Chapter 2
Lidocaine oxidation by electrogenerated reactive oxygen species in the light of oxidative drug metabolism

The study of oxidative drug metabolism by Cytochrome P450s (CYP) is important in the earlier stages of drug development. For this purpose, automated analytical techniques are needed for fast and accurate estimation of oxidative drug metabolism. Previous studies have shown that electrochemistry in combination with mass spectrometry is a versatile analytical technique to generate drug metabolites that result from direct electron transfer. Here we show that electrochemical generation of reactive oxygen species (ROS), a process reminiscent of the catalytic cycle of CYP, extends the applicability of electrochemistry in drug metabolism research. Oxidation products of lidocaine from one and two-compartment electrochemical cells, operated under various conditions were analyzed by LC-MS and metabolite structures were elucidated by collision-induced (LC-MS/MS), and thermally-induced (APCI) fragmentation. Direct oxidation of lidocaine at the anode resulted in N-dealkylation while reaction with H₂O₂ generated at the cathode, produced the N-oxide, both known in vivo lidocaine metabolites. Catalytic activation of hydrogen peroxide, using the Fenton reaction, resulted in benzylic and aromatic hydroxylations thus covering all of the known in vivo phase-I metabolites of lidocaine. This study extends the applicability of electrochemistry combined with mass spectrometry as a valuable technique in assessing oxidative drug metabolism related to CYP.

2.1 Introduction

Evaluation of oxidative drug metabolism is important in the earlier stages of drug development and requires fast, and accurate analytical techniques. This aspect of drug stability is presently studied through enzymatic reactions with Cytochrome P450s (CYP), either in pure, recombinant form or as part of liver microsomes [1, 2]. Electrochemistry coupled with mass spectrometry has been shown to be a fast technique that provides oxidation products generated by direct electron transfer [3-7]. Electrochemistry can also be used to generate reactive oxygen species (ROS), in analogy to the catalytic activation of molecular oxygen by CYP [8]. Lidocaine, a common local-anesthetic drug, is a convenient test compound, whose in vivo oxidative metabolism leads to N-dealkylation, N-oxidation, aromatic hydroxylation and benzylic hydroxylation products [4, 9]. The reactions involve a putative oxo-ferryl radical cation intermediate (a reactive electrophile) as part of the heme prosthetic group of CYP [10].

N-dealkylation of lidocaine by CYP is proposed to be initiated by two mechanisms: hydrogen atom transfer (HAT) from the carbon atom adjacent to the nitrogen, and single electron transfer (SET) from nitrogen to CYP (Scheme 1-a) [10]. The available experimental evidence supports the SET mechanism [10]. N-oxidation could proceed via initial charge transfer to the oxo-ferryl group, resulting in a radical cation, followed by homolysis of the iron-oxygen bond to generate the new N-O bond. Alternatively, this reaction may proceed through a concerted oxygen insertion process [10]. While the HAT mechanism may explain benzylic hydroxylation, it is not applicable for the hydroxylation of aromatic substrates, because of the energy that is required for the homolytic cleavage of the aromatic C-H bond [10]. In this case, it is proposed that the oxo-ferryl radical cation attacks the aromatic ring to generate an intermediate which reacts to the hydroxylation product through a hydrogen shift (NIH (National Institute of Health) mechanism, Scheme 1-b) [10]. New studies provide evidence for the presence of other electrophilic oxidants, e.g. perhydroxo-iron or iron-complexed hydrogen peroxide, in the catalytic cycle of CYP which may generate protonated alcohols (alternative electrophilic oxidants, Scheme 1-b) [11].

The majority of organic compounds is not readily oxidized by molecular oxygen, because the triplet electronic structure of molecular oxygen prevents its direct reaction with (mostly singlet) organic compounds. In the catalytic cycle of CYP, molecular oxygen gets activated through a sequence of reductions and protonations. Similarly, electrochemical reduction of molecular oxygen in organic solutions has been shown to lead to the oxidation of various organic substrates [8,
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In aprotic solutions, molecular oxygen is reduced to the superoxide anion via a one-electron process (reaction 1), and reduced further at more negative potentials to singlet peroxide anions (reaction 2). The electrode material could affect the reduction of molecular oxygen. It has been shown that metallic electrodes, especially in acetonitrile, show a broader reduction peak due to interactions between electrogenerated superoxide anions and a metallic surface [13].

