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Electrochemical Oxidation of Tamoxifen Revisited

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Tamoxifen is a selective estrogen receptor modulator that is used as an adjuvant and/or chemotherapeutic agent for the treatment of all stages of hormone-dependent breast cancer. Currently there is a deep interest in the study of tamoxifen biotransformation and identification of metabolites since they can significantly contribute to the overall pharmacological or adverse effects of the drug. Accordingly, the study of the electrochemical behavior of tamoxifen in aqueous solution is reported. To clarify the occurring oxidative process and to assess the influence of the functional groups on the oxidation mechanism, the voltammetric assessment was extended to the study of tamoxifen's analogues (*E*)-tamoxifen and dihydrotamoxifen, and to its main phase I oxidative metabolite, *N*-desmethyl tamoxifen. The data found shows that the oxidative processes occurring in tamoxifen are essentially related with the two chemical moieties present in the molecule: the substituted aromatic nucleus and the tertiary amine group. Moreover, the results obtained suggest that the ethylenic linkage is not critical for tamoxifen's oxidation although it could play an important role in the course of the oxidation process. These results could contribute to highlight some remaining questions regarding tamoxifen's metabolic behavior and to the development of new analytical strategies, based on electrochemical approaches.

Keywords: Tamoxifen, *N*-desmethyltamoxifen, dihydrotamoxifen, voltammetry, oxidation.

1. INTRODUCTION

The antiestrogen tamoxifen, [TAM, (*Z*)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-N,N-dimethylethanamine], is the drug most often used for the long-term treatment of early breast cancer [1, 2].

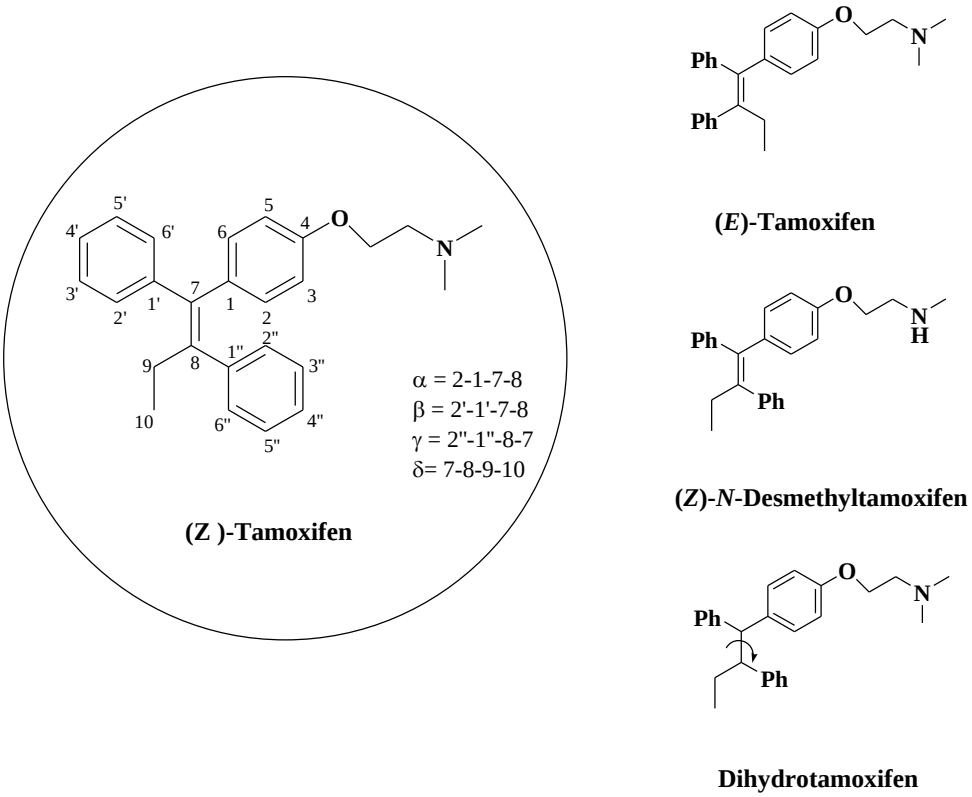
Despite recent advances in the use of aromatase inhibitors for the treatment of breast cancer disease, tamoxifen still remains the endocrine treatment of choice and has been used successfully by millions of women [2]. The widespread use of this drug has been attributed to a 25 to 30 percent reduction in breast cancer deaths in the USA and UK respectively in 2000, compared with the late 1980s [3]. Tamoxifen may also act as a chemopreventive agent in healthy 'at risk' women, reducing the overall risk of invasive breast cancer [4]. In addition to antitumour activity, tamoxifen exerts beneficial effects on osteoporosis, reduces the incidence of cardiovascular diseases and lowers circulating serum cholesterol [5, 6].

Despite tamoxifen therapeutic value, various adverse effects have been recognized related with its use. Among them, the development of uterine and endometrial cancer and venous thrombotic events are the most critical [7].

