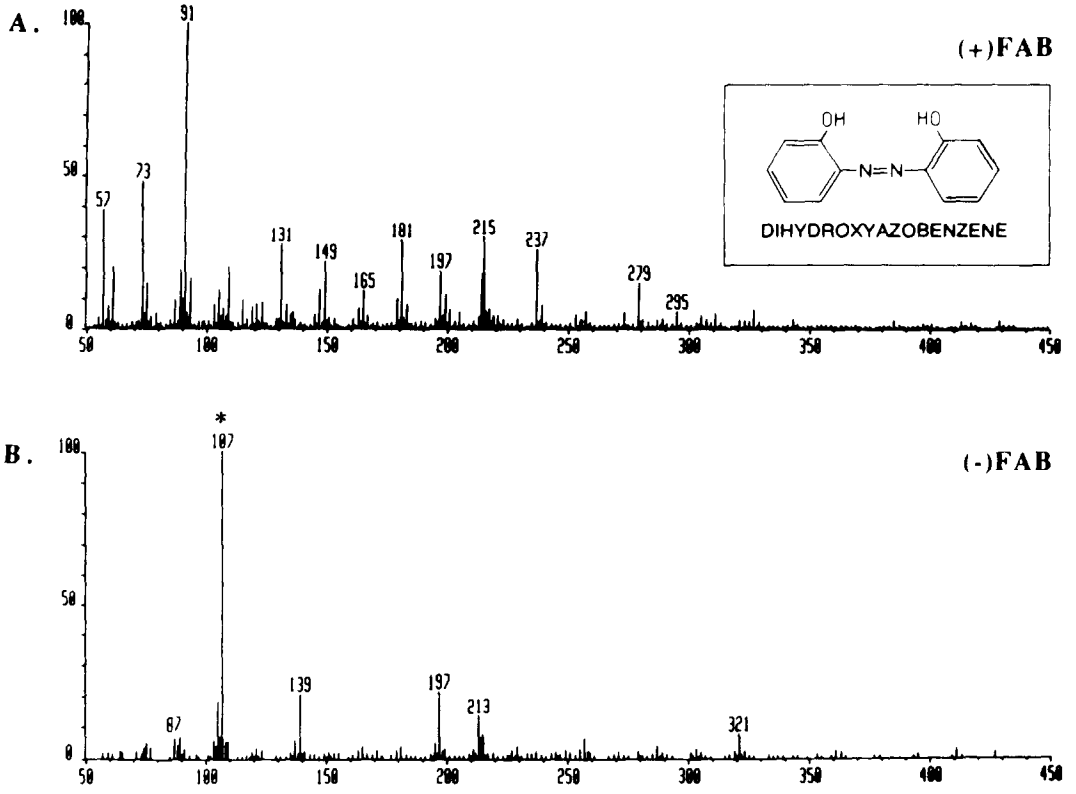

Fig. 5. (+) and (−)FAB mass spectra of *ortho,ortho'*-dihydroxyazobenzene dissolved in thioglycerol as matrix. Peaks denoted by an asterisk are due to ions originating from the matrix used.

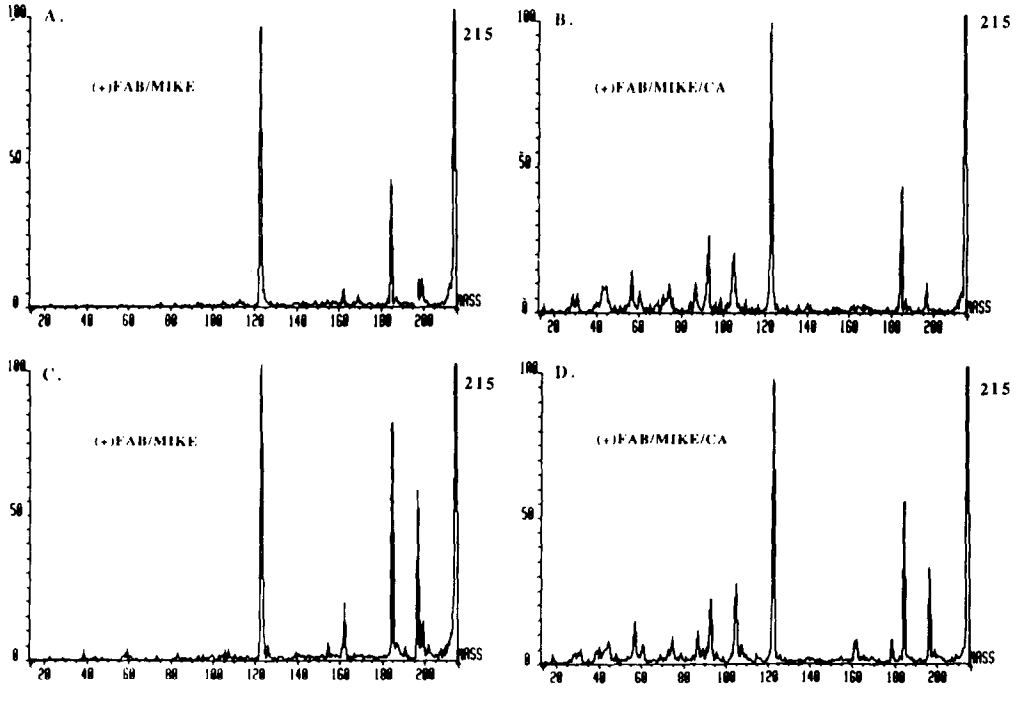

Fig. 6. (+)FAB/MIKE and FAB/MIKE/CA mass spectra of the orange-coloured oxidation product of diclofenac (A and B) and *ortho,ortho'*-dihydroxyazobenzene (C and D).

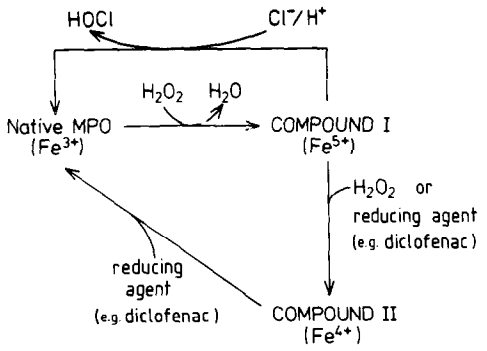

Fig. 7. A schematic representation of possible reactions of myeloperoxidase with H_2O_2, chloride ions and/or reducing agent(s).

much lower m/z 197 = $(M + H - H_2O)^+$ ion peak, as compared to that from the reference compound *ortho,ortho'*-dihydroxyazobenzene (see Fig. 6A–D).

The chemical reaction pathway which finally leads to the formation of dihydroxyazobenzene from diclofenac is as yet unknown. Concerning enzymatic formation of azobenzenes it is known that substituted aniline compounds can be converted to substituted azobenzenes by peroxidases [35]. However, an azo compound, or more precisely dihydroxyazobenzene, is not known as a diclofenac metabolite. Only hydroxylated metabolites of diclofenac, with hydroxylations at various positions of the phenyl rings, have been found in man and in animals receiving the drug [9, 10, 36]. It is still unclear whether dihydroxyazobenzene or other products of the myeloperoxidase catalysed oxidation of diclofenac are produced *in vivo* after administration of the drug, where the plasma concentration is in the 1 to 10 μM range [10]. Nevertheless, azo compounds and other derivates of arylalkanoic agents are well recognized causes of hepatotoxicity [37]. Therefore, it may well be that metabolites of diclofenac resulting from the oxidation of the drug catalysed by myeloperoxidase, are the cause of the rare hepatotoxic side effects found in some patients receiving diclofenac [11–13, 38].

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