$$\text{O}_2 + e^- \rightarrow \text{O}_2^- \quad E^0 = -0.75 \text{ V vs SCE} \quad (1)$$

$$\text{O}_2^- + e^- \rightarrow \text{O}_2^{2-} \quad E^0 = -2.05 \text{ V vs SCE} \quad (2)$$

In the presence of residual water electrogenerated superoxide anions undergo dismutation, leading to the generation of perhydroxyl radicals (reaction 3), which are strong oxidizers capable of rapidly abstracting an electron from the electrode or...
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the electrogenerated superoxide anion to form perhydroxyl anions (reactions 4 and 5) [8]. Perhydroxyl radicals are unstable, and decompose spontaneously to hydrogen peroxide and molecular oxygen (reaction 6) [8]. In addition, disproportionation of the electrogenerated superoxide anions with water produces perhydroxyl anions and molecular oxygen (reaction 7) [8]. The formation of hydrogen peroxide by protonation of the perhydroxyl anion generated in reactions 4, 5, and 7 has been proven spectroscopically [14]. Generation of hydroxyl radicals through reduction of hydrogen peroxide by superoxide anions (reaction 8), has also been reported [15, 16].

\[
\begin{align*}
O_2^- + H_2O & \rightarrow HO_2^- + OH^- \\
HO_2^- + e^- & \rightarrow HO_2^- \\
HO_2^- + O_2^- & \rightarrow HO_2^- + O_2 \\
HO_2^- + OH_2^- & \rightarrow H_2O_2 + O_2 \\
2 O_2^- + H_2O & \rightarrow HO_2^- + O_2 + OH^- \\
H_2O_2 + O_2^- & \rightarrow OH^- + O_2^- + O_2
\end{align*}
\]

(3) (4) (5) (6) (7) (8)

In the present study, we produce electrogenerated ROS in the presence of lidocaine, to examine their applicability in the generation of metabolites by CYP. We utilize cyclic voltammetry to follow the reactions, and LC-MS/MS to identify reaction products.

2.2 Experimental Procedures

Reagents. Tetrabutylammonium perchlorate (TBAP, 86893), iron chloride (FeCl₃, 157740), ethylenediaminetetraacetic acid (EDTA, 431788), ascorbic acid (A5960), m-chloroperbenzoic acid (m-CPBA, 273031), sodium carbonate (204420), hydrogen peroxide 30 vol % (31642) and lidocaine (L7757) were purchased from Sigma-Aldrich. Water was purified by a Maxima Ultrapure water system (ELGA, High Wycombe, Bucks, UK). Ultra-pure HPLC grade acetonitrile (ACN) was purchased from Merck. 3-Hydroxylidocaine (CAS Nr. 34604-55-2) was purchased from Toronto Research Chemicals Inc.

Electrode preparation. The surface of a gold disk electrode with 1.6 mm diameter (MF-2014, Bioanalytical Systems (BASi), West Lafayette, IN, USA) was polished with a lapping sheet (Micromesh grade 3200) prior to each experiment. After mechanical polishing the surface was washed with ethanol and air-dried.

Electrochemical measurements. Electrochemical experiments were performed with a home-made potentiostat controlled by a MacLab system (ADInstruments, Castle Hill, NSW, Australia) and EChem v.1.52 software (eDAQ, Denistone East, 46
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NSW, Australia). The electrochemical cell was a conventional three electrode cell in which the working electrode was a gold disk and the auxiliary electrode a platinum wire (MW-4130, BASi). Potentials were measured against a silver wire pseudo-reference electrode (MF-2017, BASi), instead of conventional reference electrodes, to eliminate the possibility of chloride contamination of the working solution during prolonged electrolysis. For the calibration of the silver reference electrode, the half-peak redox potentials of Fe$^{2+}$/Fe$^{3+}$ against the silver electrode and a Ag/AgCl 3 M reference electrode (MF-2052, BASi) in an aqueous 0.1 M NaCl solution with 10 mM potassium hexacyanoferrate were measured at 0.2 and 0.6 V, respectively. The potential shift of 0.4 V was also measured for the reduction of molecular oxygen from 0.1 M TBAP dissolved in ACN/H$_2$O 99/1 (v/v) (Figure 1).