The metabolism of tamoxifen in humans or rodents has been described to encompass oxidation and bioconjugation pathways. Tamoxifen is bioactivated by cytochrome P450 enzymes mainly through demethylation and hydroxylation reactions, and by flavin-containing monooxygenase producing a *N*-oxygenated metabolite. The major phase I tamoxifen metabolites found in human plasma are: hydroxytamoxifen, *N*-desmethyl tamoxifen (*N*-demTAM), tamoxifen *N*-oxide and 4-hydroxytamoxifen [7-10]. Demethylation of the aminoethoxy side chain of TAM to *N*-demTAM appears to be the main route of TAM metabolism [4, 7, 11]. Although the adverse effects of tamoxifen have been recognized to be related with its hormonal properties, there is currently a deep interest in the study of its biotransformation and identification of metabolites since they could act as chemical carcinogens.

The polarographic behaviour of tamoxifen and other triphenylethene derivatives have been studied in the end of 80's in view of its quantitative determination in pure form and in pharmaceutical tablets [12]. The anodic signal observed was attributed to a cyclization reaction to form the corresponding phenanthrene derivative [12]. A similar mechanistic pathway was proposed for the oxidation of tamoxifen at various types of electrodes [13, 14] and follows the generally accepted oxidative mechanism proposed for phenylethenes derivatives [15, 16]. Although the occurrence of the proposed cyclization reaction is plausible there are other reactive functional groups at tamoxifen's molecular structure (Scheme 1) that can also undergo oxidative reactions. In fact, molecules containing functional groups such as oxygen ether linked to an aromatic nucleus and/or aliphatic tertiary amine have been extensively described in literature as electroactive moieties [17-20]. In addition, the anodic waves found for the mentioned functions occur at potentials similar to those found for tamoxifen [17-20]. Furthermore, considering the recognized likeness between electrochemical and biological reactions it can be assumed that the oxidation mechanisms taking place at the electrode and in the body can share similar principles. In fact, *N*-desmethyl tamoxifen (*N*-demTAM) is the major TAM oxidative metabolite that is obtained in a process that involves the *N*-desalkylation of its aminoethoxy side chain.

In order to clarify the electrochemical oxidation mechanism of tamoxifen, a comprehensive voltammetric study of tamoxifen, (*E*)-tamoxifen, *N*-desmethyl tamoxifen and dihydrotamoxifen (Scheme 1) was accomplished. Furthermore, this study could contribute to highlight the structure-affinity or -activity relationship for the main targets that are primarily associated with tamoxifen's structural features.



Scheme 1. Chemical structures of tamoxifen, (*E*)-tamoxifen, dihydrotamoxifen and *N*-desmethyl tamoxifen.

2. EXPERIMENTAL

2.1. Chemicals

Tamoxifen citrate, (*E*)-tamoxifen and *N*-desmethyl tamoxifen were kindly provided by AstraZeneca (Lisbon, Portugal).

All other reagents and solvents were *pro analysis* grade and were acquired from Merck (Lisbon, Portugal) and used without additional purification. Deionised water (conductivity < 0.1 μS cm^{-1}) was used throughout all the experiments. Buffer solutions employed for voltammetric determinations were 0.2 M in the pH range 1.2-12.2.

2.2. Synthesis

^{1}H NMR spectra were recorded on a Brüker DPX (250 MHz), using TMS as internal standard (chemical shifts as δ in ppm, J in Hz). Mass spectra (EI, 70eV)) were obtained using a Hewlett Packard 5988A spectrometer; the data are reported as *m/z* (% of relative intensity of the most important fragments). HPLC was performed using a semipreparative column (Partisil Si, 5μm, Whatman). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck

60 F254, 0.25 mm). The spots were visualised under UV detection (254 and 366 nm) and iodine vapour.

2.2.1. Synthesis of Dihydrotamoxifen (2-[4-(1R,2R)-1,2-di(phenyl)butyl]phenoxy]-N,N-dimethylethanamine)

(2-[4-(1R,2R)-1,2-di(phenyl)butyl]phenoxy]-*N*,*N*-dimethylethanamine). To a solution of tamoxifen (0.2 g, 0.538 mmol) in EtOH (20 mL) was added 10% palladium on charcoal (25 mg). The mixture was shaken under 55 psi of H_2 for 10 h. The mixture was filtered on Celite and the filtrate was concentrated in vacuum to give a yellow oil. This was purified by HPLC using $CHCl_3$/*i*PrOH (9:1) as eluent to give dihydrotamoxifen (123 mg, 61%) as yellow oil. The oil was identified as dihydrotamoxifen.

^{1}H NMR ($CDCl_3$): 0.64 (t, *J* = 7.3, 3H, CH_3), 1.43 (m, 1H), 1.65 (m, 1H), 1.78 (m, 2H), 2.30 (s, 6H, NMe_2), 2.69 (t, *J* = 5.7, 2H, NCH_2), 3.25 (dt, *J* = 3.2, 11.0, 1H, CH_3CH_2CH), 4.03 (t, *J* = 5.7, 2H CH_2O), 4.06 (d, *J* = 11, 1H, $CHAr_2$), 6.88 (m, 3H), 7.05 (m, 9H), 7.28 (m, 2H).

MS-EI (*m/z*): 373 (M^+), 254 (19), 72 (100).

2.3. Electrochemical studies

Voltammetric studies were performed using an Autolab PGSTAT 12 potentiostat/galvanostat (Metrohm AutolabEco-Chemie, Netherlands) and a one-compartment glass electrochemical cell. Voltammetric curves were recorded at room temperature using a three-electrode system. A glassy carbon working electrode (GCE) (*d* = 2 mm), a platinum wire counter electrode and an Ag/AgCl saturated KCl reference electrode were used. The working electrode was polished manually with aqueous slurry of alumina powder (BDH) on a microcloth pad and rinsed with water before use.

