Electrochemical Generation and Interaction Study of the Nitro Radical Anion from Nimesulide

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INTRODUCTION

Nimesulide, N-(4-nitro-2-phenoxyphenyl) methanesulfonamide, is a nonsteroidal anti-inflammatory drug with analgesic and antipyretic activity (1). Nimesulide exerts its actions by means of a series of mechanisms, including the selective inhibition of the prostaglandin synthesis, inhibitory activity on superoxide anion production from polimorfonuclears leukocytes, platelet aggregant factor synthesis inhibition and hypochlorous acid trapping (1,2).

The redox behavior of this drug is a relevant matter in order to clarify the metabolism or action mechanism. There is only one paper related to the electrochemistry of nimesulide showing that nimesulide was electrooxidable and electroreducible in a wide range of pH (3). The electroreducible group was the nitro aromatic moiety. Apparently, from the data reported in the literature, there is no proof that the nitro group reduction is directly involved in the pharmacological action.

Biotransformation pathways of nitroaromatic compounds are believed to result from nitroreductases that have the ability to use nitro as either one- or two-electron acceptors. One-electron acceptance by the nitro compounds results in the production of the nitro radical anion. This nitro radical anion becomes one of the most aggressive species in biological systems because of its reaction on endogenous molecules (DNA bases) and its well-known catalytic ability to transfer one electron to molecular oxygen with superoxide anion formation (4).

The aim of this study is to determine the feasibility of nitro radical anion formation from nimesulide through cyclic voltammetry. In addition, we report the reactivity of the nimesulide nitro radical anion with relevant biological targets. The reactivities were quantitatively assessed using cyclic voltammetry and a quantitative procedure to calculate the interaction constants between the free radicals and the xeno/endobiotics are also provided.

EXPERIMENTAL

Reagents and Solutions

Nimesulide (100% chromatographically pure) was obtained from Pharma Investi Laboratory, Santiago, Chile. Dimethylformamide (DMF) and ethanol were spectroscopic grade from Merck and were used without previous purification procedures. Reduced glutathione (GSH), cysteamine, uracil and adenine were obtained from Aldrich.

Trisodium citrate buffer (0.015 M) and Britton Robinson buffer (0.04 M) with different ethanol contents, as a protic medium, were used. Trisodium citrate buffer (0.015 M) with different DMF contents, as a mixed media, was used. The ionic strength was kept constant at 0.3 M KCl. Some solutions, in mixed media, also contain tetrabutylammonium iodide as supporting electrolyte.

Apparatus and Methods

Electrochemical measurements were carried out using an Inelecsa assembly similar to the previously described (10). A Metrohm hanging mercury drop electrode (HMDE) was used as the working electrode and the counter electrode was a platinum wire. All potentials were measured against a silver-silver chloride reference electrode. All cyclic voltammograms were recorded at a constant temperature of 25°C and the solutions were purged with pure nitrogen for ten minutes before the voltammetric runs. The return-to-forward peak current ratio, $i_{p,a}/i_{p,c}$, for the reversible first-electron transfer (the $ArNO_2/ArNO_2^-$ couple) was measured for each cyclic voltammogram according to the procedure described by Nicholson (11). The sweep rate was varied between 0.1 to 10 V/s. Experiments at sweep rate constant were generally done at 1V/s.

RESULTS AND DISCUSSION

Nimesulide (Nime-NO_2) is reducible at the mercury electrode due to the well-known ability of nitro aromatic group in accepting electrons from the electrode (7). The nimesulide reduction process is strongly dependent on the nature of the media, following different reduction behavior. On the other hand, our interest is focused to found the electrochemical and chemical conditions to detect, by cyclic voltammetry, a nimesulide nitro radical anion (Nime-NO_2^-). This radical would be formed as an intermediate in the reduction pathway of the drug.