All experiments were performed at ambient temperature. For deaeration and aeration, argon and synthetic air, respectively, were bubbled at 25 mL/min via a sparge tube (MW-4145, BASi) through the 1 mL solution for 10 minutes prior to each experiment. Cyclic voltammetry measurements were started at 0 V, swept toward negative potentials, and the steady state scans were selected for analysis. A two-compartment electrochemical cell was constructed by using a porous Vycor tip with Teflon heat shrink (MF-2064, BASi) to separate working and auxiliary half-cells. The atmosphere in the both compartments was controlled by sparging with argon or synthetic air. The reference electrode was placed in the working compartment.

Solutions containing 10 mM lidocaine and 0.1 M TBAP dissolved in ACN or ACN/H$_2$O 99/1 (v/v) (0.1 M TBAP used as electrolyte to provide sufficient conductivity for electrochemical experiments) were subjected to constant potentials ranging from 0 to -2.4 V vs. Ag, in 0.2 V steps, with continuous gas flow, for 10 minutes prior to LC-MS and LC-MS/MS analysis. Electrochemical control experiments were done under argon atmosphere. Reaction with 1 vol % hydrogen peroxide (no electrochemistry) was done for one hour. Samples were collected and diluted 100 times in water containing 10 $\mu$M acetaminophen, as an internal standard for LC-MS signal normalization, immediately after the batch oxidations and stored at room temperature until LC-MS analysis. Oxidation mixtures from the two-compartment cell were collected separately. Stability of the oxidation products during storage was examined by analyzing the solution immediately after oxidation and again after storage for two days at room temperature. No changes were observed in the 100 times diluted samples. Three independent batch potentiostatic oxidation experiments were performed, each of which was analyzed twice by LC-MS.
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Figure 1. (a) Redox couple of Fe²⁺/Fe³⁺ recorded in an aqueous 0.1 M NaCl solution with 10 mM potassium hexa-cyanoferrate and (b) reduction of molecular oxygen from ACN/H₂O 99/1 (v/v) solution with 0.1 M TBAP, versus silver wire (solid line) and versus a Ag/AgCl 3 M KCl reference electrode (dashed line). Scan rate 100 mV/s.

Fenton reaction. In a glass vial, 0.1 mL of 10 mM FeCl₃, and 10 mM EDTA in ACN/H₂O 50/50 (v/v) was added to 0.8 mL ascorbic acid (6 mM) in ACN/H₂O 50/50 (v/v). Subsequently, 2 μL H₂O₂ (30 % in water) was added, followed by 0.1 mL of 100 mM lidocaine to give a final lidocaine concentration of 10 mM. The mixture was kept for ten hours at 50 °C prior to analysis.

N-oxide synthesis. 3.44 mg of m-chloroperbenzoic acid was added to a 1 mL solution of 10 mM lidocaine in dichloromethane and the mixture was stirred for 1 h at room temperature. Saturated Na₂CO₃ solution (1 mL) was added and the
mixture was stirred for an additional hour [17]. For the LC-MS analysis, the sample was taken from the dichloromethane layer.

**LC-MS analysis.** LC-MS experiments on 100-times diluted samples were carried out on an LC-Packings Ultimate HPLC system (LC-Packings, Amsterdam, the Netherlands) coupled to an API 365 triple quadrupole mass spectrometer (MDS Sciex, Concord, ON, Canada) with electrospray ionization in the positive mode. The original API 365 was used for experiments shown in Figure 3. All other LC-MS experiments were performed on an API 365 triple quadrupole upgraded to EP10+ (Ionics, Bolton, ON, Canada). The MS parameters, for the API 365, were as follows: ion spray voltage 5200 V, orifice (OR) voltage 5 V, and ring (RNG) voltage 50 V, with scans between m/z 150-300 (step size 1.0 amu, dwell time 2 ms). The MS parameters, for the EP 10+, were as follows: ion spray voltage 4800 V, OR 40 V, and RNG 120 V, with scans between m/z 50-600 (step size 1.0 amu, dwell time 1 ms). MS/MS parameters, for EP 10+, were as follows: ion spray voltage 5000 V, OR 40 V, RNG 170 V, and collision energy 20 eV, with product ion scans between m/z 50-300 (step size 1.0 amu, dwell time 1 ms). Heated turbo gas (nitrogen) with a flow rate of 1.0 L/min was used at 450 °C for LC-MS and LC-MS/MS experiments. Atmospheric Pressure Chemical Ionization (APCI) experiments were done with OR 5 V, and RNG 170 V, with scans between m/z 50-600 (step size 1.0 amu, dwell time 1 ms); the discharge current of the APCI source was 3 μA, and the heated nebulizer temperature was 450 °C.

