Electrochemistry in the mimicry of oxidative drug metabolism
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Chapter 4
Electrochemical Oxidation by Square-Wave Potential Pulses in the Imitation of Phenacetin to Acetaminophen Biotransformation

Electrochemistry in combination with mass spectrometry is emerging as a versatile analytical technique in the imitation of oxidative drug metabolism during the early stages of drug discovery and development. Here, we present electrochemical O-dealkylation of phenacetin to acetaminophen by square-wave potential pulses consisting of consecutive sub-second oxidation and reduction steps. This O-dealkylation could not be achieved by oxidation at constant potential or longer potential pulses because of the fast hydrolysis of the reactive intermediates. Electrochemical conversion by square-wave potential pulses can thus widen the scope of electrochemical synthesis of metabolites and imitation of in vivo drug metabolism.

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4.1 Introduction

New analytical techniques capable of imitating various in vivo oxidation reactions are required to study oxidative drug metabolism during the early stages of drug discovery and development [1, 2]. Direct electrochemical oxidation in combination with mass spectrometry has been shown to be a versatile analytical technique in the imitation of in vivo oxidation reactions that are initiated by single electron transfer (SET), such as N-dealkylations, whereas it proved to be difficult to promote oxidation reactions that are initiated by oxygen insertion or hydrogen atom transfer (HAT), such as O-dealkylations [3, 4]. Biotransformation of phenacetin to the therapeutically active acetaminophen, a known O-dealkylation reaction catalyzed by CYP1A2, a member of Cytochrome P450 family, is considered to be initiated by HAT from the alpha carbon of the ethoxy group [5, 6]. We have recently shown that the use of square-wave potential pulses could imitate the oxidation reactions initiated by SET, by higher yields and selectivity based on the applied cycle time [7]. In this study we show that though the transformation of phenacetin to acetaminophen by direct electrochemical oxidation is not possible, the electrochemical oxidation by sub-second square-wave potential pulses promotes the transformation of phenacetin to acetaminophen. The reaction mechanism will be studied by isotope labelling and stabilization and characterization of the intermediates.

As proposed by Kissinger and colleagues, the electrochemical oxidation of phenacetin involves formation of a quinone-imine cation intermediate, where the oxygen remains substituted (Scheme 1, compound 1) [8]. Water or hydroxide anions attack rapidly, liberating ethanol and producing N-acetyl-p-benzo-quinone imine (NAPQI) [8]. Similarly, the peroxidase-catalyzed O-demethylation of 9-methoxyellipticine was shown to proceed through the formation of a quinone-imine derivative intermediate followed by demethoxylation [9]. In aqueous solutions a rate constant of 2500 s⁻¹ for the decomposition of 1 to NAPQI at pH 6.7 was measured by double potential step chronoamperometry [8]. NAPQI is the major in vivo and in vitro oxidation product of acetaminophen, but electrochemical oxidation of acetaminophen in water results in dimerization between pH 5 and 7, aromatic hydroxylation at basic pH, and oxidation to p-quinone at acidic pH [10]. NAPQI reacts in vivo with nucleophilic antioxidants such as glutathione (GSH) during phase II drug metabolism.

4.2 Experimental Procedure

Reagents. All reagents were from electrochemical and analytical grade and purchased from Sigma-Aldrich. Water was purified by a Maxima Ultrapure water system (ELGA, High Wycombe, Bucks, UK).
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**Electrode preparation.** The surface of a platinum disk electrode with 2.0 mm diameter (MF-2071, 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 set up.** 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, NSW, Australia). The electrochemical cell was a three electrode cell in which the working electrode was a platinum disk and the auxiliary electrode a platinum wire (MW-4130, BASi). It was constructed as a two-compartment cell by using a porous Vycor tip with Teflon heat shrink (MF-2064, BASi) to separate working and auxiliary half-cells. The reference electrode was placed in the working compartment. The auxiliary compartment was always under continuous argon atmosphere. 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. All experiments were performed at ambient temperature.

