J Mol Model (2009) 15:945–952
DOI 10.1007/s00894-009-0451-z

ORIGINAL PAPER

DFT and electrochemical studies on nortriptyline oxidation sites

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Received: 7 October 2008 / Accepted: 8 December 2008 / Published online: 27 January 2009

Abstract A study on the possible sites of oxidation and epoxidation of nortriptyline was performed using electrochemical and quantum chemical methods; these sites are involved in the biological responses (for example, hepatotoxicity) of nortriptyline and other similar antidepressants. Quantum chemical studies and electrochemical experiments demonstrated that the oxidation and epoxidation sites are located on the apolar region of nortriptyline, which will useful for understanding the molecule's activity. Also, for the determination of the compound in biological fluids or in pharmaceutical formulations, we propose a useful analytical methodology using a graphite-polyurethane composite electrode, which exhibited the best performance when compared with boron-doped diamond or glassy carbon surfaces.

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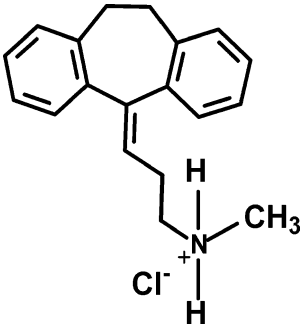
Keywords Antidepressant · DFT studies · Electrochemical oxidation · Nortriptyline

Introduction

Tricyclic antidepressants (TCA)—one of the oldest classes of antidepressants—are still extensively used for the treatment of psychiatric disorders. They act by blocking the reuptake of the neurotransmitters norepinephrine and serotonin in the central nervous system [1]. However, these drugs appear with new therapeutic indicators for other illnesses besides depression [2]. Nortriptyline [1-propanamine, 3-(10,11-dihydro, 5H-dibenzo [a, d] cyclohepten- 5-ylidene)-N-methyl; Fig. 1] is derived from the demethylation of amitriptyline. It is used as a sedative and is useful in depressed patients with insomnia and nervousness. It has also been found to be helpful for treating chronic pain [3]. The development of new types of modified electrodes that can be applied to many types of biologically important compounds brings new perspectives to the analysis of nortriptyline [4].

Due to its biological relevance, we investigated the possible oxidation sites of nortriptyline using electrochemical and quantum chemical methods. Additionally, we studied the possible epoxidation sites, since the epoxidate form of nortriptyline is a proposed intermediate in the hepatotoxicity mechanism [5]. This work also describes the use of a graphite-polyurethane composite electrode (GPU) as an alternative tool for the study of the electrochemical reactions of nortriptyline, as well as the use of the electrochemical impedance spectroscopy technique. Both of these techniques can be valuable methodologies in determining the oxidative behavior of this interesting biological compound.

Fig. 1 Structure of nortriptyline



In electrochemical terms, nortriptyline and other compounds with no heteroatom in the cycloheptene fragment were previously reported as being electrochemically inactive under common solid electrodes [6]. More recently, application of the wide potential window of boron-doped diamond (BDD) electrodes has allowed the detection of nortriptyline [7]. Several studies have indicated that the BDD, if adequately pre-treated, can constitute a good feasible alternative for the analysis of organic compounds [8, 9]. On the other hand, analysis of the hydrophobic sites of reaction is not amenable to this material, due to the high hydrophilic character of the BDD electrodes after cathodic pre-treatment. In this context, the use of a GPU can permit the analysis of hydrophobic sites due to the hydrophobic character of the resin [10].

To establish the charge transfer resistances in aqueous processes, several authors have used electrochemical impedance spectroscopy (EIS). For example, Smiechowski and Lvovich [11] have studied the mechanism of carbon black interactions in a dispersant, and the role of these interactions in the formation and stabilization of colloidal dispersions. Sine et al. [12] have studied the deposition of nanoparticles in conductive diamond electrodes, measuring the charge-transfer resistances of all synthesized materials. In our studies, EIS was used to determine the hydrophilic/hydrophobic behavior of each electrodic material. On the other hand, cyclic voltammetry can yield some information about the nortriptyline oxidation process. The presence of oxidation currents as a function of the applied potential indicates that the oxidation process is carried out adequately. The absence of current is an indication that the oxidation process is unfavorable under some conditions.