A Crison pH-meter with glass electrode was used for the pH measurements (Crison, Spain).

2.4. Ionization Potentials (IP)

The Ionization Potentials (IP) were determined as the energy difference between the corresponding neutral and ionized compounds, as presented in Eq. (1):

$$IP = E_{compound} - E_{ionized\ compound} \quad (1)$$

where $E_{compound}$ stands for the gas phase electronic energy of the optimized neutral compound and $E_{ionized\ compound}$ for the gas phase electronic energy of the optimized geometry of the ionized compound.

Further, the electronic energies of the neutral and ionized compounds were determined through Density Functional Theory (DFT) calculations using the B3LYP hybrid functional as implemented in

Gaussian03 computer code [21]. This hybrid functional includes Hartree Fock (HF) and DFT exchange-correlation terms as it was proposed and parameterized by Becke [22, 23], being the DFT terms from the gradient-corrected LYP density functional [24, 25]. On the other hand, the compounds were described using the atom-centered Gaussian basis set 6-31++G** for all the atoms. During the calculations, the structures were fully relaxed according with the Berny algorithm.

3. RESULTS AND DISCUSSION

Biotransformation is the enzyme - catalyzed process of chemically modifying drugs and other xenobiotics to increase their water solubility in order to facilitate their elimination from the body. Biotransformation phase I reactions are functionalization reactions that introduce polar chemical moieties either by inserting new polar functional groups or by interchanging or unmasking existing functional groups via oxidation, reduction and hydrolytic reactions.

Electrochemical studies could furnish an enormous amount of evidence regarding the mechanisms of biological electron-transfer processes. In fact, electrochemical simulation of oxidative phase I reactions are of considerable interest since the information about oxidatively labile sites in a molecule is easily accessible. Although metabolites are chemically distinct from the parent drug, they have jointly structural similarities that might help to understand the oxidative profile of the precursors. With the aim of clarifying the oxidative mechanism of tamoxifen, several metabolites and specific congeners were studied using different voltammetric techniques.

3.1. Chemistry

The hydrogenation of TAM carbon-carbon double bond was accomplished by using hydrogen and heterogeneous transition metal catalysis (Pd/C). After the preliminary work-up the hydrogenated compound was purified using semi-preparative HPLC. After NMR analysis, and in accordance with the literature [26], high values were observed for the coupling constants between the benzylic hydrogen atoms a fact that indicate that these atoms are in an antiperiplanar orientation.

3.2. Voltammetric studies

Tamoxifen [(*Z*)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-N,N-dimethylethanamine] is the *Z* isomer of a triphenylethylene derivative (Scheme 1). The drug is prescribed to humans in the form of a citrate salt. Tamoxifen citrate is an orally administered non-steroidal antiestrogen agent used to treat estrogen-dependent metastatic breast cancer [7]. On the other hand, the tamoxifen (*E*)-isomer has estrogenic activity and stimulates the proliferation of hormone-responsive breast cancer cells.

To understand tamoxifen oxidation pathways and to assess the influence of pH on its oxidative profile, a comprehensive study on the electrochemical properties of both isomers was therefore carried

out, in a broad pH range (1.2-12.2), at a glassy carbon electrode, using different voltammetric techniques.