In protic medium, Britton Robinson buffer, 0.3 M KCl/ethanol: 70/30, the Nime-NO_2^- is observed only in strong alkaline media. In Figure 1a, the effect of changing the pH from pH 9 to pH 12 is observed. At pH 9, a typical cyclic voltammogram of nitroaromatics in protic media with only one irreversible cathodic peak ($E_{p,c} = -690$ mV) corresponding to the four-electron reduction of the nitro group to form the hydroxylamine nimesulide derivative (Nime-NHOH), can be observed. The anodic peak observed at -210 mV is due to the further oxidation of the Nime-NHOH derivative to the nitroso nimesulide derivative (Nime-NO). At pH 12 the situation is totally different, wherein three cathodic peaks ($E^{I}_{p,c} = -820$ mV, $E^{II}_{p,c} = -1040$ mV and $E^{III}_{p,c} = -1250$ mV) and two anodic ones ($E^{I}_{p,a} = -780$ mV and $E^{III}_{p,a} = -410$ mV) are observed (Fig. 1a). In the above condition, the Nime-NO_2^-/Nime-NO_2 couple ($E^{I}_{p,a}$, $E^{I}_{p,c}$) was observed but not well resolved. The second cathodic peak ($E^{II}_{p,c}$) corresponds to the further one-electron reduction of the Nime-NO_2^- to form the corresponding dianion (8) and the last cathodic peak ($E^{III}_{p,c}$) involves the two-electron reduction of the dianion to form the corresponding Nime-NHOH derivative (8). The anodic peak ($E^{III}_{p,a}$) corresponds to

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the further oxidation of the Nime-NHOH derivative formed in the cathodic sweep. The above assignments are well summarized by the following:

$$\text{Nime-NO}_2 + e^- \leftrightarrows \text{Nime-NO}_2^-$$

$$E^{I}_{p,c} = -820, \quad E^{I}_{p,a} = -780 \text{ mV} \qquad (1)$$

$$\text{Nime-NO}_2^- + e^- \rightarrow \text{Nime-NO}_2^{2-} \; E^{II}_{p,c} = -1040 \text{ mV} \qquad (2)$$

$$\text{Nime-NO}_2^{2-} + 2e^- + 2\,H_2O \rightarrow \text{Nime-NHO}^- + 3\,OH^-$$

$$E^{III}_{p,c} = -1250 \text{ mV} \qquad (3)$$

$$\text{Nime-NHO}^- + OH^- \rightarrow \text{Nime-NO} + 2e^- + H_2O$$

$$E^{III}_{p,a} = -410 \text{ mV} \qquad (4)$$

We tried with several other buffer conditions and it was not possible to obtain better resolution for the couple in protic media. The above results are in accord with the well known fact that the protic character of the media hinder the stabilization of the nitro radical anion by protonation of the free radical (8). Consequently, the use of a more aprotic solvent would obtain a better resolution for the couple. In fact, the use of a mixed media containing citrate buffer, 0.3 M KCl/DMF was examined for the influence of different DMF percentages. When the mixed media contains 60% DMF the voltammogram changes to (a) a well-resolved couple ($E^{I}_{p,c} = -950$ mV, $E^{I}_{p,a} = -880$ mV) corresponding to the one-electron reduction of Nime-NO_2 to the Nime-NO_2^- derivative, (b) an irreversible cathodic peak ($E^{II}_{p,c} = -1450$ mV) corresponding to the further three-electron reduction of the Nime-NO_2^- derivative to the hydroxylamine derivative (Fig. 1b). Therefore, in this condition, we obtained a good resolution for the couple in order to obtain the kinetic parameters. Results show that as the scan rate increased, the $I_{p,a}/I_{p,c}$ ratio increased towards unity, typical behavior for an irreversible chemical reaction following a charge-transfer step, i.e. the EC process (9). Furthermore, an increase in the nimesulide concentration resulted in a decreased $I_{p,a}/I_{p,c}$ value and the cathodic peak potential depends on nimesulide concentrations with a $\delta E_{p,c}/\delta \log C$ value of 20 mV. These results are in agreement with a second order for the chemical step. According to previous works (10) this second order for the kinetic of the nitro radical anion decay corresponds to a dismutation reaction. Consequently this EC_2 type behavior can be well explained by the following equations:

$$\text{Nime-NO}_2 + e^- \rightleftharpoons \text{Nime-NO}_2^- \qquad (1)$$

$$2\,\text{Nime-NO}_2^- + 2H^+ \rightarrow \text{Nime-NO}_2 + \text{Nime-NO} + H_2O \qquad (5)$$

The decay constant for the nimesulide free radical can be calculated according to the procedure described by Olmstead et al. (11) for a second order kinetic. A k_2 value of 240.1 $Lmol^{-1}\,s^{-1}$ was obtained for the decay. From this k_2 value, a half-life time, $t_{1/2}$, of 0.83 s, for an electrochemically generated radical equivalent to $5*10^{-3}$ M nimesulide concentration was calculated. The results show that in mixed media a stable free radical is generated. The description of this free radical from nimesulide is a new experimental fact providing *in vitro* evidences that could justify the investigation of new metabolic routes (reductive pathways) for this compound. The calculation of the decay