To establish the relationship between the oxidation currents (including the charge transfer processes) and the hydrophilic/hydrophobic behavior of each electrodic material, we performed several theoretical studies in an attempt to explain why the nortriptyline oxidation process is favorable when using electrodes with a hydrophobic configuration.

In the last few decades, quantum chemical methods have been employed to explain several physical-chemical phenomena. Progress in computational hardware and the development of efficient algorithms have encouraged the development of new routines for molecular quantum mechanical calculations, giving realistic quantum-chemical molecular quantities in a relatively short computational time frame [13]. These quantum-chemical molecular properties/descriptors can express all of the electronic and geometric properties of molecules and their interactions. Quantum chemistry provides a more accurate and detailed description of electronic effects than empirical methods [13]. Therefore, quantum chemical methods were applied in this work, with the objective of obtaining valuable evidence of the possible oxidation and epoxidation sites of the antidepressant nortriptyline.

Materials and methods

Cyclic voltammetric experiments were carried out in a polarograph analyzer (PAR model 174 A) automated with an A/D & D/A IBM compatible interface. A conventional three-electrode cell was employed, and the electrodes were inserted into the cell through its Teflon cover. The working electrode was a laboratory-made graphite-polyurethane composite (GPU, geometric area=0.07 cm^2). The composite electrode was prepared by mixing polyurethane resin and graphite according to the method published by Mendes et al. [14]. In order to compare the voltammetric responses for nortriptyline oxidation, we used (1) a glassy carbon electrode (GC, geometric area=0.20 cm^2), and (2) a boron doped diamond electrode (BDD, geometric area=0.6 cm^2). The auxiliary electrode was a Pt foil (area=1.0 cm^2). All potentials are referenced to the $Ag/AgCl_{(s)}$ (3.0 mol L^{-1} KCl) reference electrode.

EIS experiments were carried out in a nortriptyline (5.6×10^{-4} mol L^{-1}) solution using a Voltalab potentiostat (mod. PGZ 402) controlled by Voltamaster 4.0 Software. The experiments were performed at a fixed potential of 950 mV vs Ag/AgCl, and in the frequency range of 1mHz to 100 kHz, using the three carbon surfaces—GPU, BDD and GC—as working electrodes.

All reagents used in this work were of analytical grade and were used without further purification. The buffer solution, 0.1 mol L^{-1} Briton Robinson (BR), was prepared by adding boric acid (0.04 mol L^{-1}), glacial acetic acid (0.04 mol L^{-1}), phosphoric acid (0.04 mol L^{-1}) (all from Merck) and sodium perchlorate (0.1 mol L^{-1}) (Sigma) in the appropriate concentrations. The pH of the solution was adjusted to the desired value by small additions of 0.1 mol L^{-1} NaOH. All solutions were prepared using water purified in a Milli-Q system (Millipore). The 3.3×10^{-3} mol L^{-1} nortriptyline stock solution (nortriptyline hydrochloride; Sigma) was prepared in ethanol due to its low solubility in aqueous media.

Computationally, we performed a study using density functional theory (DFT) [15–17] in order to identify the nortriptyline oxidation and epoxidation sites. To perform the quantum chemical calculations, geometry optimization and electronic properties calculations were performed for two species of nortriptyline: the species with charge +1 and the oxidized species (charge+2).

The calculation methodology consisted of the use of the B3LYP [18–20] functional, 6-31G(d) [21–32] basis set and the IEF-PCM (integral equation formulation version of the polarizable continuum model) [33–37] to simulate the solvent (water), as implemented in the Gaussian 03 code [38]. The cavity in which the solute (nortriptyline) is embedded was obtained as a superposition of overlapping spheres centered on heavy atoms defined in terms of van der Waals radii [39] multiplied by a factor (f=1.2) [40]: only the hydrogen atoms bonded to nitrogen have their proper sphere. The resulting radii we used were 2.40 for $–CH_3$ and $–CH_2$, 2.28 for –CH, 2.04 for –C, 1.92 for –N and 1.44 for –H bonded to N. The absence of imaginary frequencies was used as a criterion to ensure that the optimized structure represents the minimum of the potential energy surface. This methodology has been applied successfully to the study other tricyclic molecules, namely cationic dyes [41].

