

Use of Electrochemical Flow Cells to Facilitate Early Stage Characterization of Drug Metabolites and Reactive Species

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Introduction

New U.S. FDA guidelines on Metabolites in Safety Testing (MIST) have led pharmaceutical companies to reassess the timing of drug metabolite studies within the development process. To facilitate effective decision making, information on metabolite identity, quantity, pharmacological, and toxicological effects is now often required at earlier stages. Oxidation is a primary route of drug metabolism, and can result in the production of reactive species that may lead to adverse effects. Previous studies have shown that electrochemical (ECE) oxidation can be used to produce species that correspond to biological oxidative metabolites. Here, ECE flow cells were used on line with liquid chromatography for ECE synthesis of products from model compounds. A variety of approaches were used, including on-line coupling with mass spectrometry (MS) for rapid characterization of both stable and reactive products. Nucleophilic reagents (e.g., glutathione) were also used to trap reactive electrophilic species, further facilitating their characterization. High-capacity ECE flow cells were also used for semipreparative scale synthesis. The results shown here indicate that ECE allows rapid, controlled production of species that correspond to biological metabolites, including those involving dehydrogenation, secondary and tertiary N-dealkylation, S-oxidation, N-oxidation, and O-dealkylation. Furthermore, ECE formation of reactive species is demonstrated with either direct or indirect (i.e., trapped electrophile) determination by MS. These data demonstrate that ECE flow cells may facilitate early-stage metabolite studies in a variety of ways, including the generation of product in sufficient quantity for use as standards for quantitation, to study biological effects, and to perform more in-depth structural elucidation. A particular advantage of this purely instrumental approach may be its ability to study certain reactive species where nonspecific binding may prevent detection in biological systems.

Experimental

Electrochemical Instrumentation

Thermo Scientific Dionex Coulochem™ III Electrochemical Detector for MS with Model 5021A Conditioning Cell (P/N 70-6045A) or Model 5125 Synthesis Cell (P/N 70-7700)

Thermo Scientific Dionex CoulArray™ Multi-Channel (8) Electrochemical Detector (P/N 70-4325)

ECE cells were used in various configurations in combination with LC-MS including:

Flow Injection Analysis (FIA): Pump → Autosampler → ECE → MS

Pre-column ECE: Pump → Autosampler → ECE → Column → MS (and ECE array)*

**For some studies, postcolumn flow was split between MS and multichannel ECE array detection (i.e., CoulArray Detector).*

LC-MS Instrumentation

HP 1100 LC/MSD single quadrupole mass spectrometer (Agilent Technologies)

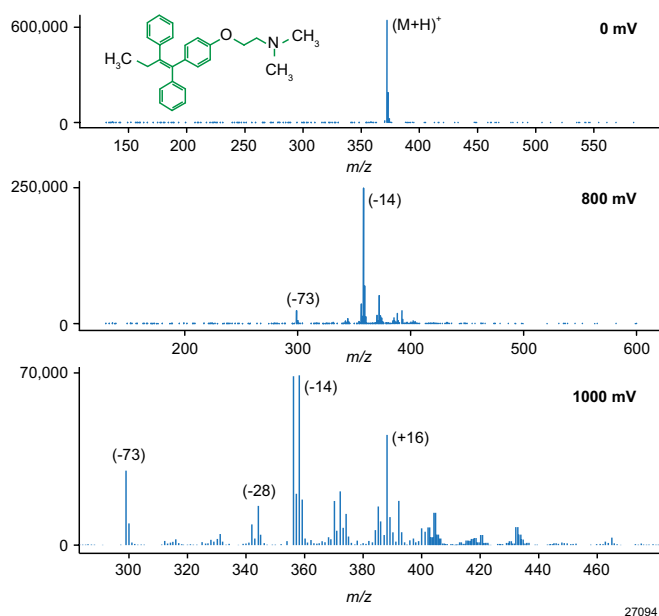
MS conditions (unless otherwise noted): Positive ESI, scan 80 to 500 m/z . Fragmentor 70, gain 1.0, threshold 150, step 0.25, drying gas flow 12 L/min, Nebulizer pressure 35 psig, drying gas temperature 350 °C, capillary voltage 3500 V.

Specific chromatographic and ECE conditions are described with results.

Flow Injection Analysis (FIA)

Figure 1 shows positive electrospray ionization (ESI) mass spectra for a representative compound, tamoxifen, analyzed by flow-injection analysis (FIA) with an on-line ECE cell at three different potentials. Each FIA result was obtained in <30 s. With the ECE cell at 0 mV, the most abundant ion (m/z 372) corresponded to protonated tamoxifen. At 800 mV, the most abundant ion was m/z 358. This mass shift of -14 is consistent with N-demethylation, a primary in-vivo Phase I oxidative reaction of tamoxifen. At 1000 mV, additional product ions were observed with mass shifts corresponding to other common enzymatic oxidative reactions: N-dealkylation (-28), O-dealkylation (-73), and N- or C-oxidation (+16).

FIGURE 1. ESI mass spectra from FIA of 10 μ L of 20 μ g/mL tamoxifen diluted in mobile phase (50% methanol in 20 mM ammonium acetate). Flow rate = 0.1 mL/min. Model 5021 EC cell potentials of 0, 800, or 1000 mV (vs Pd reference electrode).

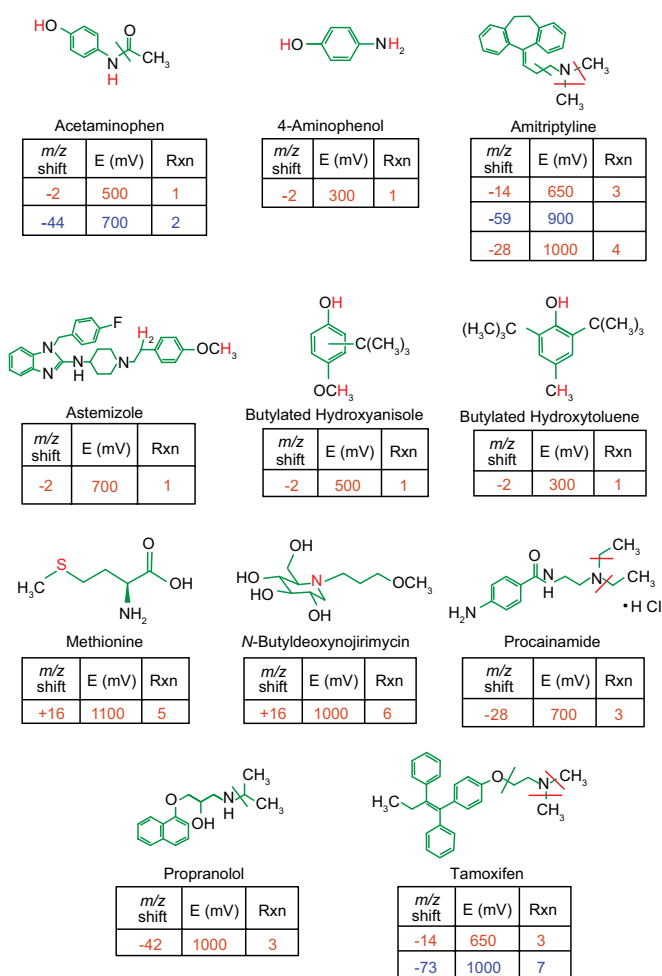


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Comparison studies conducted by Jurva, et al.¹ have shown that ECE oxidation using a porous carbon working electrode (WE) generally leads to the formation of similar products for cytochrome P-450 (CYP-450) catalyzed reactions that are supposed to proceed through a mechanism initiated by a one-electron transfer oxidation. This includes dehydrogenation, N-deacetylation, N-dealkylation, S-oxidation and aromatic O-dealkylation. CYP-450 reactions initiated by H-atom abstraction, i.e., aliphatic C-oxidation and hydroxylation of aromatic rings without electron-donating groups are, however, generally not mimicked by ECE oxidation.

Despite this limitation, the instrumental LC-ECE-MS technique provides a simple and rapid means of obtaining information on relative ease and likely chemical sites of oxidation, as well as the nature of products obtained from these on-line reactions. These data are typically generated to provide input to lead optimization strategies or to provide structural alerts for oxidatively unstable compounds. An example of this is shown in Figure 2, which summarizes results from FIA of a series of compounds analyzed using ECE potentials of 0, 400, 800, and 1200 mV vs Pd.

FIGURE 2. Representative compounds, mass shifts, and likely sites of EC oxidation. Estimated redox potential vs Pd (E[mV]) and likely reactions (Rxn) are: 1) dehydrogenation; 2) N-deacetylation; 3) 3° N-dealkylation; 4) 2° N-dealkylation; 5) S-oxidation; 6) N-oxidation; 7) O-dealkylation.

