

ADME/Tox Profiling

Using Coulometric Electrochemistry and Electrospray Ionization Mass Spectrometry

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This article describes the use of serial coulometric flow cells coupled online with electrospray ionization mass spectrometry for rapid electrochemical study of biologically relevant oxidation-reduction reactions.

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A major focus in today's pharmaceutical development process is reducing the incidence of late-stage compound failure. Such attrition is commonly a result of unfavorable properties including absorption, distribution, metabolism, excretion, toxicity (ADME/Tox), and stability. To address this concern, predictive ADME/Tox assays are now being implemented at very early stages of drug discovery and development. In vitro methods of studying metabolism and toxicity are very useful for understanding the metabolic pathways and potential interactions of candidate compounds. Data obtained with assays that involve use of biological extracts, however, are sometimes compromised by changes in absolute and relative enzymatic activity, loss of cofactors, non-specific reactions, and other variables. These issues limit the generation of broadly applicable, modeling-friendly ADME/Tox data for high-throughput profiling of large compound libraries. Alternative and complementary techniques are thus actively being explored with a goal of more timely prediction of drug-like properties and more highly focused discovery and development efforts (1).

Oxidative and reductive (redox) reactions are clearly important to the chemi-

cal and biological properties of most pharmaceuticals:

- the most common Phase I metabolic biotransformation reactions of xenobiotic compounds are oxidative (2)
- chemical degradation often proceeds through oxidative mechanisms (3)
- the toxicity of many organic chemicals is linked to redox-based metabolic activation to form reactive electrophilic species (4)
- the actions of therapeutic agents and the physiological conditions they target are very frequently associated with redox metabolism and oxidative stress (4).

It is interesting to note that, although only a small percentage of organic chemicals undergo facile electrochemical (EC) reactions, most approved pharmaceutical compounds can be easily oxidized or reduced (5).

Coulometric EC flow cells have gained widespread use as high performance liquid chromatography detectors (HPLC-ECD) for the study of redox-active chemicals based on their ability to produce highly specific (potential-dependent) and reproducible EC reactions. The primary use of coulometric ECD has been for quantitative bioanalysis of antioxidants, markers of oxidative stress, neurotransmitters, pharmaceuticals, and vitamins (6). Additional application of coulometric cells involves their use as a simple means of producing redox reactions, the products of which may then be studied by mass spectrometry (MS) and various forms of spectroscopy and MS. Serial coupling of EC with MS (EC-MS) has been previously described as a very useful tool to provide insight into biological and chemical redox processes. Examples of this approach include direct infusion, flow injection analysis (FIA) and pre- and post-column HPLC. Jurva et al. used

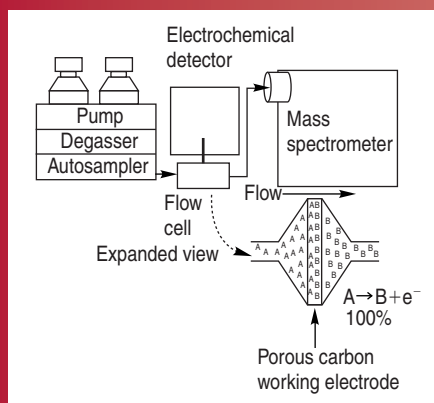


Figure 1. EC-MS system diagram with an expanded representation of the EC flow cell with a porous graphitic carbon working electrode. The 3-D configuration and turbulent flow within this porous structure leads to high electrolytic efficiency. Conversion of reduced analyte species (A) to oxidized species (B) with associated loss of one electron is shown.

coulometric EC to mimic N-dealkylation, sulfoxidation, and desulfuration (7). Volk et al. used a coulometric cell to mimic purine metabolism (8). Deng and Van Berkel used thin-layer EC to study the oxidation products of dopamine and their subsequent reaction with benzene thiol (9). Getek et al. studied the oxidative formation of the reactive intermediate, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), from acetaminophen and follow-up chemical addition reactions with infused nucleophile (10). In this article, we further evaluate the extent to which coulometric EC may be used to assess drug-like properties of pharmaceutical library compounds. A primary focus of this work is on modeling of biological redox reactions in the context of high-throughput prediction of oxidative metabolism and metabolic activation that may lead to toxic effects.