We calculated the CHELPG (charges from electrostatic potentials using a grid-based method) atomic charges [42]. From the CHELPG option, we can evaluate the electrostatic potential of a molecule from the calculation of a set of point atomic charges so that it represents the best quantum molecular potential for a set of points defined around the molecule [42]. The charges from the electrostatic potential represent an advantage because they are, in general, physically more satisfactory than Mulliken charges [43], especially when working with compounds that exhibit biological activity [44].

Results and discussion

Experimental studies

As previously mentioned, the voltammetric oxidation of nortriptyline was observed only at the BDD electrode, due to its wide electrochemical potential window [7]. In the case of the GPU electrode (Fig. 2a), nortriptyline oxidation was characterized by a well-defined peak at lower potentials (1,005 mV vs $E_{Ag/AgCl}$) than that observed for the BDD electrode. This behavior may be explained by the active sites, i.e., likely functional groups of the polyurethane polymer on the electrode surface that interact with the molecule. The GPU electrode is known to be capable of promoting the electrochemical oxidation of ascorbic acid and dopamine [10] during the dopamine oxidation process at lower potentials than on other electrode materials. This feature is related to a catalytic effect promoted by active sites on the surface of the GPU electrode.

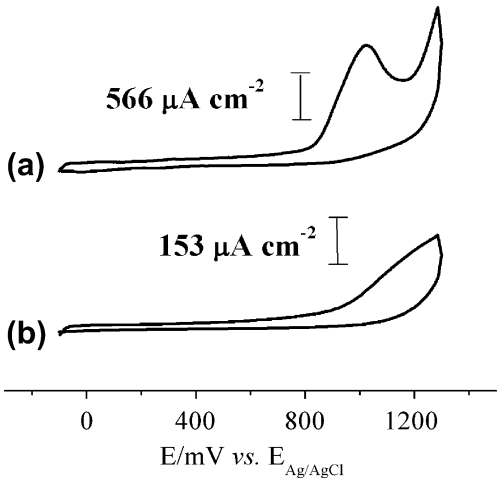


Fig. 2 Cyclic voltammetry (CV) curves for nortriptyline (1.54×10^{-4} mol L^{-1}) in 0.10 mol L^{-1} Briton Robinson (BR) buffer (pH 7.0) at: **a** a graphite-polyurethane composite electrode (GPU; geometric area=0.07 cm^2), and **b** a glassy carbon (GC) electrode (geometric area =0.20 cm^2). ν=100 mV s^{-1}

The voltammetric response of the compound was also analyzed under the GC electrode (Fig. 2b). Notably, the potential window of the GC electrode was limited by the oxygen evolution reaction (values of potential higher than 1.2 V vs Ag/AgCl), which hinders observation of nortriptyline voltammetric oxidation. This suggested that the GPU electrode can be successfully employed for both the study of electrochemical behavior and the development of new analytical methodologies to be applied for the detection of nortriptyline.

Subsequently, the influence of pH on the peak current (I_p) and peak potential (E_p) was also studied using a pH range between 6 and 11. Cyclic voltammograms of a 1.54 x 10^{-4} mol L^{-1} nortriptyline solution in 0.1 mol L^{-1} BR buffer are presented in Fig. 3. Since the highest anodic peak current value was observed at pH 7.0, this pH was chosen for all subsequent experiments. The peak potentials shifted linearly to less positive potentials with an increase in pH, showing that the mechanism of the electrode reaction is pH dependent. The slope of the graph E_p vs pH corresponds to 103.20 mV per unit of pH (Fig. 3), indicating the participation of equal numbers of protons and electrons in the oxidation reaction. An estimation of the number of electrons consumed in the rate-determining step of the oxidation process was made by comparing the high similarity between the structures of nortriptyline (secondary amine) and amitriptyline (tertiary amine). An oxidation study of the tricyclic antidepressant imipramine was performed, and the oxidation mechanism was determined.