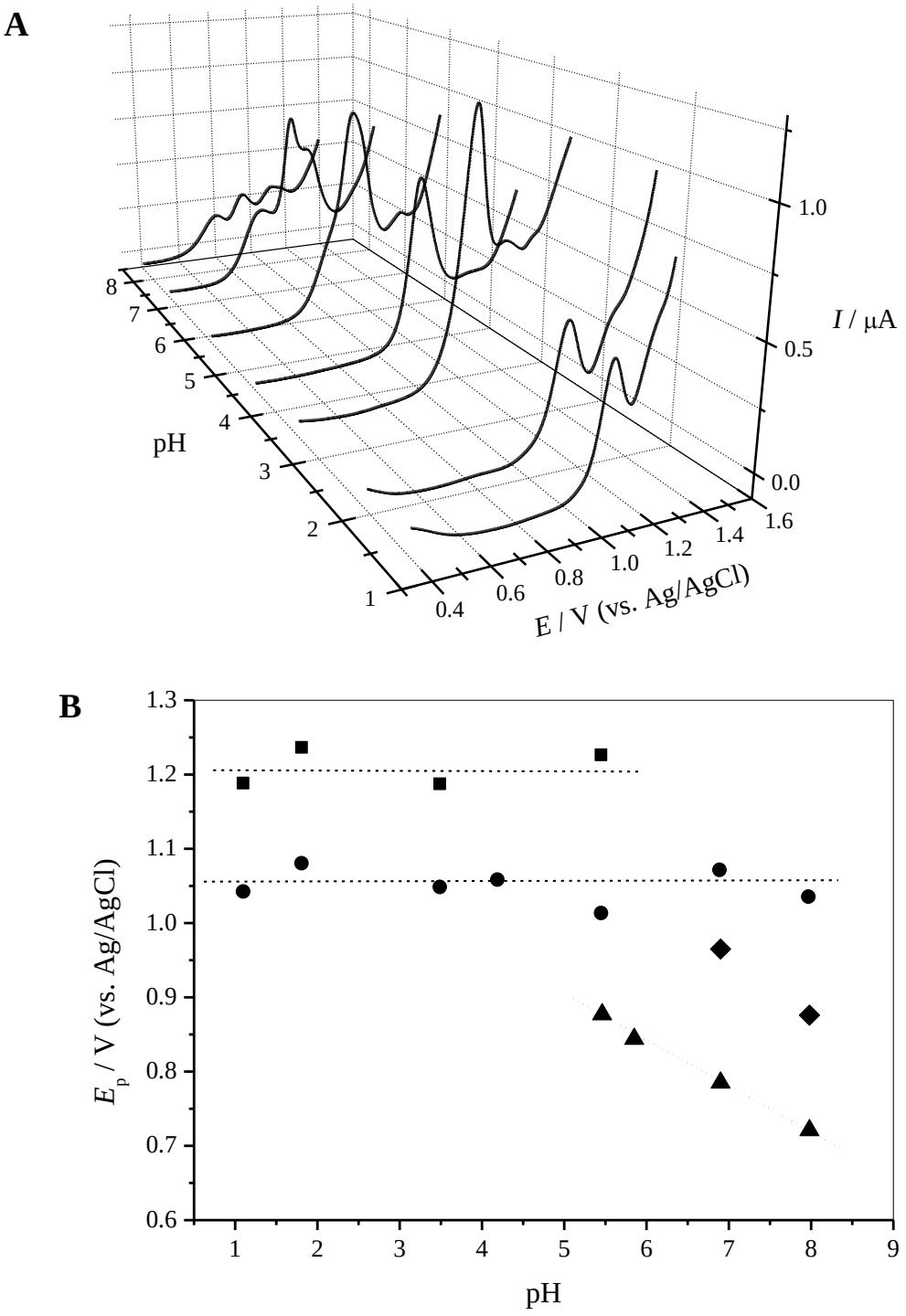
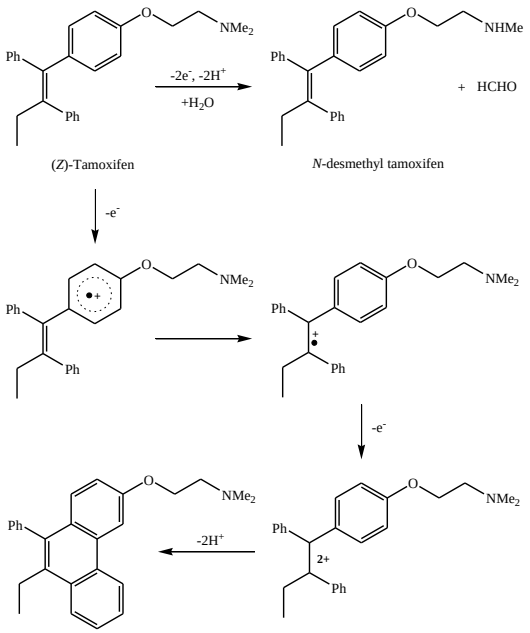


Figure 1. A) 3D plot and B) plots of E_p (filled symbols) and I_p (open symbols) vs. pH from DP voltammograms of 0.1 mM solutions of tamoxifen in different buffer electrolytes as a function of pH. (●) first peak, (■) second peak; (▲) third peak and (♦) fourth peak (error bars are constructed for five measurements). Scan rate 5 mV s^{-1}.

Differential pulse (DP) voltammetry of tamoxifen showed the first anodic peak, at E_p = +1.04 V, starting at pH 1, (Fig. 1A) corresponding to the loss of a π electron with the subsequent formation of a cation radical (Scheme 2). From the E_p-pH plot (Fig. 1B) one can conclude that the electrode process is pH-independent over the whole studied pH range. It has been established that the first anodic charge transfer from an aromatic compound and other compounds with extensively delocalised π electrons, usually involves the removal of an electron from the highest energy molecular orbital occupied. Previous work on similar alkoxybenzenes and literature data indicate that the oxidation process proceeds at the aromatic nucleus, through an electron transfer, via the formation of a cation radical intermediate that could undergo further subsequent reactions [15, 18, 20, 27]. In fact, a second poorly resolved wave, at E_p = +1.19 V starts appearing also at pH 1 (Fig. 1A), and is a consequence of further oxidation of the product formed at the first peak, being the most likely a cyclization reaction to form the corresponding phenanthrene derivative [15, 27] (Scheme 2). The E_p-pH plot (Fig. 1B) shows that this electrode process is pH-independent over the whole pH range. Further discussion on the appearance of these anodic peaks will be presented below.

Starting at pH 5 a well-defined shoulder prior to the main peak is observed, E_p = +0.88 V (Fig. 1A), corresponding to the oxidation of the tertiary amine group present in the tamoxifen's molecule (Scheme 1). The E_p-pH plot (Fig. 1B) shows that this electrode process is pH-dependent over the whole pH range; E_p decreases linearly with increasing pH. The slope of the dotted line, ca. 60 mV per pH unit (eq. 2), shows that the mechanism of this oxidation process, in aqueous media involves the same number of electrons and protons (Scheme 2).

$$E_p \text{ / V} = (1.20 \pm 0.05) \text{ / V} - (0.060 \pm 0.007) \text{ pH} \quad (R^2 = 0.998) \qquad (2)$$



Scheme 2. Proposed mechanism for the electrochemical oxidation of tamoxifen.

Another well defined shoulder is seen at pH 7 and above, E_p = +0.97 V (Fig. 1A), and occurs as a result of the oxidation of the secondary amine obtained from the oxidative process involving the tertiary amine group present in the tamoxifen's molecule (Scheme 1). Evidence for this comes from the voltammetric study of *N*-desmethyl tamoxifen and will be discussed later. Moreover, this pattern is consistent with the voltammetric behaviour described in the literature for other molecules containing tertiary aliphatic amines [18, 28, 29].

At pHs higher than 9, a significant decrease in the current magnitude of the oxidative waves was observed. This fact seems to be directly related with the ionization constant of tamoxifen (pKa = 8.85).

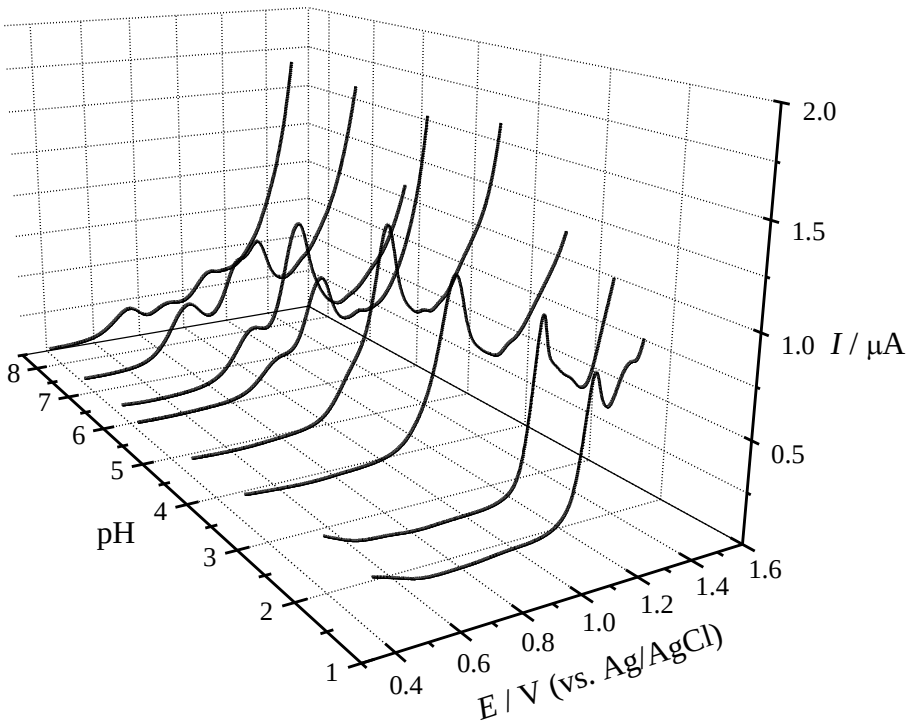


Figure 2. 3D plot from DP voltammograms of 0.1 mM solutions of (*E*)-tamoxifen in different buffer electrolytes as a function of pH. Scan rate 5 mV s^{-1}.

Cyclic voltammograms recorded at different pHs indicated the irreversibility of the tamoxifen's redox processes since no peaks have been observed in the cathodic branch. These data evidence that very fast subsequent chemical reactions of the species generated on the anodic oxidation are taking place and that their reduction will not occur in the time scale of the experiment. This behavior is well-documented for related compounds [19, 20, 27]. However, at high scan rates the first anodic wave can exhibit reversible character, as was demonstrated using ESR spectroscopy [15].

DP voltammetric data obtained of tamoxifen (*E*)-isomer suggests an analogous oxidative profile to that found for the (*Z*) isomer (Fig. 2). This behaviour is expected considering that both stereoisomers are very close in energy [30]. Moreover, the ionization potentials calculated for both molecules are quite comparable (Table 1).

Table 1. Structural and electronic parameters for tamoxifen, (*E*)-tamoxifen, *N*-desmethyl tamoxifen and dihydrotamoxifen.

Molecule	Torsional angle (degrees)*				C=C bond length (Å)	Vertical IP (eV)
	α	β	γ	δ[JG1]		
TAM	53.48	64.13	74.41	114.73	1.358	6.49
(*E*)-TAM	47.69	60.55	56.02	-115.48	1.362	6.57
N-desmethylTAM	48.52	60.53	56.41	-115.76	1.361	6.56
dihydroTAM	-41.00	74.23	124.37	-92.86	1.568	7.04

* Torsional angles are shown in Scheme 1

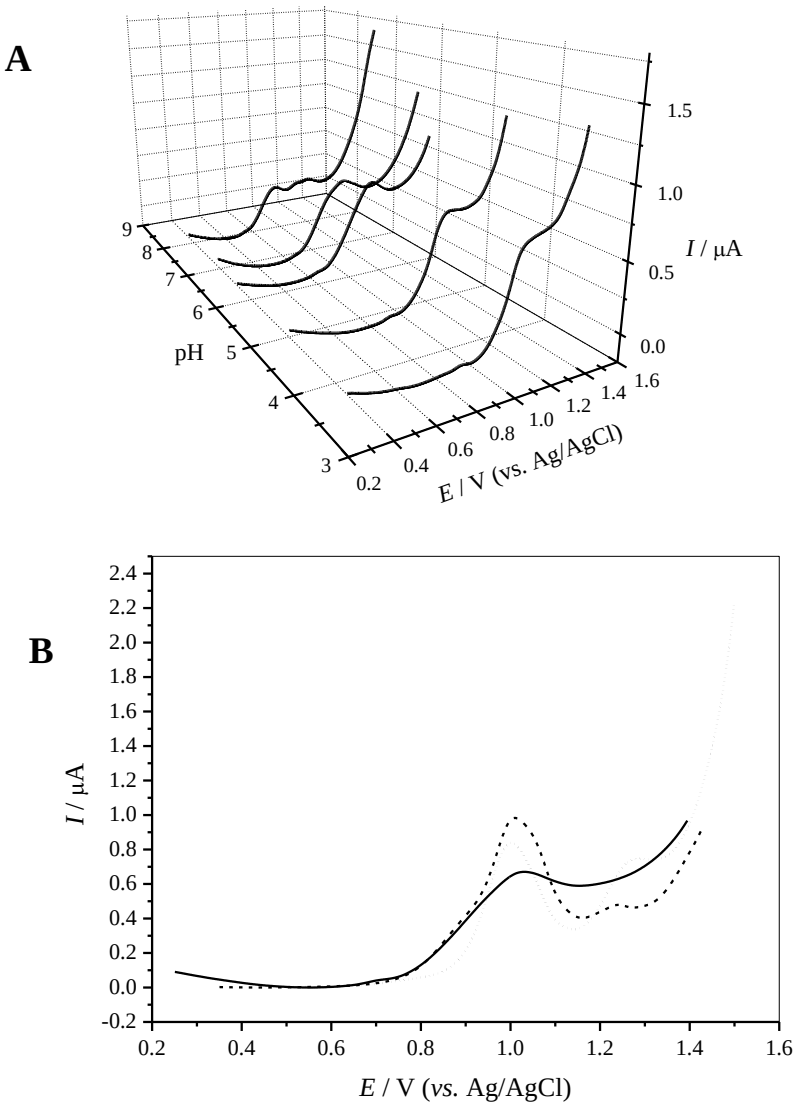


Figure 3. A) 3D plot from DP voltammograms of 0.1 mM solutions of dihydrotamoxifen in different buffer electrolytes as a function of pH. Scan rate 5 mV s^{-1}. B) DP voltammograms of 0.1 mM solutions of (▬) dihydrotamoxifen, (▪▪▪) tamoxifen and (•••) *N*-Desmethyl tamoxifen in pH 5 buffer electrolyte. Scan rate 5 mV s^{-1}.

To better comprehend the oxidative profile and to assess the influence of the ethylenic linkage on its oxidation mechanism, the voltammetric studies were extended to the analysis of a tamoxifen's analogue, dihydrotamoxifen, and one of its main Phase I metabolites, *N*-desmethyl tamoxifen.

Differential pulse voltammetry of dihydrotamoxifen showed a first anodic peak, E_p = +1.05 V, starting at pH 3 (Fig. 3A) corresponding to the loss of a π electron with the subsequent formation of a cation radical. The E_p-pH plot shows that the electrode process is pH-independent over the whole pH range. Although the hydrogenation of the ethylenic double bound in tamoxifen did not influence the energetic of the electron transfer process, as can be ascribed from the similarity of the anodic potentials, a significant drop in the intensity of cation radicals was attained, evidenced by the decrease of the voltammetric signal that corresponds to this oxidation step (Fig. 3A).

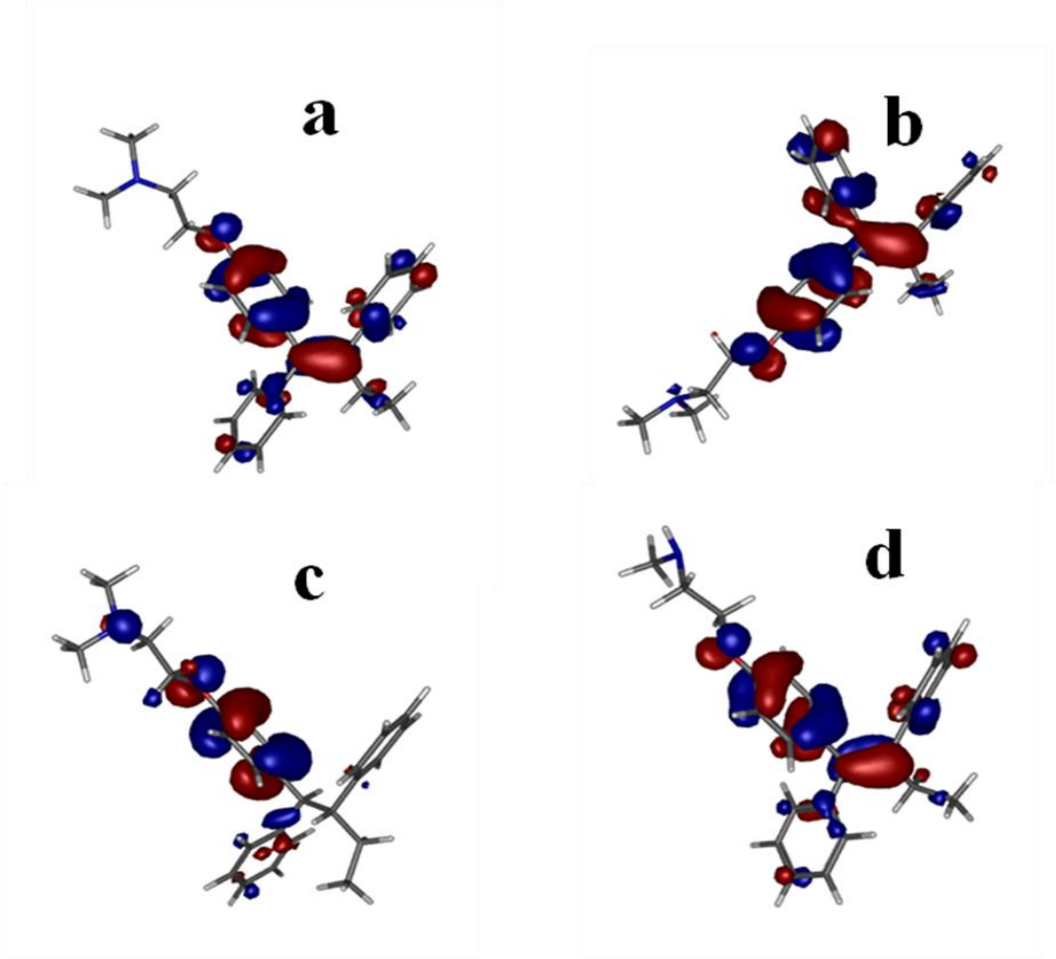


Figure 4. Representation of the Highest Occupied Molecular Orbital (HOMO) for a) tamoxifen, b) (*E*)-tamoxifen, c) dihydrotamoxifen and d) *N*-Desmethyl tamoxifen. Determined at the B3LYP/6-31++G** level of theory.

The absence of a concurrent wave, starting at pH 1, for dihydrotamoxifen (Fig. 3B) seems to support the assumption made for tamoxifen that a subsequent oxidation of the product formed at the first peak occur resulting in the formation of a phenanthrene derivative. In fact, a decrease in the stability of the generated cation radicals formed is expected, since the conjugation of the phenyl ring with the ethylenic double bond could not occur, leading to a decrease of their lifetime. Actually, the

main contributors to the HOMO of tamoxifen are p_z orbitals located at the ethylene bond system as well as p_x, p_y and p_z components of the para-substituted phenyl ring and accompanying 4-substituted oxygen (Fig. 4). This ring is rotated approximately 50° out of the ethylene bond plane, maintaining some electronic overlap with the double bond system (Table 1). The gathered results suggest that the presence of the ethylenic double bond is not crucial for tamoxifen's oxidative profile but plays an important role in the course of the oxidation mechanism (Scheme 2).

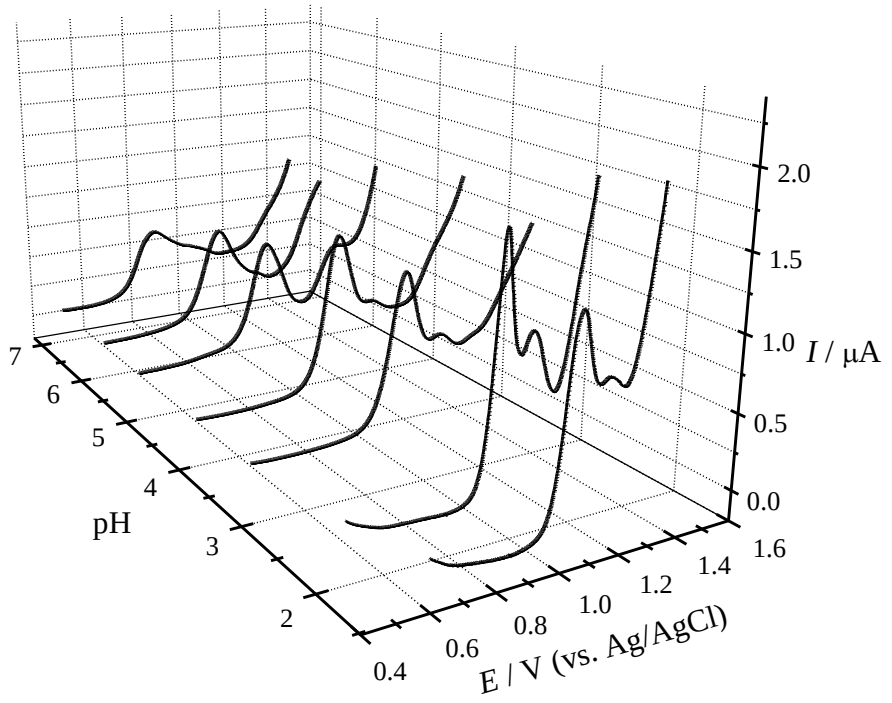


Figure 5. 3D plot from DP voltammograms of 0.1 mM solutions of *N*-Desmethyl tamoxifen in different buffer electrolytes as a function of pH. Scan rate 5 mV s^{-1}.

Starting at pH 5 a well-defined shoulder prior to the main peak is observed, E_p = +0.89 V (Fig. 3), corresponding to the oxidation of the tertiary amine group present in the dihydrotamoxifen's molecule (Scheme 1). Another well-defined shoulder is seen above pH 7, E_p = +0.94 V (Fig. 3A), and occurs as a result of the oxidation of the secondary amine formed by the oxidative process that involved the tertiary amine group (Scheme 2). Regarding the oxidation of the tertiary amine group a very close match was observed between tamoxifen and dihydrotamoxifen as was expected considering the similarity of their structures.