Experimental

A model 1100 LC/MSD single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA) was used in combination with a Coulochem III ECD and model 5021 coulometric cell (ESA, Chelmsford, MA), as shown in Figure 1.

Compounds (200 ng each), diluted in mobile phase (50% aqueous methanol

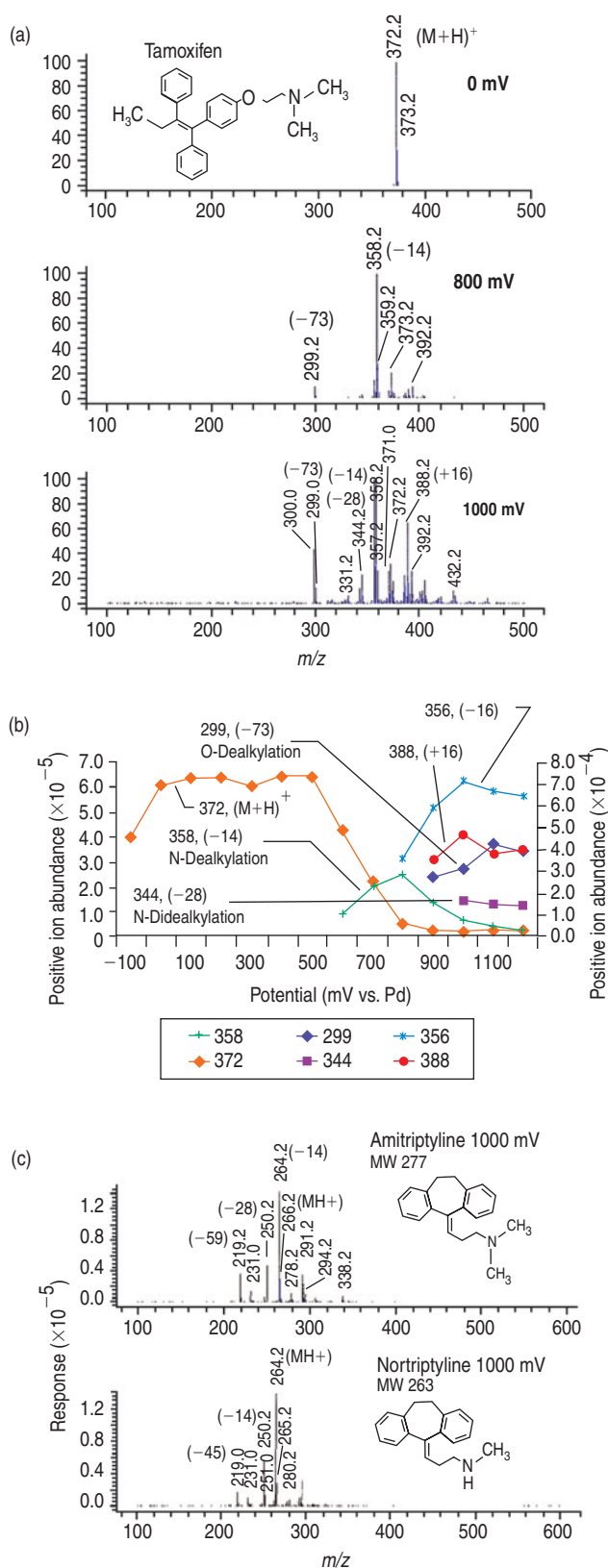
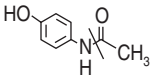
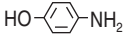
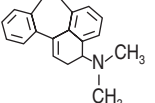
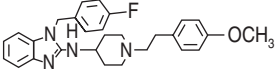
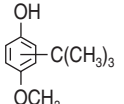
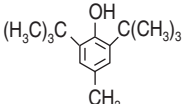
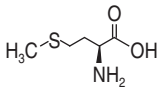
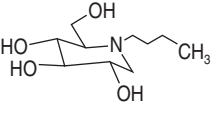
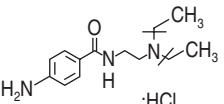
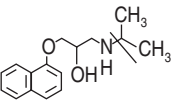
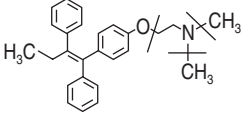


Figure 2. Examples of Phase I-type oxidative reactions. (a) Positive full scan ESI mass spectra of tamoxifen with EC = 0, 800, and 1000 mV vs. Pd. (b) Voltammetric mass spectral plot summarizing tamoxifen data. Left axis scale refers to 372 and 358 ion abundance; all other ions correspond to right axis scale. (c) Mass spectra of amitriptyline and nortriptyline with EC cell at 1000 mV.

			
Acetaminophen	4-Aminophenol	Amitriptyline	Astemizole
m/z shift	m/z shift	m/z shift	m/z shift
E (mV)	E (mV)	E (mV)	E (mV)
Rxn	Rxn	Rxn	Rxn
-2	-2	-14	-2
500	300	650	700
1	1	3	1
-44		-59	
700		900	
2		-28	
		1000	
4			
			
Butylated hydroxyanisole	Butylated hydroxytoluene	Methionine	N-Butyldeoxyjirimycin
m/z shift	m/z shift	m/z shift	m/z shift
E (mV)	E (mV)	E (mV)	E (mV)
Rxn	Rxn	Rxn	Rxn
-2	-2	+16	+16
500	300	1100	1000
1	1	5	6
			
Procainamide	Propranolol	Tamoxifen	
m/z shift	m/z shift	m/z shift	
E (mV)	E (mV)	E (mV)	
Rxn	Rxn	Rxn	
-28	-42	-14	
700	1000	650	
3	3	3	
		-73	
		1000	
		7	

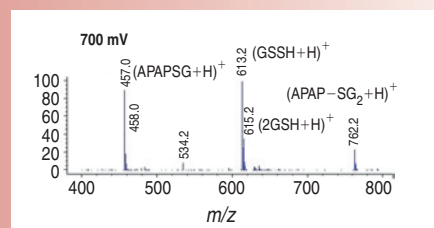


Figure 3 (left). Representative substrates, mass shifts, and likely sites ("soft spots") of EC oxidation. Rxn – likely reaction based on m/z : 1 = dehydrogenation, 2 = N-deacetylation, 3 = tertiary N-dealkylation, 4 = secondary N-dealkylation, 5 = S-oxidation, 6 = N-oxidation, 7 = O-dealkylation.

Figure 4 (above). Full-scan mass spectrum of a mixture containing acetaminophen and glutathione. The ions correspond to EC formation, at 700 mV vs. Pd, of protonated acetaminophen-monogluthionyl conjugate (APAP-SG), glutathione disulfide (GSSG), and acetaminophen-digluthionyl conjugate. Proton-associated dimer of glutathione (2GSH + H) was evident at all potentials.

with added electrolyte 20 mM ammonium acetate), were introduced into the system by FIA with a solvent flow rate of 0.1 mL/min. Compounds underwent rapid electrolysis and products were monitored by MS. Each compound was analyzed at several oxidative potentials (for example, 100, 300, 700, and 1100 mV vs. Pd reference). Substrate–product pairs were used to aid in the study of Phase I–type product formation as a function of potential (that is, voltammetric MS [VMS]). Study of Phase II type conjugation (chemical follow-up addition reactions of electrochemically generated electrophiles) was accomplished by co-injecting nucleophilic compounds (for example, glutathione [GSH]) at potentials selected based on the results of the previously mentioned voltammetric experiments.

Results and Discussion

The model 5021 coulometric EC cell used in these studies allowed reproducible and highly efficient (>95%) electrolysis at flow rates of as much as 1 mL/min. Electrolytic efficiency is afforded by the 3-D surface area of the