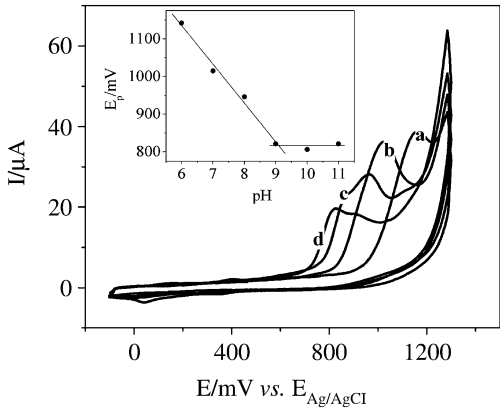


Fig. 3 CV curves for nortriptyline (1.54×10^{-4} mol L^{-1}) in 0.10 mol L^{-1} BR buffer solutions at the GPU electrode. pH: *a* 6.0, *b* 7.0, *c* 8.0, *d* 9.0. ν=100 mV s^{-1}. *Inset* E_p vs pH curve (dE_p/dpH=103.20 mV/pH)

In this case, a one-electron transfer was obtained [45]. Thus, it seems reasonable that the mechanism of nortriptyline oxidation involves the participation of one electron and one proton. The pK_a of the compound (9.2), obtained by the intercept of the E_p vs pH graph, was very close to the value found in the literature (9.7) [46].

In order to obtain the nature of the mass transport, the effect of the scan rate was also investigated in the range of 10 to 200 mV s^{-1}. The linearity of I_{pa} vs ν (R=0.9973) suggests that the electron transfer is an adsorption-controlled process [47]. The plot of log (I_{pa}) vs log (ν) was a straight line (R=0.9998) with a slope value of 0.79, which is close to the theoretical value of 1.0, which is expected for an ideal electrode reaction with adsorbing species [12]. From the slope ofI_{pa} vs log(ν) graph, it was possible to estimate the nα value as equal to 0.43. Considering the number of electrons (n=1), the transfer coefficient (α) obtained was 0.43, which is indicative of a product-like transition state, and suggests a concurrent alteration in the structure of the product [12].

A cyclic voltammetry (CV) study using different nortriptyline concentrations (1.66×10^{-5} mol L^{-1}–17.36×10^{-5} mol L^{-1}) was performed to verify the applicability of the GPU electrode for analytical purposes. The good linearity of I_p vs C_{NOR} (R=0.9982) suggested that the GPU electrode could be utilized for the development of new analytical methodologies. In fact, the GPU has already been used successfully for the determination of dopamine in a synthetic cerebrospinal fluid sample with good sensitivity and selectivity; therefore, the GPU may be a valuable material that could be applied in the development of other analytical methodologies for biological molecules, such as other tricyclic antidepressants [10]. An increment in sensitivity can be obtained using pulse techniques such as square wave voltammetry. Besides the use of pulse techniques, an enhancement in the current response could be achieved by using a pre-concentration step, since there is an adsorption process during nortriptyline oxidation.

Finally, we performed EIS studies in order to understand the charge-transfer processes as a function of the hydrophilic/hydrophobic behavior of each material. The charge-transfer resistances for the nortriptyline oxidation process, presented in Figs. 4 and 5, were studied at a fixed potential of 950 mV vs Ag/AgCl, using three different carbon-based working electrodes: GPU, GC and BDD. For the first case, presented in Fig. 4a, it can be observed that after six consecutive measurements, the charge transfer resistance increased from 80 to 500 kΩ cm^{-2}, indicating a continuous adsorption process that is determined by the affinity of the resin with the reaction site, which is hydrophobic. In fact, polyurethanes have been studied extensively due to their characteristics such as hydrophobicity and stability in water [10, 48, 49]. Figure 4b shows that the increase in the charge-transfer resistances is limited to a value of 380 kΩ cm^{-2}, likely due to saturation of reactant near the electrode surface. Thus, polyurethane is important for approximating the hydrophobic site of reaction to the electrode surface, and the electrochemical reaction occurs in the graphite contained in the composite. Figure 5a shows that a pure carbon surface, such as GC, presents a charge transfer resistance of about 1,100 kΩ cm^{-2} for the same reaction as that presented in

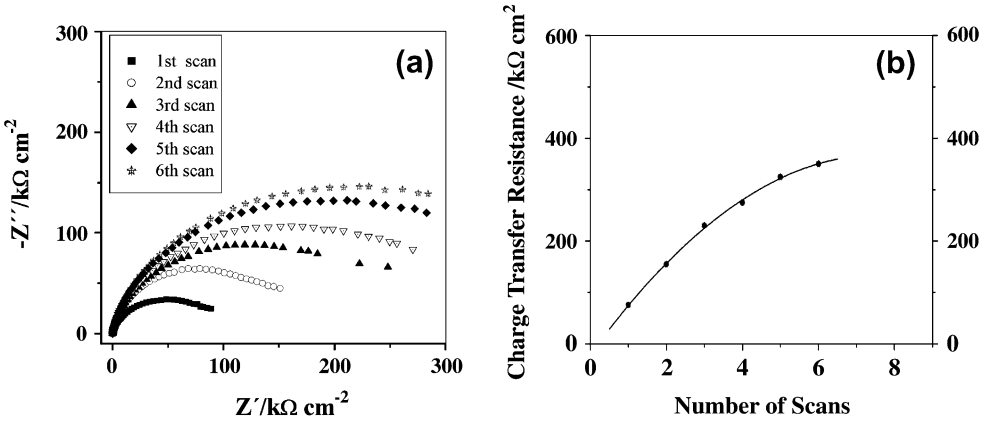


Fig. 4 **a** Successive scans (Nyquist Plots) for a GPU electrode in a 5.6×10^{-4} mol L^{-1} nortriptyline solution. Measurements were carried out at a fixed potential of 950 mV vs Ag/AgCl in a frequency range from 1 mHz to 100 kHz. **b** The limitation of charge transfer resistance as a function of number of scans for the reaction presented in **a**

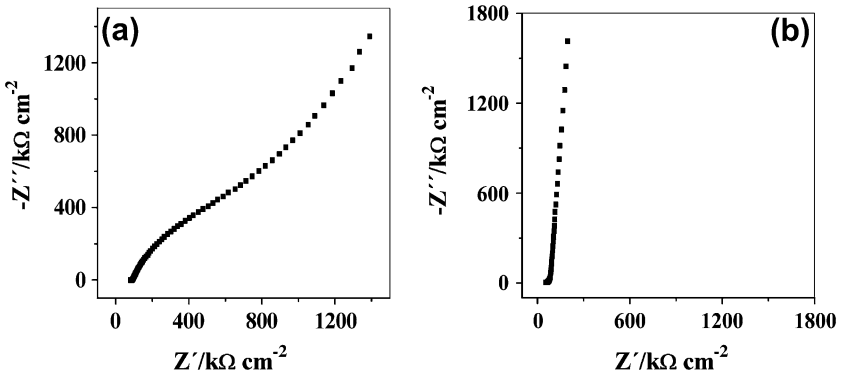


Fig. 5 Nyquist plots for **a** GC and **b** BDD electrodes pre-treated at −3,000 mV vs Ag/AgCl for 30 s. Measurements were carried out in the same solution used in Fig. 4

Fig. 4a for the polyurethane composite electrode. Figure 5b shows a typical capacitive response for a BDD electrode in the oxidation process [50]. It is important to note that this capacitive response was obtained in the presence of nortriptyline, and after a cathodic surface pre-treatment that made the BDD surface hydrophilic [8]. It can be concluded that the hydrophobic character is related directly to the possibility of nortriptyline analysis.

Theoretical studies

Quantum chemical studies using DFT methodology were performed in order to elucidate the oxidation and epoxidation sites of nortriptyline. As mentioned before, we have optimized the geometry and calculated several electronic properties for two states of nortriptyline, namely, species with charges +1 and +2. Figure 6 presents the results obtained for the species with a +1 charge, i.e., the optimized geometry along with the numbering system adopted in the calculations, the dipole moment vector and the CHELPG atomic charges.

Table 1 lists some important structural parameters for both optimized structures, i.e., species with charge +1 and +2. The system containing three rings is folded in the middle of the central ring. However, the species with charge +2 is more planar (the 25C–4C–3C–7C torsion angle is smaller, and the 8C–25C–4C angle is greater when compared to the same angles of the species with charge +1), and the 7C–10C bond length is greater than the same bond in the species with charge +1, i.e., the character of the double bond decreases. Therefore, the oxidation process particularly affects the system with three rings, decreasing its stability due to the decrease in resonance character.

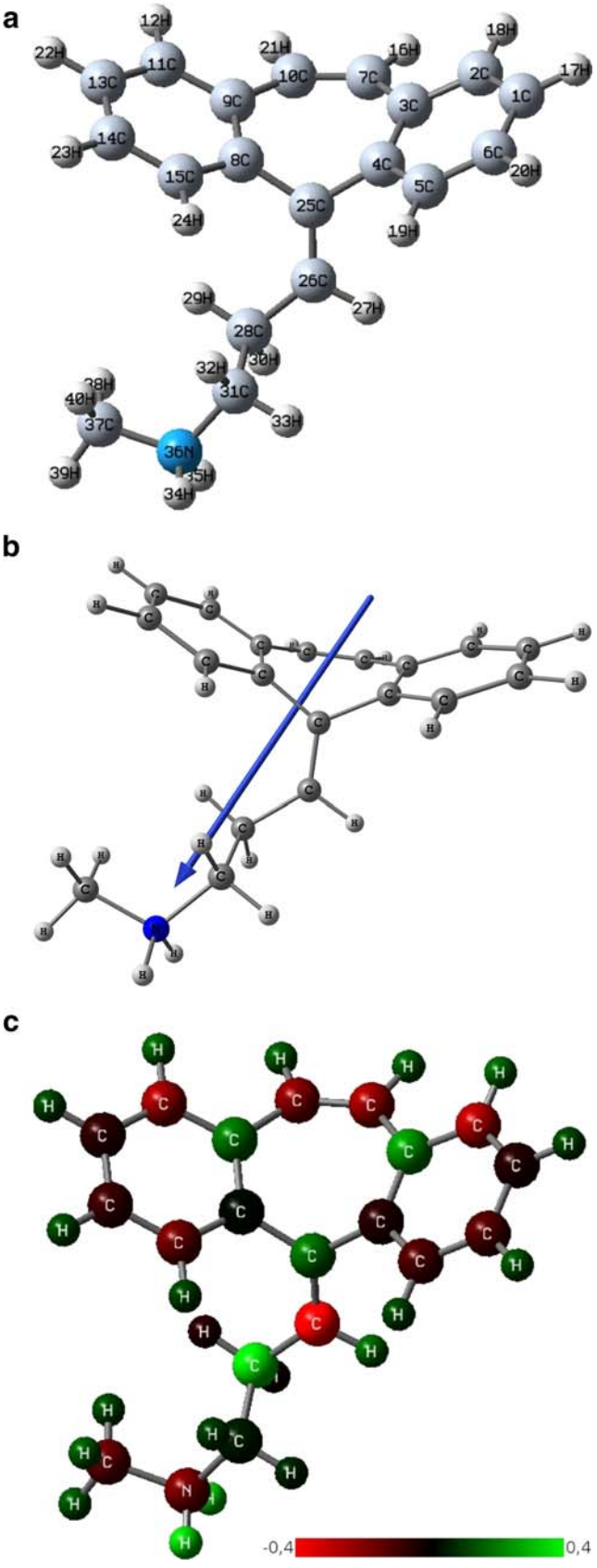
The atomic charges (see Fig. 6c) indicate a high charge concentration (negative and positive) in the aliphatic group, while the ring system is almost neutral, corroborating the dipole moment data. The dipole moment obtained from DFT calculations for nortriptyline with charge +1 was equal to 22 Debye, and as seen in Fig. 6b, the dipole is oriented along the aliphatic portion of the molecule. It is interesting to note that the species after oxidation (charge +2) presents a dipole moment equal to 15 Debye, indicating that this species is less polar than the initial species. Since nortriptyline is highly polar, it should interact with hydrophilic electrodes. As the electrochemical results show that this interaction does not occur, we performed further analysis of the theoretical results.

Table 2 shows the CHELPG atomic charges for the nortriptyline molecule with charge +1 and the species after oxidation (charge +2, according to electrochemical results). From Table 2, we can see important variations in the whole molecule when one electron is removed, especially at 4C, 6C, 7C, 8C, 14C, 10C, 25C (all them located in the system containing three rings) and 26C, indicating the molecular symmetry and resonance effect.

Another way to determine the possible oxidation sites is to analyze the graphical representation of the HOMO (highest occupied molecular orbital), since this is the molecular orbital that loses the electron [51]. Figure 7 shows that the HOMO of nortriptyline is formed mainly by the rings, i.e., the apolar region of the molecule, indicating that this region should interact with the electrode for oxidation to occur. Therefore, to promote the oxidation of nortriptyline, the electrodic materials must be hydrophobic.