A C18 reversed-phase column (GraceSmart RP 18 5 μm, 2.1×150 mm; Grace Davison, Lokeren, Belgium) was used at a flow rate of 200 μL/min. Solvent A: H2O/ACN 95/5 (v/v) with 0.1 % formic acid; Solvent B: ACN/H2O 95/5 (v/v) with 0.1 % formic acid. 50 μL of a diluted oxidation product mixture was injected and a linear gradient of 5-50 % B in 20 minutes was used for elution. For APCI experiments, a 5 μL injection was used. Peak heights were normalized with respect to the peak height of the acetaminophen internal reference compound.
2.3 Results and discussion

2.3.1 Electrochemistry of molecular oxygen in the presence and absence of water

Cyclic voltammetry (CV) was used to follow the electrochemical reduction of molecular oxygen in an acetonitrile/tetrabutylammonium perchlorate (TBAP) solution in the absence and presence of water, as shown in Figure 2. In the absence of water, CV shows the reduction of molecular oxygen to superoxide anions and the oxidation of the generated superoxide anions back to molecular oxygen in the potential region between -0.5 and -1.5 V (Figure 2, solid line). The difference between the reduction and oxidation half-peak potentials is diagnostic for a quasi-reversible reaction [18], and this difference is probably due to the adsorption of the electrogenerated superoxide anions on the electrode surface [19]. At more negative potentials (below -2.0 V), a totally irreversible reduction was observed, which corresponds to the generation of peroxide anions. These reductions have been previously described according to reactions 1 and 2 in dimethyl sulfoxide on a gold electrode [20]. The reduction potentials in dimethyl sulfoxide differ from those observed here, due to the different solvent [21].

CV experiments performed in the presence of 0.2 and 1 vol % water (Figure 2, dashed and dotted lines) showed that increasing the water content shifts the cathodic peak toward less negative potentials with a two times higher current. The suppression of the corresponding anodic peak indicates the instability of the electrogenerated superoxide anions in the presence of water. Presumably, superoxide anions undergo dismutation through protonation by water (reaction 3), leading to the generation of perhydroxyl radicals (HO$_2^*$) [22-24], which react further to hydrogen peroxide or its conjugated base according to reactions 4 to 6. The higher current in the presence of water is attributed to the reactions 4 to 6. An ECE (Electrochemical-Chemical-Electrochemical) mechanism for the reduction of molecular oxygen in the presence of water has been proposed previously [25].
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Figure 2. Cyclic voltammetry of molecular oxygen reduction on a gold electrode in 0.1 M tetrabutylammonium perchlorate in acetonitrile (solid line), after addition of 0.2 (dashed line), and 1 vol % water (dotted line), under air atmosphere (scan rate 100 mV/s).

2.3.2 Electrochemistry of lidocaine in the presence and absence of molecular oxygen

The electrochemical behavior of a solution of lidocaine in acetonitrile/TBAP was studied by CV in the presence or absence of dissolved molecular oxygen (Figure 3). An intense oxidation peak observed at the potentials above 1.0 V in the absence of molecular oxygen (Figure 3-a) is due to the direct oxidation of lidocaine, leading to radical cations, as has been reported for tertiary amines [6]. Direct oxidation of lidocaine on a porous graphite electrode at potentials above 800 mV vs Pd/H₂ has been shown by Jurva et al [5].

In the presence of dissolved molecular oxygen, several additional oxidation peaks of lidocaine were observed (Figure 3-a, solid line), which were subsequently studied by CV over various potential windows (Figure 3-b and c). Between -0.8 and 1.5 V (Figure 3-b, dashed line), an oxidation peak (Ox₁) and an associated reduction peak (Red₁) were observed at peak potentials of -0.5 and 1.0 V, respectively. Ox₁ is due to the direct oxidation of lidocaine and Red₁ is its subsequent reduction in the presence of dissolved molecular oxygen. In the potential window between -1.5 and 0.5 V (Figure 3-c, solid line). Another redox...
couple was observed (Ox$_2$ and Red$_2$) in the same potential region as for the superoxide anion and molecular oxygen redox couple (Figure 2, solid line). This indicates that the generation of superoxide anions and their subsequent reoxidation proceeds also in the presence of lidocaine. When extending the scan range to -2.5 V to 0.5 V (Figure 3-c, dashed line), another reduction peak was observed at potentials more negative than -2.0 V (Red$_3$), indicating the generation of peroxide anions (Figure 2, solid line). The reaction of peroxide anions with lidocaine leads apparently to a product that can be reoxidized at 0.25 V (Ox$_3$), since Ox$_3$ was not observed in the absence of lidocaine.

**Figure 3.** Cyclic voltammetry on a gold electrode with 10 mM lidocaine (a) in 0.1 M tetrabutylammonium perchlorate in acetonitrile under aerated (solid line) and deaerated (dashed line) atmosphere. Cyclic voltammetry of the same solution under aerated atmosphere across different potential windows: (b) -2.5 and 1.5 V (solid line), -0.8 and 1.5 V (dashed), and (c) -1.5 and 0.5 V (solid), -2.5 and 0.5 V (dashed); Scan rate 100 mV/s. (d) Peak current versus the square root of scan rate ($I_p$ vs $U^{1/2}$) representations for reoxidation of superoxide anions (Ox$_2$) in the absence (top line, squares) and presence of 10 mM lidocaine (bottom line, open circles)
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The chemical reaction between superoxide anions and lidocaine was studied by determining the peak current of the $Ox_2$ region, which corresponds to the number of reoxidized superoxide anions (see Figure 3-b) at different scan rates in the presence and absence of lidocaine. $I_p - I_0 \sqrt{v}$ (peak current vs. the square root of the scan rate) curves (data taken from Figure 4) showed that the presence of lidocaine decreased the number of reoxidized superoxide anions, indicating reaction with lidocaine (Figure 3-d). The nature of the lidocaine reaction products that are formed in the presence of molecular oxygen was determined by collection of samples at different potentials and their analysis by LC-ESI/MS.

2.3.3 Oxidation of lidocaine: analysis of the reaction products by LC-ESI/MS

LC-ESI/MS of lidocaine after reaction at constant potential of -1.0 V in the presence of molecular oxygen showed one major reaction product at $m/z$ 251 and a minor reaction product at $m/z$ 207. The product at $m/z$ 207 results from the N-dealkylation of lidocaine, as previously reported by Jurva et al. [5], whereas the component at $m/z$ 251 is a product with one additional oxygen atom (+16 Da) compared to lidocaine ($m/z$ 235). A more detailed product assignment is given in the following section.

The relative amount of both products was studied as a function of potential, in the presence or absence of molecular oxygen (Figure 5). In acetonitrile solution, the presence of dissolved molecular oxygen is required for formation of $m/z$ 251 (Figure 5-a), but there is a large variation in the $m/z$ 251 signal in three separate experiments. The reason could be that, although dry acetonitrile was used, absorption of trace amounts of water is difficult to avoid, resulting in varying water content across experiments. To control this, a known concentration of water (1 vol %) was added showing that the presence of both oxygen and water is essential for a reproducible production of both reaction products (Figure 5-c and d). The highest amount of the $m/z$ 251 reaction product is observed at -1.0 V, which corresponds to the peak potential for superoxide anion formation (Figure 2). These conditions were used for further studies.

Since the $m/z$ 207 N-dealkylation product is known to be the major product upon direct electrochemical oxidation of lidocaine, and since CV at positive potentials (Figure 3-a) suggested direct oxidation of lidocaine, we investigated the possibility whether a reaction takes place at the auxiliary electrode when applying a negative potential to the working electrode. Potential measurements on the auxiliary electrode showed indeed a sufficiently positive potential (+1.1 V), when a potential of -1.0 V was applied to the working electrode.
Figure 4. Cyclic voltammetry in 0.1 M tetrabutylammonium perchlorate in acetonitrile under air atmosphere (a) in the absence of lidocaine and (b) in the presence of 10 mM lidocaine across different potential windows: -1.8 to 0.0 V, and (c) -2.5 to 0.5 V. Scan rates were 20, 50, 100, 200, 500, and 1000 mV/s (the arrow indicates the direction of increasing scan rate.)
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Figure 5. Ion intensities of the m/z 251 and 207 oxidation products of lidocaine, obtained on a gold electrode at different potentials, from a solution of 10 mM lidocaine under air (squares) and argon atmosphere (open circles) in 0.1 M tetrabutylammonium perchlorate (a) m/z 251 in acetonitrile, (b) m/z 207 in acetonitrile, (c) m/z 251 in acetonitrile/water 99/1 v/v, and (d) m/z 207 in acetonitrile/water 99/1 v/v. Ion intensities were normalized relative to the intensity of the signal for acetaminophen, which was added as internal standard to all LC-MS analyses. Batch oxidations of aerated solutions were performed in triplicate, and all LC-MS analyses in duplicate.