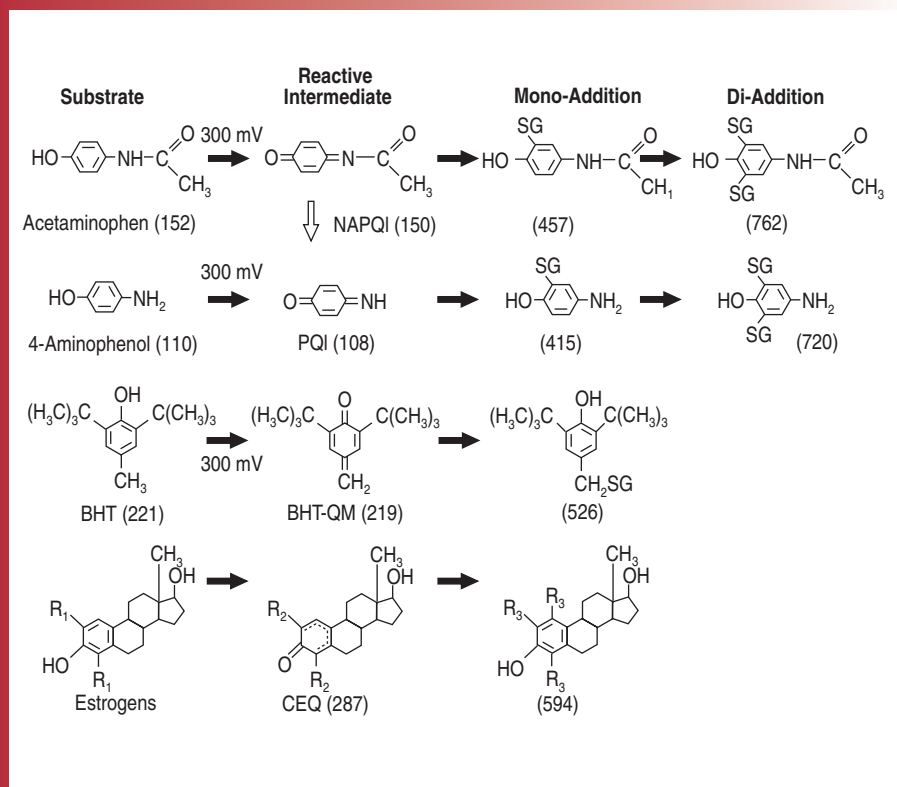


Figure 5. Proposed EC-generated reactive intermediates and addition products. Proposed structures are based on m/z and literature (10, 12, 13). Numbers in parentheses refer to m/z of protonated molecule. NAPQI = *N*-acetyl-*p*-benzoquinoneimine; PQI = *p*-benzoquinoneimine; BHT-QM = Butylated hydroxytoluene quinone methide; Estrogens include: 2- and 4-hydroxyestradiol ($R_1 = \text{H, OH}$), 2- and 4-methoxyestradiol ($R_1 = \text{H, OCH}_3$) and 17 β -estradiol ($R_1 = \text{H}$). CEQ = catecholesterogen quinones ($R_2 = \text{H, = O}$). For addition products, $R_3 = \text{H, SG}$. CEQ products were observed at 300 mV for 2- and 4-hydroxyestradiol, 700 mV for 2- and 4-methoxyestradiol, and 900 mV for 17 β -estradiol.

porous carbon working electrode (Figure 1). These properties and the cell's high-pressure capabilities provide versatility for use in FIA and pre- or post-column LC-MS with flow rates suitable for use with electrospray ionization (ESI), atmospheric pressure chemical ionization, and other LC-MS interfaces. For Phase I-type EC transformation studies, the changes in EC current, the corresponding consumption (oxidation) of substrate, and associated product formation were monitored as a function of electrode potential. In these studies, a solvent flow rate of 0.1 mL/min was used with ESI and a single analysis was complete in less than 2.5 min. To study both Phase I oxidative reactions and potential-dependent covalent binding (Phase II), total time for characterization of each compound required approximately 15 min (6 analyses/compound).

Given the efficiency of the EC cell, much higher throughput is theoretically possible at higher flow rates.

Phase I-type Oxidative Reactions

Product ions associated with EC oxidation of tamoxifen are evident in Figure 2a. These data are summarized in the VMS plot (Figure 2b). The most abundant ion, observed at low potential, corresponded in mass to protonated tamoxifen (m/z 372). At 500–800 mV, the abundance of protonated tamoxifen decreased with concomitant increase in ion abundance at m/z 358. Additional ions, as m/z , were evident at higher potentials. These mass shifts are likely to correspond respectively with oxidative N-demethylation (-14), N-deethylation (-28), O-dealkylation (-73), and N or C oxidation ($+16$). The mass spectra in Figure 2c provide

evidence that the most abundant, low potential, oxidation product of amitriptyline was its primary biological metabolite nortriptyline. Subsequent reaction of this product at higher potential led to product ions similar to nortriptyline itself.

Figure 3 summarizes results for the most abundant product ions obtained from oxidation of representative compounds. The type and relative ease (potential) of reaction are indicated, along with likely oxidative sites of each molecule. For most compounds studied, mass shifts corresponded to expected enzymatic Phase I oxidative reactions. As expected, certain biological reactions (for example, O-dealkylation of 7-ethoxycoumarin and aliphatic C-oxidation) were not mimicked electrochemically. However, these data, in agreement with literature, show significant overall relevance to the study of biological redox metabolism. Furthermore, the nature of this EC-MS approach is very applicable to assessment of liabilities related to chemical oxidative degradation. This on-line coulometric EC-MS approach therefore represents a rapid means of studying relative compound reactivity, resultant formation of related substances (metabolites and degradants) and determination of metabolic and chemical soft spots for early profiling of drug candidates.

Phase II-type Reactions (Potential-Dependent Covalent Modifications)

Acetaminophen is a frequently administered drug, and its oxidative metabolic activation to form reactive NAPQI is widely regarded as an essential component of its hepatotoxic effects in humans (11). Numerous retrospective studies have suggested that reactive electrophiles are thought to arise, via redox metabolism, from a wide range of chemical structures and to act in a diverse array of toxic processes that typically involve covalent binding or other modifications to small and large molecules (for example, DNA, proteins, peptides, and lipids), redox cycling, antioxidant-scavenger depletion, and other elements of oxidative stress. The propensity of library candidate com-



pounds to similarly undergo redox-based metabolic activation to form reactive electrophilic species is therefore a major consideration in pharmaceutical development. As indicated in Figure 3, EC oxidation of acetaminophen and several other compounds resulted in mass shifts suggestive of formation of electrophilic quinoid species. To further examine these products, nucleophilic compounds were added to each solution and coinjected with test compounds to investigate the capability of EC-MS to model metabolic activation and resultant reactions with nucleophiles. The mass spectrum in Figure 4 shows clear evidence of ions indicative of mono- (m/z 457) and di-glutathionyl (m/z 762) addition products obtained from oxidation of acetaminophen to NAPQI (m/z 150). Figure 5 summarizes the likely EC reaction products for acetaminophen and additional compounds studied. Oxidation of structural analog, 4-aminophenol, resulted in product ions indicative of the reactive quinoneimine (m/z 108), mono-glutathionyl (m/z 415), and di-glutathionyl (m/z 720) addition products. BHT oxidation resulted in formation of ions indicative of quinone methide (m/z 219) and a mono-glutathionyl addition product (m/z 526). For estradiol and metabolites, positive (m/z +594) and negative (m/z +592) ions corresponding to formation of protonated and deprotonated catecholestrogen-glutathione (CE-SG) conjugates reached maximal abundance with EC potentials of 300 mV for CE, 700 mV for their methyl ether metabolites, and 900 mV for estradiol. These reactions were demonstrated for estradiol (E2), 2-methoxyestradiol (2ME), 4-methoxyestradiol (4ME), 2-hydroxyestradiol (2HE) and 4-hydroxyestradiol (4HE). The results demonstrate that this technique is capa-

ble of producing biologically relevant chemical reactions including the proposed biotransformation reactions related to acetaminophen (11), 4-aminophenol (11), and BHT (12) cytotoxicity and estrogen-dependent carcinogenesis (13).

Conclusion

Compound bioactivity, metabolism, chemical stability, and toxicity frequently involve redox reactions. A rapid assessment of a library component's electrochemical activity and reaction products is therefore highly relevant to assessment of its drug-like properties. These data provide significant additional evidence that serial coulometric EC-MS allows rapid study of relative compound reactivity, resultant formation of related substances, and determination of metabolic and chemical soft spots. This technique is more readily standardized, has higher throughput potential than biological assays, and can

be readily integrated with LC-MS-based systems including FIA, pre-column, and postcolumn techniques. Coulometric EC-MS may therefore represent a mechanistic probe that can be consistently and reproducibly applied to large compound libraries to generate modeling-friendly data for prediction and assessment of drug-like properties. Future studies planned by our group include examination of coulometric EC-MS to predict and assess chemical stability to oxidative degradation and for selective production and subsequent identification of related substances (metabolites and degradants).

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