DP voltammetry of *N*-desmethyl tamoxifen showed the first anodic peak, at E_p = +1.03 V, starting at pH 1 (Fig. 5) corresponding to the loss of a π electron to form a cation radical. A second wave, E_p = +1.13 V, starts appearing also at pH 1 (Fig. 5) as a consequence of further oxidation of the cation radical formed at the first peak. No further peak was seen until pH 7 (Figs. 3B and 5). This behaviour is consistent and justified by the single structural difference between tamoxifen and *N*-desmethyl tamoxifen, the amine group (a tertiary and a secondary amine group, respectively). At pH 7

a well-defined shoulder is seen, E_p = +0.98 V (Fig. 6), and occurs as a result of the oxidation of the secondary amine group present in *N*-desmethyl tamoxifen (Scheme 1). Actually, other compounds described in the literature containing secondary aliphatic amines disclose a similar oxidation peak at this E_p value [20, 28, 29].

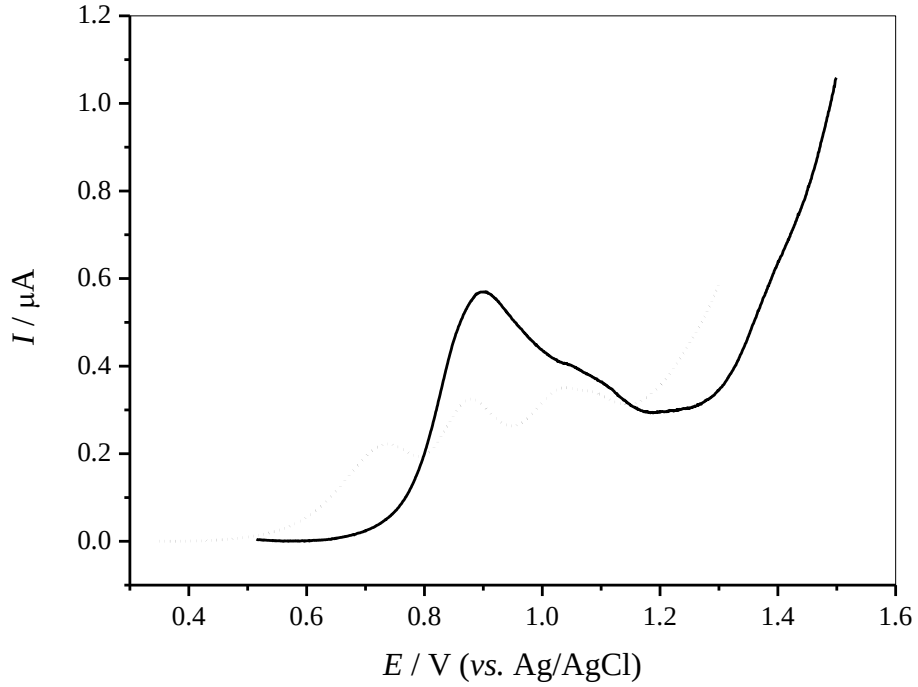


Figure 6. DP voltammograms of 0.1 mM solutions of (**—**) *N*-Desmethyl tamoxifen and (**•••**) tamoxifen in pH 7 buffer electrolyte. Scan rate 5 mV s^{-1}.

The collected data provided evidence that the mechanism of oxidation of tamoxifen is a complex process involving the generation of a dication radical which cyclizes to form the corresponding phenanthrene derivative, as was already proposed for the oxidation of tamoxifen's related compounds [15, 16, 27], and also the oxidation of the tertiary amine moiety yielding the corresponding secondary amine that could be subsequently oxidized.

4. CONCLUSION

Regarding tamoxifen pharmaceutical interest as a nonsteroidal antiestrogen very few electrochemical studies have been performed to clarify remaining questions concerning its oxidative profile. Therefore, the oxidation behaviour of tamoxifen was studied over a large pH interval and it was verified that in aqueous solution its oxidation follow a complex mechanism. To understand and clarify its oxidative profile, a series of analogues and metabolites of tamoxifen were obtained and their voltammetric behaviour studied.

Analysing the overall data obtained it was concluded that the oxidation process of tamoxifen proceeds at the aromatic nucleus through an electron transfer process, from which a cation radical intermediate is generated. This intermediate could undergo a subsequent reaction, being the most likely related to a cyclization reaction with formation of a phenanthrene derivative. The gathered results suggest that the ethylenic double bond is not critical for the occurrence of the tamoxifen's main oxidation process. However, it could play an important role in the course of the oxidation mechanism or in the subsequent oxidative pathways. Furthermore, it was concluded that the tertiary amine group has also a predominant action on the oxidative process as well as its oxidation product (a secondary amine) that was found to undergo further oxidation at the electrode surface.

These results could contribute to answer many questions about tamoxifen's metabolic behaviour and to the development of new analytical methodologies, based on electrochemical detection, either for its monitoring or for the detection of specific interactions. As the major products from the electrochemical oxidation and from tamoxifen metabolic activation are the same one can conclude that these two oxidation processes may produce the same type of reactive intermediates. The overall data support the importance of electrochemistry to facilitate the early stage characterization of phase I biotransformation processes.

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