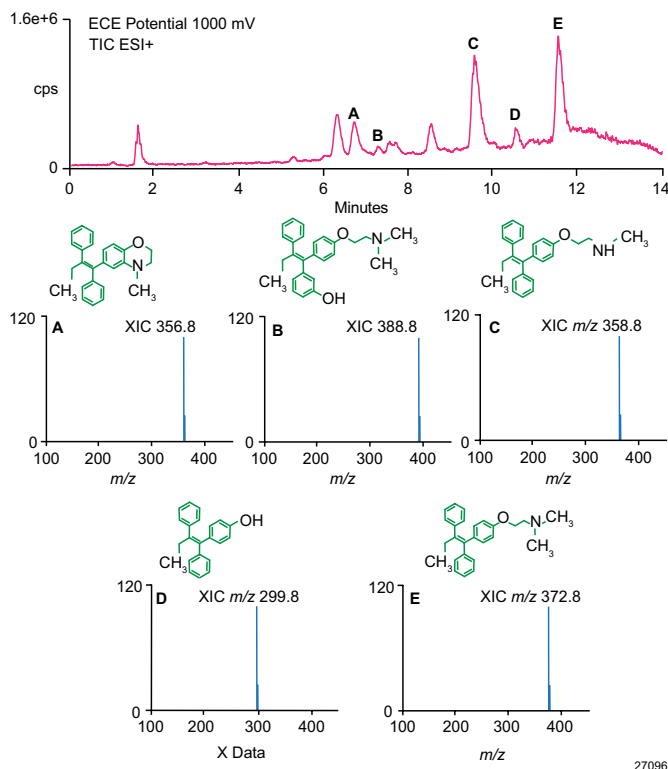


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Precolumn ECE Synthesis

A logical extension of FIA is to incorporate an LC separation step to allow more detailed characterization of ECE-generated products. Figure 3 shows a total ion chromatogram obtained for tamoxifen with precolumn ECE oxidation at 1000 mV. Several peaks corresponding to oxidation products are evident. This example illustrates on-line generation and analysis of ECE products using LC-MS conditions that are typical of metabolic studies (e.g., in vitro microsomal analysis). Serial LC-ECE-MS can thus be used with neat parent compound solutions for preliminary optimization of LC and MS/MS conditions for subsequent metabolite analysis in biological samples. By using identical conditions, ECE data may then be used as input to automated metabolite identification software to aid in finding metabolites present in more complex biological matrices.²

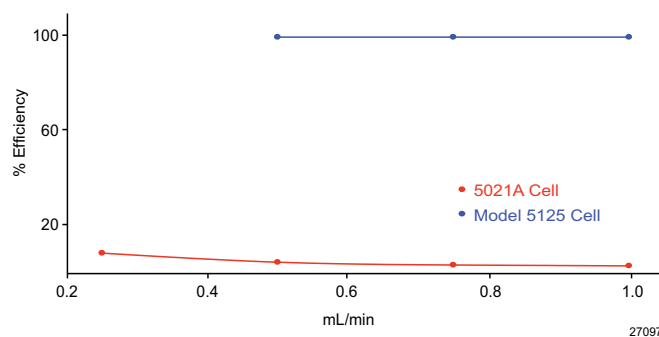
FIGURE 3. On-line oxidation of tamoxifen (50 μ L of 20 μ g/mL diluted in mobile phase) using precolumn ESA Model 5021 EC cell (500 mV vs Pd). Binary gradient from 16 to 80% acetonitrile over 10 min, 20 mM ammonium acetate as supporting electrolyte. Shiseido C18 MG 3 μ 75 \times 4.6 mm i.d. column, 1 mL/min, 200 μ L/min split to MS.



Furthermore, when the data from an ECE-generated product correspond to that of a biological metabolite, the ECE technique may then be viewed as a selective and rapid synthetic route to small quantities of this metabolite. In this example, a Model 5021A cell was used to produce estimated ng quantities of metabolites.

The recent development of higher-capacity coulometric synthesis cells provides the ability to produce larger quantities for more detailed structural elucidation studies. Figure 4 shows that high efficiency is maintained for oxidation of 2 μ g quantities of amitriptyline over a range of flow rates, thus facilitating the production of quantities that may be analyzed by NMR.

FIGURE 4. Effect of flow rate on % efficiency for a given cell. Oxidation efficiencies were measured for 2 μ g AMI on column (10 μ L injection).



Generation and Characterization of Reactive Species

Many studies suggest that redox metabolism of a wide range of chemical structures leads to formation of reactive electrophiles which participate in a diverse array of toxic processes that typically involve covalent binding or other modifications to small and large molecules. The propensity of compounds to undergo redox-based metabolic activation to form reactive electrophilic species is therefore a major consideration in pharmaceutical development. Several reports have shown LC-ECE-MS useful in the study of reactive intermediate metabolites.^{3,4,5}

Figure 5 provides a basic example of precolumn ECE oxidation using the widely studied compound, acetaminophen (APAP). Oxidative metabolic activation of APAP to form *N*-acetyl, *p*-benzoquinoneimine (NAPQI) is widely regarded as an essential component of its hepatotoxic effects in humans. MS data indicate that ECE oxidation of APAP in the presence of GSH (co-injected) resulted in two separate peaks corresponding to monogluthionyl conjugates, and one peak indicative of a di-gluthionyl conjugate.

FIGURE 5. Precolumn oxidation of APAP with GSH as trapping agent. 10 μ L of 20 μ g/mL APAP, 1 mM GSH mixture with precolumn ESA Model 5021 cell at 500 mV vs Pd. Binary gradient elution from 1 to 80% CH_3CN in 5 min, 20 mM ammonium acetate as supporting electrolyte. Shiseido C18 MG 3 μ m 50 \times 4.6 mm i.d. column, 1 mL/min, 200 μ L/min split to MS. MS conditions as described in the Experimental section, except scan range was 80 to 1000 m/z .

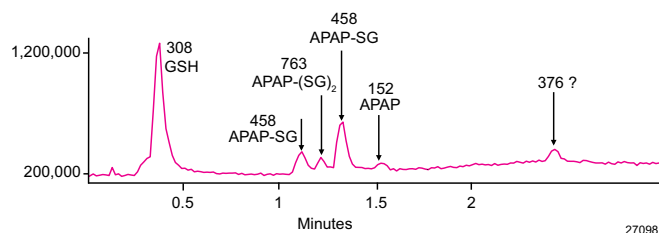
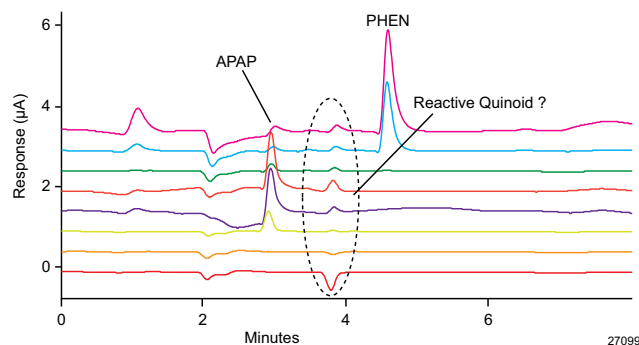


Figure 6 illustrates the precolumn oxidation of a phenacetin solution with LC-ECE-Array detection. A peak, which eluted at 3.8 min, shows a characteristic voltammetric profile (i.e., reduction followed by oxidation) of a quinone species. Based on ECE Array and MS data (not shown), this peak has been identified as NAPQI, the expected reactive intermediate. This peak was not evident in a microsomal incubate of phenacetin analyzed using the same conditions (not shown). A possible explanation for this is that nonspecific binding of this reactive species occurred in the biological preparation.

FIGURE 6. Precolumn oxidation of phenacetin. Conditions as in Figure 3 except LC detector was an 8-channel CoulArray detector with Model 6210 EC cell potentials of 0 to 840 mV vs Pd in 120 mV increments.



Conclusion

These results show that ECE coupled with MS is capable of mimicking certain Phase I enzymatic reactions, including dehydrogenation, N-deacetylation, N-dealkylation, S-oxidation, and aromatic O-dealkylation. Furthermore, ECE formation of reactive intermediates is demonstrated from acetaminophen and phenacetin, either directly or indirectly (i.e., trapped electrophile) determined by MS and ECE. These data demonstrate that coulometric ECE flow cells provide significant flexibility and simplicity in experimental design to facilitate a variety of studies relevant to metabolism and toxicity. Furthermore, this purely instrumental technique may provide significant advantages for studying certain reactive species where nonspecific binding (i.e., interaction with endogenous biochemicals) may prevent detection in biological systems.

References

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