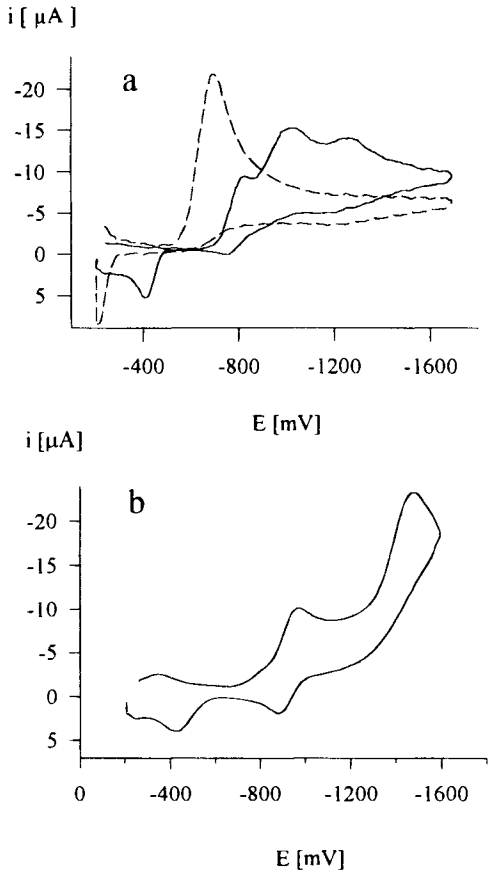

Fig. 1. Cyclic voltammograms of 1 mM nimesulide in (a) Britton Robinson buffer, 0.3 M KCl/ethanol: 70/30 at pH 9.0 (dashed line) and pH 12.0 (full line), and (b) Citrate buffer, 0.3 M KCl/DMF: 40/60.

constant was carried out at alkaline pH to obtain better resolution in the first sweep voltammogram, what is necessary for the appropriate quantification. However, also it is possible to generate the free radical under stationary conditions of multiple sweeps at the physiologic pH of 7.4.

One of the main advantages of the cyclic voltammetry in the study of free radicals is that this radical can be generated and then studying its reactivity *in situ*. In this case we study the reactivity of the radical anion of nimesulide electrochemically generated with some endobiotics or xenobiotics compounds like adenine, uracil, cysteamine and reduced glutathione. All the previously mentioned compounds didn't show electrochemical signals in the potential range of between −600 and −1100 mV, thus allowing studying the influence of the endo/xenobiotics on the free radical couple. When adding growing concentrations of the endo/xenobiotic compounds to the nimesulide solution, a significant effect was observed on the voltammetric peak of the radical anion. Specifically, a remarkable decrease of the peak corresponding to the oxidation of the radical, indicating an interaction between the free radical and the endo/xenobiotic, is observed. This effect can be well quantified by the change in the current ratio $I_{p,a}/I_{p,c}$, at all the experimental sweep rates. In the 3-D graphics of the Fig. 2, the decrease of the current ratio with the increase of the endo/xenobiotics concentration can be observed. This same effect was observed at all the sweep rates. All the curves maintain the same form wherein the current

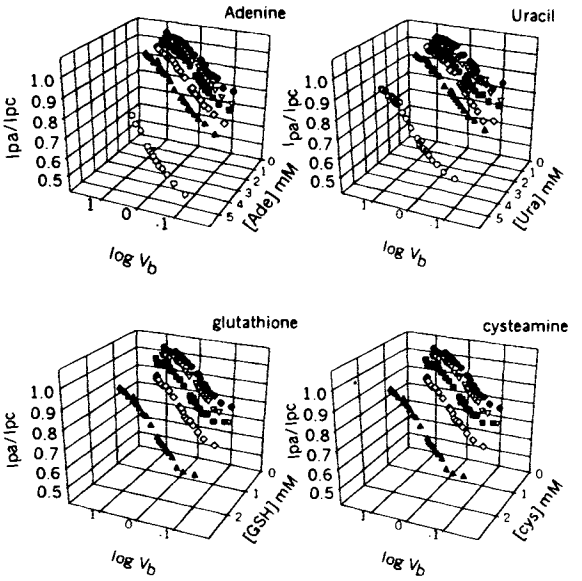

Fig. 2. Decrease of the current ratio $I_{p,a}/I_{p,c}$ with the increase of the endo/xenobiotic concentration at different sweep rates.