It is interesting to note that the HOMO map (see Fig. 7) and the atomic charges (Table 2) are highly related, i.e., the atoms that have the most charge variations are the same ones that contribute to the HOMO. Therefore, according to the results displayed in Table 2 and Fig. 7, we can assume that the most probable regions of oxidation are 4C, 6C, 7C (or the atoms symmetrical to them: 8C, 14C and 10C, respectively), 25C and 26C.

Amitriptyline and nortriptyline have been associated with incidences of hepatotoxicity in patients. Wen et al. [5] carried out chromatographic and mass spectroscopic analysis to determine the intermediates of drug bioactivation in human liver microsomes. Their findings are consistent with a bioactivation mechanism that involves an epoxide as the intermediate (structure presented in



◀ **Fig. 6** **a** Optimized geometry, **b** dipole moment vector, and **c** atomic charges derived from electrostatic surface potential for nortriptyline obtained with B3LYP/6-31 g(d)/IEF-PCM (integral equation formulation version of the polarizable continuum model) calculations

Fig. 8). Although the epoxide structure has not been identified (it was determined only indirectly), its formation has been implicated in the bioactivation of another tricyclic antidepressant (imipramine). As a result, considering that the nortriptyline molecule is a structurally similar N-dealkylated metabolite of amitriptyline, the formation of an epoxide intermediate from nortriptyline could contribute to the observed hepatotoxicity caused by amitriptyline and nortriptyline.

In this context, we reanalyzed the quantum chemical results for the nortriptyline with charge +1 to confirm epoxide formation. A general epoxidation mechanism is presented in Fig. 9. From this mechanism, it should be noted that a high electronic density at the double bond facilitates the epoxidation process, since this characteristic makes the double bond more nucleophilic. In this work, for the nortriptyline with charge=+1, the 2C atom has a high negative charge and is located at a double bond, indicating that it could be the epoxidation site (see Table 2), which corroborates the epoxide intermediate proposed by Wen et al. (Fig. 8) [5].

Table 1 Main structural parameters for the nortriptyline molecule with charge= +1 and charge= +2 (after oxidation)

Parameter	Nortriptyline (charge+1)	Nortriptyline (charge+2)
Bond length (Å)		
1C-2C	1.39	1.38
7C-10C	1.35	1.39
11C-13C	1.39	1.38
1C-6C	1.40	1.41
3C-4C	1.42	1.44
8C-9C	1.42	1.44
4C-25C	1.49	1.48
25C-26C	1.35	1.36
26C-28C	1.51	1.50
Angle (°)		
8C-25C-4C	116.8	120.7
9C-10C-7C	128.4	129.8
10C-7C-3C	128.8	130.2
4C-25C-26C	119.7	117.2
25C-26C-28C	127.4	128.7
31C-36N-37C	114.9	115.1
Torsion Angle (°)		
25C-4C-3C-7C	4.5	7.4
25C-4C-3C-7C	28.8	19.5

Table 2 Atomic charges for the nortriptyline molecule with charge= +1 and charge=+2 (after oxidation) and the difference between both states

Atom	Nortriptyline (charge + 1)	Nortriptyline (charge + 2)	Difference
1 C	-0.056	-0.071	0.015
2 C	-0.271	-0.177	-0.094
3 C	0.224	0.175	0.049
4 C	-0.059	0.050	-0.109
5 C	-0.120	-0.150	0.030
6 C	-0.127	0.017	-0.144
7 C	-0.240	-0.097	-0.143
8 C	0.013	0.120	-0.107
9 C	0.177	0.112	0.065
10 C	-0.206	-0.051	-0.155
11 C	-0.239	-0.142	-0.097
12 H	0.144	0.152	-0.008
13 C	-0.071	-0.087	0.016
14 C	-0.090	0.040	-0.130
15 C	-0.170	-0.196	0.026
16 H	0.137	0.158	-0.021
17 H	0.105	0.130	-0.025
18 H	0.151	0.159	-0.008
19 H	0.101	0.128	-0.027
20 H	0.119	0.127	-0.008
21 H	0.132	0.151	-0.019
22 H	0.106	0.130	-0.024
23 H	0.113	0.123	-0.010
24 H	0.101	0.126	-0.025
25 C	0.169	0.045	0.124
26 C	-0.440	-0.262	-0.178
27 H	0.154	0.152	0.002
28 C	0.361	0.263	0.098
29 H	-0.059	-0.036	-0.023
30 H	-0.035	0.008	-0.043
31 C	0.044	0.008	0.036
32 H	0.088	0.104	-0.016
33 H	0.058	0.076	-0.018
34 H	0.328	0.330	-0.002
35 H	0.330	0.330	0.000
36 N	-0.188	-0.163	-0.025
37 C	-0.160	-0.176	0.016
38 H	0.109	0.118	-0.009
39 H	0.130	0.136	-0.006
40 H	0.135	0.139	-0.004

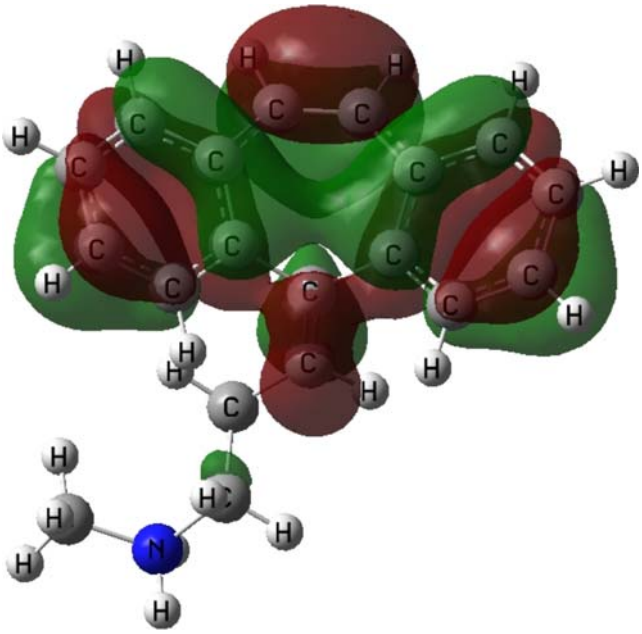


Fig. 7 Highest occupied molecular orbital (HOMO) plot calculated for nortriptyline

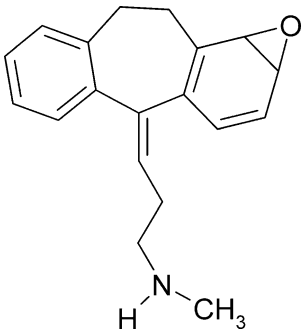


Fig. 8 Epoxide intermediate in nortriptyline bioactivation proposed by Wen et al. [5]

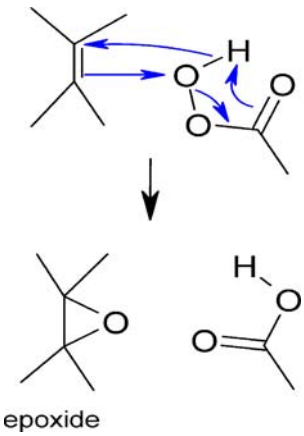


Fig. 9 General mechanism for epoxidation of an alkene

Conclusions

In this work, for the first time, electrochemical and DFT studies were carried out on the antidepressant nortriptyline, in order to locate its oxidation and epoxidation sites, which are related to its biological responses. The results obtained are important because nortriptyline is the principal metabolite of amitriptyline and also exhibits antidepressant activity. EIS studies and quantum chemical calculations demonstrate that the hydrophobic character of the electrodic materials

may promote the oxidation of nortriptyline, since oxidation sites are located on the tricyclic atoms instead of the aliphatic region of the molecule. We have also proposed a new sensitive analytical methodology for the nortriptyline determination based on a graphite-polyurethane composite, which exhibited the best performance due to its high hydrophobic character. Finally, from DFT studies, it was possible to elucidate some important aspects of nortriptyline epoxidation (at the 2C atom), which could be important in understanding its hepatotoxicity.

Acknowledgments The authors wish to thank the Brazilian Funding Institutions CNPq (The National Council for Scientific and Technological Development) and FAPESP (The State of São Paulo Research Foundation), processes numbers 01/14629-0, 01/06029-3, 05/59992-6, 07/05370-0 and 07/05155-1, for the financial support of this research.

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