Solutions containing 10 mM phenacetin and 0.1 M tetrabutylammonium perchlorate (TBAP) dissolved in acetonitrile/water 99/1 (v/v) (0.1 M TBAP used as electrolyte to provide sufficient conductivity for electrochemical experiments) were subjected to constant potential and potential pulse oxidation, for 30 minutes prior to LC-MS and LC-MS/MS analysis. Samples were collected and diluted 100 times in water containing 0.5 μM lidocaine, as an internal standard for LC-MS signal normalization, immediately after the batch oxidations and stored at room temperature until LC-MS analysis.

**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) upgraded to EP10+ (Ionics, Bolton, ON, Canada) with electrospray ionization in the positive mode. The MS parameters were as follows: ion spray voltage 4800 V, OR 40 V, and RNG 120 V, with scans between m/z 100-600 (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. Negative ion Atmospheric Pressure Chemical Ionization (APCI) experiments were done with OR -5 V, and RNG -30 V, with scans between m/z 50-500 (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 250 °C. APCI generated the molecular anion of p-quinone at m/z 108. 

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Scheme 1. Electrochemical oxidation of phenacetin to p-quinone (path A) and to acetaminophen (path B) in a solution of 0.1 M tetrabutylammonium perchlorate in acetonitrile/water 99/1 (v/v). After initial oxidation, the quinone-imine intermediate 1, is hydrolyzed to NAPQI. In the presence of pyridine, 1 reacts instead to generate 2. Under constant potential oxidation, NAPQI is hydrolyzed further to p-quinone (path A), and reaction of p-quinone after addition of glutathione (GSH) generates 3. Under square-wave potential pulses, generation of NAPQI was followed by reduction to acetaminophen (path B) during the reduction step of the potential pulse.
A C18 reversed-phase column (GraceSmart RP 18 5μm, 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. 5 μL of a diluted oxidation product mixture was injected and a linear gradient of 5-50 % B in 20 minutes was used for elution.

4.3 Results and discussion

The products of electrochemical oxidation of phenacetin at a constant potential of +3.0 V in 0.1 M tetrabutylammonium (TBA) perchlorate in acetonitrile/water 99/1 (v/v) were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). No oxidation product of phenacetin was observed by positive ion electrospray ionization (ESI). However, p-quinone was detected by negative ion atmospheric pressure chemical ionization (APCI). The absence of detectable amounts of p-quinone in the LC-(ESI)-MS analysis is due to its very low electrospray ionization efficiency. In order to verify the formation of 1, constant potential oxidation in the presence of 1 vol % pyridine was performed, which produced a compound with m/z 257 resulting from nucleophilic attack of pyridine on 1 to generate 2 (see Scheme 1). The structure of 2 was in agreement with MS/MS analysis (Figure 1). As shown in Figure 1, the oxidation product (m/z 257) loses pyridine (a), ketene (b), and ethene (c). This confirms that nucleophilic attack of pyridine on 1 makes it detectable by LC-MS/(MS).

Compound 1 is expected to be hydrolyzed rapidly to NAPQI and NAPQI can be further hydrolyzed to p-quinone with a half-life of a few seconds (path A in Scheme 1) [11]. In order to reveal the presence of NAPQI as an intermediate and confirm p-quinone as the final oxidation product, reaction mixture was diluted in an aqueous solution of 1 mM GSH to capture them in analogy to phase II drug metabolism. LC-MS/(MS) confirmed generation of the reaction product of GSH with p-quinone (compound 3 in Scheme 1 and Figure 2). As shown in Figure 2, in order to reveal the generation of p-quinone during constant potential oxidation, an experiment was performed to capture p-quinone by using glutathione (a reaction that occurs also in vivo). LC-MS analysis showed a reaction product at m/z 416 after dilution of the reaction mixture in an aqueous solution of 1 mM glutathione. Collision induced dissociation (CID) of this reaction product showed the y$_2$, b$_2$, and z$_2$ fragments. The reaction of p-quinone standard with glutathione, as a control experiment, showed the same product at m/z 416 with the same CID fragmentation pattern. There was no detectable amount of the reaction product of GSH with NAPQI.
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Figure 1. Collision induced dissociation (CID) at a collision energy of 30 eV of the oxidation product at \( m/z \) 257, 2 in Scheme 1, generated during electrochemical oxidation at constant potential of +3.0 V from a solution of 10 mM phenacetin in 0.1 M tetrabutylammonium perchlorate dissolved in acetonitrile/water 99/1 (v/v) in the presence of 1 vol % pyridine.