We separated the compartments at the working (cathodic half-cell) and auxiliary (anodic half-cell) electrodes with a porous Vycor frit, which allows passage of ions and small organic molecules, but prevents extensive mixing of the solutions in the two compartments. No significant difference was observed between the magnitude of currents passing through the electrodes in the presence and absence of the Vycor frit. The product distributions between the anodic and cathodic compartments are shown in Figure 6. The m/z 207 product was observed almost
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exclusively in the anodic compartment, while the m/z 251 product was present in both parts, but mainly in the cathodic compartment. This shows that the N-dealkylation product of lidocaine (m/z 207) is produced by direct oxidation at the auxiliary electrode. The presence of the m/z 251 in both compartments indicates that a reactive (oxygen) species is involved, that can migrate or diffuse from the cathodic to the anodic half-cell.

2.3.4 LC-MS/MS analysis of oxidation products at m/z 207 and 251

The structures of the oxidation products at m/z 207 and 251 were characterized by LC-MS/MS. Figure 7 shows product ion scans for lidocaine, m/z 235, and its two oxidation products. The product ion scan for the unmodified lidocaine peak (parent ion at m/z 235) showed an intense fragment ion at m/z 86 corresponding to the tertiary amine group (Figure 7-a). The corresponding fragments containing the aromatic ring did not give rise to detectable ions. The product ion scan for m/z 207 showed an intense signal at m/z 58 (Figure 7-b) corresponding to the absence of one ethyl chain from the original tertiary amine group, confirming that this is the N-dealkylation product. It has been demonstrated that anodic oxidation of amines proceeds via electron transfer and deprotonation, to give the iminium intermediate that, after hydrolysis and intramolecular rearrangement, leads to N-dealkylation (Scheme 2) [26].

![Scheme 2. Proposed reaction mechanisms for the electrochemical N-dealkylation initiated by single electron transfer (SET) on the auxiliary electrode.](image-url)
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Figure 6. LC-MS analysis of oxidation products obtained from 10 mM lidocaine oxidized in an aerated solution of 0.1 M tetrabutylammonium perchlorate in acetonitrile/water 99/1 (v/v) on a gold electrode at -1.0 V for one hour in a two-compartment cell. (a) Cathodic compartment (working electrode), and (b) anodic compartment (auxiliary electrode).
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Figure 7. Positive product ion scans (LC-ESI-MS/MS) of (a) lidocaine (m/z 235), (b) its m/z 207 N-dealkylation product, and (c) its m/z 251 N-oxide product. (d) APCI-MS spectrum for the N-oxide, showing thermal degradation in the heated nebulizer.

The product ion scan for m/z 251 showed fragments at m/z 86, 88 and 130 (Figure 7-c). Incorporation of an oxygen atom in lidocaine can take place in the following positions: the aromatic ring (hydroxylation on carbon 3 or 4), one of the methyl groups (benzylic hydroxylation), and on the amino-nitrogen (N-oxide formation). Fragment ion m/z 130 points to incorporation of oxygen on the right hand side of the amide bond, which rules out hydroxylation in the ring or in the benzylic position. Fragment ion m/z 86 seems to exclude formation of the N-oxide. However, the fragment ion at m/z 88 supports the assumption of N-oxide formation (see Scheme 3-a). Formation of the fragment ion m/z 86 can be rationalized by OH group migration in the protonated N-oxide. Since the combination of electrospray ionization and CID does not provide a distinction between N-oxide...
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and some other oxidation product, the sample was subjected to atmospheric pressure chemical ionization (APCI). During APCI, N-oxides readily undergo thermal degradation prior to ionization, leading to diagnostic ions [27]. Figure 7-d shows the thermally-induced degradation of the MW=250 Da oxidation product, with peaks at m/z 235, 223 and 207 which correspond to oxygen atom loss, Cope elimination, and Meisenheimer rearrangement, respectively, as illustrated in Scheme 3-b, and described by Ma et al. [27]. This was confirmed in a control experiment, where the N-oxide was synthesized by reaction of lidocaine with m-chloroperbenzoic acid. LC retention time and MS/MS spectrum of the chemically synthesized N-oxide were identical to those obtained for the electrochemical reaction product.

2.3.5 N-oxide formation and hydroxylation

Since electrochemical reduction of molecular oxygen may generate radical species and hydrogen peroxide, we incubated lidocaine with hydrogen peroxide and subjected lidocaine to the Fenton reaction, which catalytically activates hydrogen peroxide to form hydroxyl radicals.

Incubation of lidocaine with 1 vol % hydrogen peroxide for one hour under the same solvent conditions as used for electrochemistry resulted in a significant amount of the m/z 251 product, with the same retention time and MS/MS spectrum as the electrochemically-generated N-oxide. No measurable amount of other oxidation products was detected. Apparently, the electrochemically generated ROS is hydrogen peroxide, produced as follows: superoxide anions generated at the working electrode at -1.0 V abstract a proton from water, or lidocaine itself in the absence of water, to generate perhydroxyl radicals (reaction 3), followed by formation of hydrogen peroxide in reactions 4 and 7. The reaction between hydrogen peroxide and tertiary amines has been documented to lead to the formation of N-oxides [28]. The relatively high amount of the N-oxide in the anodic compartment is explained by diffusion of hydrogen peroxide or migration of its anion from the cathodic to the anodic compartment.

The Fenton reaction in the presence of lidocaine leads to a number of products, including five LC peaks at m/z 251 (Figure 8). Co-injection with 3-hydroxylidocaine shows that this compound co-elutes with peak c (Figure 8-a), and that its MS/MS spectrum (Figure 8-a) is identical to that of 3-hydroxylidocaine. According to the MS/MS spectra (Figure 8-b and c), it is likely that the other two major m/z 251 peaks are 4-hydroxylidocaine (peak b) and lidocaine that is hydroxylated on the benzylic position (peak d). Benzylic hydroxylation leads to facile water loss as indicated by the fragment at m/z 233. The short retention times of peaks b, c, and d are consistent with benzylic and aromatic hydroxylation, which reduce the hydrophobicity of lidocaine. The small peak e has the same MS/MS spectrum as peak d, but cannot be easily assigned. Peak f has an identical MS/MS spectrum and retention time as the N-oxide generated by electrochemistry.
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(a) Collision-induced fragmentation

(b) Thermally-induced degradation

Scheme 3. (a) Proposed collision-induced fragmentation during MS/MS for the protonated N-oxide of lidocaine (see Figure 7-c); (b) Proposed thermally-induced degradation during APCI for the N-oxide of lidocaine (see Figure 7-d).
Figure 8. (a) Extracted ion chromatogram for m/z 251, obtained from the Fenton reaction in the presence of lidocaine (solid line), the same sample with co-injected 3-hydroxylidocaine (dashed line), and (b-f) the corresponding MS/MS spectra for m/z 251 peaks labeled b to f.
These results indicate that hydroxyl radicals, generated by the Fenton reaction, lead primarily to hydroxylation. These hydroxylation products were not observed in the EC cell. Therefore, reduction of molecular oxygen, under our conditions does not generate hydroxyl radicals in sufficient amounts to produce detectable hydroxylation of lidocaine.

2.4 Conclusions

Electrochemical reduction of molecular oxygen on a gold electrode led to ROS intermediates, as shown by CV. The major reactive species was hydrogen peroxide as indicated by formation of the N-oxide of lidocaine as main oxidation product. Addition of 1 % water resulted in well-controlled conditions with an optimum potential for product formation of -1.0 V. A standard single-compartment cell led to a mixture of the N-oxide and the N-dealkylation product due to $\text{H}_2\text{O}_2^-$ mediated and direct electrochemical oxidation, respectively. Separation of anodic and cathodic compartments shows that direct oxidation occurs at the auxiliary electrode at a positive potential of 1.1 V. Aromatic or benzylic hydroxylation products, both in vivo metabolites of lidocaine, are not formed in the electrochemical cell. These can be produced by reaction with hydroxyl radicals using the Fenton reaction.
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2.5 References


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