ratio increases toward the unit with the increase of the sweep rate, indicating that the EC type mechanism remains. When we add the endo/xenobiotic compound, an additional reaction that competes with the natural decay of the radical will appear.

$$\text{Nime-}NO_2^- + \text{endo or xenobiotic} \rightarrow \text{Product} \quad k_i \qquad (6)$$

According to a previously developed methodology (12), all the interaction constants between the endo-xenobiotics with the radical anion of nimesulide were calculated (Table 1). In all the cases the k_i values are significantly higher than the value of the natural decay constant (k_2). In accordance with the obtained results, glutathione and adenine exhibits a high k_i value acting as a scavenger for this radical. Furthermore, from the k_i values corresponding to the interaction among the bases of the nucleic acids (adenine and uracil) and the radical anion it is possible to think in a possible toxic effect of the Nime-NO_2^-.

In Table 2 the electrochemical characteristics and reactivities towards endobiotics for the one-electron reduction products from different pharmaceutical relevant nitrocompounds are shown. From this table is apparent that the nitro anion electrochemically generated from these compounds behaves similarly, i.e. the easiness to undergo reduction reflecting on the cathodic peak potential does not exhibit significant differences, excepting nitrofurantoin, nifedipine and nisoldipine. This latter drug shows the most cathodic peak potential (−1118 mV), therefore the formation of the radical is more difficult. In spite of this difficulty, the aromatic nitro group reduction of nisoldipine as a minor biotransformation pathway in rat, dog, monkey and man has been confirmed (13). Furthermore, *in vivo* nitroreduction pathways for nimodipine and nicardipine are also reported (14). The above results indicate that *in vivo* nitroreduction can happen in compounds whose reduction potentials *in vitro* are as high as that shown in the Table II. In consequence, it seems reasonable to consider the nimesulide nitroreduction like a probable biodegradation route. Nimesulide nitroreduction has not been described in the literature, probably due to the *in vivo* radical anion generated is autoxidized rapidly in the presence of molecular oxygen to regenerate the parent compound and superoxide anion generation, thus making impossible the isolation of nitro-reduced metabolites. For example, is very well known that nitrofurantoin activation may proceed via one-electron reduction of the nitro group (15), however, the detection of the nitrofurantoin reduction products (i.e. amine and nitroso derivatives) has been extremely difficult due to autoxidation of the radical as was above discussed. On the other hand, (Table 2), it can be concluded that it doesn't exist major differences in the stability (k_2 value) of all nitrocompounds. In conclusion, both the substituent effects, i.e. electronic effects (environment) and steric effect on the free radical did not affect the stability of the tested nitrobenzenoids, such as nimesulide. Results for the reactivity toward the tested endobiotics (GSH, adenine) show that the nimesulide nitro anion presented a weaker reactivity than the others radicals which could be considered an advantage in chronic treatment, where the appearance of toxicity is more possible.

Table 1. Interaction Constants (k_i) for the Reaction Between the Nitro Free Radical from Nimesulide and Endo-Xenobiotics and Its Comparison with the Natural Decay (k_2) of the Free Radical

Endo-xenobiotics	k_i [M^{-1} s^{-1}] × 10^{-3}	k_i/k_2
Adenine	2.91	12.1
Glutathione	2.32	9.7
Uracil	1.14	4.8
Cysteamine	0.91	3.8

Table 2. Comparative Electrochemical Characteristics and Reactivity of the One-Electron Reduction Product of Different Pharmacological Relevant Nitrocompounds in Mixed Media at pH 9.0[a]

	−Epc [mV]	k_2 [$M^{-1}s^{-1}$]	$k_i \times 10^{-3}$	
			GSH	Adenine
Nimesulide	−820	240	2.32	2.91
Nisoldipine[b]	−1118	283	16.45	9.51
Nifedipine[b]	−1089	265	17.10	12.52
Nimodipine[b]	−877	294	8.14	1.74
Nitendipine[b]	−867	299	9.52	7.15
Nicardipine[b]	−856	284	8.54	3.44
Nitrofurantoin[c]	−600	201	—	2.12

[a] Mixed media: 0.015 aqueous citrate buffer/DMF: 40/60, 0.1 M TBAI, 0.3 M KCl.
[b] Ref. 16.
[c] Ref. 17.

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