We reasoned that after hydrolysis of 1, the short-lived NAPQI intermediate could be reduced to acetaminophen by an immediate reduction step in a square-wave potential pulse. Oxidation at +3.0 V was thus followed by reduction at -1.0 V with a cycle time that was varied between 20 ms and 200 s. Figure 3 shows that acetaminophen was produced with increasing yield when cycle times are shorter than 1 s. One hour electrochemical oxidation with potential pulses of 200 ms cycle time resulted in 6-7% conversion of phenacetin to acetaminophen based on LC-MS analyses of the reaction products and standard addition of acetaminophen. We expect that this yield can be further augmented by using longer reaction times and electrodes with a larger surface area.
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Figure 2. Collision induced dissociation (CID) at a collision energy of 25 eV of the reaction product at m/z 416, 3 in Scheme 1.

We hypothesize that the oxidation of phenacetin under square-wave potential pulses begins with formation of 1, which reacts with water to generate NAPQI. By rapidly changing the potential on the surface, NAPQI is reduced to acetaminophen (path B in Scheme 1). When phenacetin was subjected to square-wave potential pulses of 200 ms in the presence of pyridine, 2 was generated, while no acetaminophen was observed. Therefore, scavenging 1 with pyridine blocks the generation of acetaminophen during square-wave potential pulses in agreement with the proposed reaction mechanism.
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**Figure 3.** MH+ ion intensities of acetaminophen (relative to a lidocaine standard) extracted from LC-MS analyses of a solution of 10 mM phenacetin in 0.1 M tetrabutylammonium perchlorate in acetonitrile/water 99/1 (v/v) that was subjected to square-wave potential pulses alternating between +3.0 V and −1.0 V of varying cycle times for 30 min (individual results of triplicate reactions are shown).

Compound 1 may convert to NAPQI by reaction with water either on the alpha carbon of the ethoxy group leading to O-deethylation, or on the aromatic ring leading to O-deethoxylation through nucleophilic addition and intramolecular rearrangement (see **Scheme 2**). The latter is the proposed mechanism in O-dealkylations catalyzed by peroxidases [9]. To study the hydrolysis mechanism, H$_2^{16}$O was substituted by H$_2^{18}$O followed by mass spectrometric analysis of the reaction products. Both $^{18}$O- and $^{16}$O-labeled acetaminophen were found under pulse conditions indicating that hydrolysis proceeds via either of the two reaction pathways.
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Scheme 2. Electrochemical oxidation of phenacetin under square-wave potential pulses proceeds via the quinone-imine intermediate 1. Hydrolysis to NAPQI may occur through two parallel reaction pathways which can be verified by performing the reaction in the presence of $H_2^{18}O$. Hydrolysis on the ethoxy alpha carbon leads to O-deethylation (top reaction) without incorporation of $^{18}O$ in NAPQI. Hydrolysis on the aromatic ring leads to O-deethoxylation through nucleophilic addition and intramolecular rearrangement (bottom reaction) with incorporation of $^{18}O$ in NAPQI. In both cases the hydrolysis is followed by the reduction of NAPQI to acetaminophen during the reduction step.

4.4 Conclusions

In conclusion, the electrochemical approach using square-wave potential pulses is capable of converting phenacetin to acetaminophen, a dealkylation reaction that is not accessible by electrochemical oxidation at constant potential. Pulse time played a crucial role: only short, sub-second pulse times promoted the O-dealkylation reaction. The combination of electrochemical oxidation with a reduction step in the form of a square-wave potential pulse, thus widens the scope of electrochemistry in the imitation of oxidative drug metabolism, as well as the synthesis of drug metabolites.
4.5 REFERENCES:


