11 On-Line Electrochemical/LC-MS Techniques for Profiling and Characterizing Metabolites

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11.1 SUMMARY

Over the last decade, there has been a steady increase in the number of publications that describe on-line combination of electrochemical (EC) techniques with liquid chromatography-mass spectrometry (EC-LC-MS) [1,2]. These techniques have been shown to possess unique capabilities to both utilize and study oxidative and reductive (redox) reactions. This can provide not only analytical utility (e.g., LC detection) but also an insight that can be beneficial to drug discovery and development processes. This latter capability is largely based on the relevance of redox processes to many chemical and biochemical aspects of drug development, including chemical oxidative degradation [3], oxidative metabolism and formation of reactive species [4,5], and the relevance of oxidative stress in disease and toxicity [6]. The incorporation of rugged commercially available EC cells into the existing LC-MS systems and protocols is typically inexpensive and readily implemented. These and other factors have led to an increased use of these techniques at strategic times within the drug development process.

This chapter is an overview of several EC/LC-MS approaches, specifically those that employ three-electrode EC flow cells for controlled potential electrolysis. We describe some basic theories and a number of applications including those that use EC as reaction devices in series with MS and those involving parallel EC and MS for HPLC detection. Serial EC-MS applications include generating and characterizing products that often correspond to metabolites, degradants, or short-lived species of interest. The

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quantitative and qualitative aspects of parallel EC-Array-MS are also described for studying chemical oxidative stability, for profiling metabolites and degradants and for metabolomics. To facilitate expanded adoption of EC/LC-MS techniques, our emphasis is on basic methodology and practical considerations.

11.2 OVERVIEW OF ELECTROCHEMICAL (EC) FLOW CELLS

The common uses of three-electrode EC flow cells include HPLC detection (LCEC) [7–10] and hydrodynamic voltammetry [11]. There are many excellent sources of EC information that can be consulted for detailed description of theory and applications [12–15]. Briefly, the cell functions by establishing a specific applied potential between a reference electrode (RE) and a working electrode (WE). The auxiliary electrode (AE), through electrical feedback via a potentiostat, provides the energy necessary to maintain the potential and relies on solution conductivity (typically mobile phase with $\geq$20 mM buffer) to complete the circuit. This energy drives electrolysis of solution-phase species (i.e., analyte and reactant) at the WE surface. The majority of applications described below involve recording EC cell current and associated mass spectra as a function of, and as a result of, the applied potential.

EC cells with carbon-based WE are the most widely used for LCEC and, as such, are the primary focus of this discussion. Other WE materials (e.g., Au, Ag, and Pt), while more advantageous for certain applications [16,17], are typically more prone to surface passivation and are generally less practical for many solvent and pH conditions [16]. There are several possible EC flow cell designs, but only three basic geometries are in widespread use: thin-layer, wall-jet, and porous flow through. Thin-layer and wall-jet amperometric cells have small surface area WE, and when using typical HPLC flow rates (i.e., 0.2–2.0 mL/min), only a small percentage (typically $<$5%) of analyte comes into close-enough proximity to the WE to be oxidized or reduced. Response is therefore affected by changes in flow rate, and the overall yield of reaction products is typically low. Also, electrode maintenance is frequently required because of WE passivation, particularly, when analyzing complex matrices or a large number of samples [18]. Thin-layer and wall-jet cells are widely used in the analysis of limited volume samples, such as microdialysis perfusates. In these instances, the combination of low volume EC cells with microbore chromatography can provide limits of detection in the femtogram range. A third class of EC flow cell utilizes high surface area microporous WE to achieve higher electrolysis efficiencies (typically $>$95% at 2.0 mL/min). This design is termed *coulometric* since the integrated response (peak area) represents the charge realized (coulombs) from nearly complete electrolysis of analyte as described by Faraday's law. A disadvantage to the coulometric cell design is that the WE is not accessible to mechanical resurfacing. However, in comparison to thin-layer and wall-jet cells, coulometric cells provide higher yield and more reproducible reactions (i.e., response) and require much less maintenance [8–10]. These advantages have led to its more widespread use in the analysis of complex matrices and in higher throughput approaches including those that utilize gradient elution HPLC. Further discussion is focused on two basic implementations of coulometric EC: (i) a single cell upstream and in series with MS and (ii) multiple cells (EC-Array) parallel with MS.

11.3 ELECTROCHEMISTRY IN SERIES WITH LC-MS

11.3.1 General Considerations

Several laboratories have used single EC cells as a simple "add-on" to a typical LC-MS setup. The common experimental configurations (Fig. 11.1) include flow injection, direct infusion, and both pre- and postcolumn EC/LC-MS. Several design aspects must be considered when adding an EC cell to an LC-MS system, such as solution conductivity, electrical grounding, and cell volume. Supporting electrolyte is required to provide solution conductivity. Common LC-MS mobile phases containing, for example, 0.1% formic acid or 5–10 mM ammonium acetate may have insufficient conductivity, and the resulting *IR* drop must be compensated for by using higher applied potentials. While this may be acceptable for some experiments, the use of more conductive solutions is generally preferred. This is particularly important for LCEC detection and for generating hydrodynamic voltammograms (HDVs) where low solution conductivity may lead to poor response, peak tailing, and loss of voltammetric resolution. Volatile buffers (e.g., ammonium acetate or ammonium formate) of at least 20 mM concentration typically provide sufficient conductivity and pH buffering. While higher buffering capacity may provide improved control of chromatography and detection, it is important to consider potential adverse effects on MS detection (e.g., ionization suppression, adduct formation, background noise). A further consideration is related to the use of conductive fluids and high voltage ion sources. The liquid inlet to the ion source (e.g., electrospray) of most LC-MS instruments is electrically grounded. However, in some instruments, there exists the possibility that current flow may occur from the high voltage ion source through a conductive fluid. Since many LC eluents are somewhat conductive, grounding the fluid line is typically recommended, irrespective of the use of EC. Current flow through the fluid line may compromise analyte integrity since it can affect the interfacial potential of distal, upstream, wetted components (e.g., injector and analytical column), which can lead to unwanted redox reactions [19]. A simple way to ground the fluid line is to use a stainless steel fluidic union connected to the ground of the MS high voltage power supply, as previously described [20].

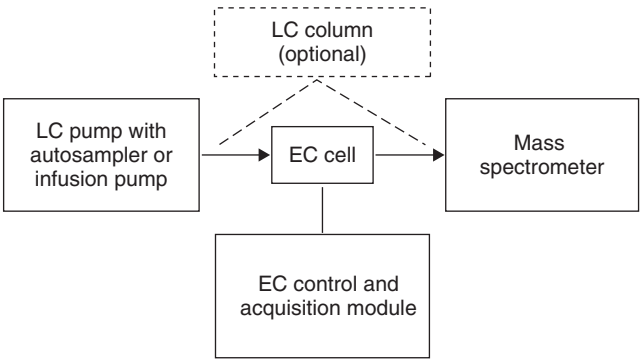

Figure 11.1 Representative serial EC/LC-MS configurations using either an ESA Model 5021A or 5030 coulometric EC cell controlled with Coulochem III for MS Detector (ESA Biosciences, Inc., Chelmsford, MA, USA).

11.3.2 Applications Using Flow Injection Analysis

Serial EC/LC-MS configurations are used in drug discovery and development to study the susceptibility of a compound to oxidation (or reduction), the nature of products and short-lived intermediates, and the associated reaction pathways. These data are used to provide insight to chemical stability, metabolism, and toxicity in a variety of contexts from rapid early-stage screening to more in-depth studies. A common approach is to use flow injection analysis (FIA) for medium throughput analysis of a series of compounds [21]. Each compound is typically analyzed at several EC potentials (e.g., 0, 400, 800, and 1200 mV vs Pd) to quickly (e.g., <30 s per injection) generate voltammetric data, which are indicative of relative ease of EC oxidation. The corresponding MS data are then used to provide information on the likely chemical sites of oxidation and the nature of products. These data are typically generated for analogous series to provide input to lead optimization strategies or to provide structural alerts for oxidatively unstable compounds. It should be noted that instantaneous voltammetric data may also be generated from a single injection by using several EC cells arranged in series, each successive cell maintained at a higher potential than the preceding cell. This technique, typically conducted in early-stage discovery for studying oxidative stability, is discussed in Section 12.4.

11.3.3 Pre- and Postcolumn EC with LC-MS

A logical extension of FIA with serial EC/LC-MS is to incorporate a separation technique to further characterize products. Figure 11.2 is an example of precolumn EC oxidation of tamoxifen. With the EC cell at 600 mV, the total ion chromatogram (TIC) shows two primary peaks corresponding to the starting material (tamoxifen) and, at an earlier retention time, an N-demethylated product. With the precolumn EC cell at 900 mV (Fig. 11.3), additional peaks are evident, including possible aromatic hydroxylation and dealkylation products. This example shows that product formation can, to some extent, be manipulated through simple control of EC cell potential and that several of these EC oxidation products correspond to biological metabolites or degradants of tamoxifen [22]. It should be noted that EC oxidations lack the steric control of enzyme-catalyzed reactions and may also involve different reaction mechanisms (see Section 12.3.6 for additional details). Therefore, the relative abundance of products generated by EC is not expected to correlate with that of biological systems. EC generation of metabolites is thus considered to be a qualitative technique.

The examples in Figs. 11.2 and 11.3 illustrate on-line generation and analysis of EC products using LC-MS conditions that are typical of metabolic studies (e.g., *in vitro* microsomal analysis). Serial EC/LC-MS can thus be used with "neat" parent compound solutions for preliminary optimization of LC and MS conditions for subsequent metabolite analysis in biological samples. By using identical conditions, EC data may then be used as an input to automated metabolite identification software to aid in finding metabolites present in more complex biological matrices [23].

11.3.4 Semipreparative EC Synthesis

When the data from an EC-generated product corresponds to that of a biological metabolite, the EC technique may then be viewed as a selective and rapid synthetic

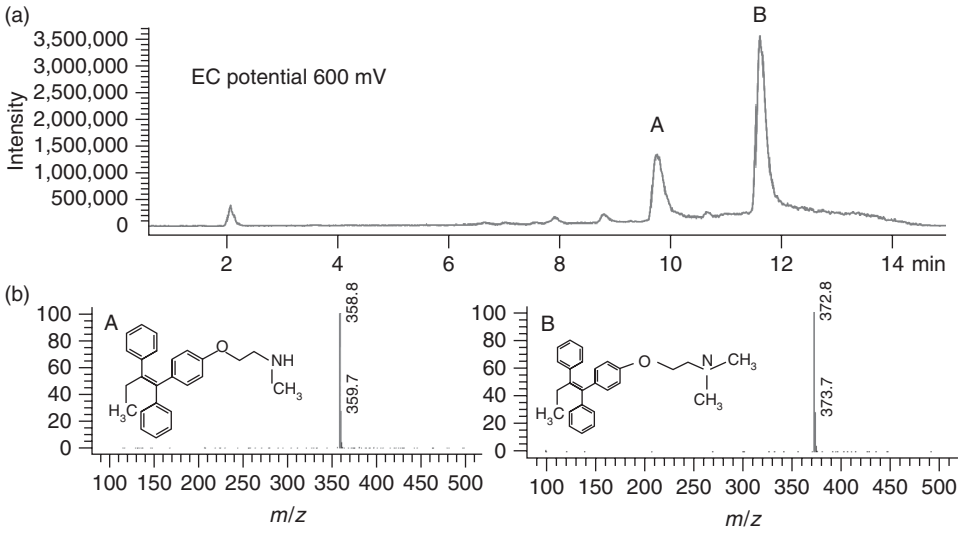

Figure 11.2 On-line oxidation of tamoxifen (50 μL of 20 μg/mL diluted in mobile phase) using precolumn ESA Model 5021 EC cell (600 mV vs Pd). (a) Total ion chromatogram and (b) full-scan mass spectra of chromatographic peaks labeled A and B. Conditions: binary gradient from 16% to 80% acetonitrile over 10 min, 20 mM ammonium acetate as supporting electrolyte. Shiseido C18MG 3 μm 75 × 4.6 mm i.d. column, 1 mL/min, 200 μL/min split to MS. Agilent 1100MSD single quad, positive ESI (electrospray ionization), 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, and capillary voltage 3500 V.

route to small quantities of this metabolite. This can then be used to facilitate structural elucidation or, more generally, to produce additional chemical entities. Earlier studies demonstrated the feasibility of scaling up on-line EC techniques to produce sufficient quantities for structural confirmation by NMR [24]. Tahara *et al*. [25] have more recently described the EC production in milligram quantities and subsequent structural elucidation of a troglitazone metabolite. By using nonaqueous conditions, Tahara and coworkers [26] furthermore demonstrated the direct EC production of a reactive metabolite of troglitazone, which was useful to investigate its mechanisms of hepatotoxicity. As discussed in the following section, the ability to use a purely instrumental technique to quickly generate EC products can provide significant advantages for studying certain reactive species where nonspecific binding (i.e., interaction with endogenous biochemicals) may prevent detection in biological systems. Madsen *et al*. [27] have also recently described a method for EC synthesis of a glutathione (GSH) conjugate of clozapine, which made it possible to obtain structural information of this potentially toxic species by NMR. Moreover, the recent development of higher capacity coulometric cells (Models 5125 and 5150 Synthesis Cells, ESA, A Dionex Company) provides the ability to produce even larger quantities of material. The simplicity and speed of on-line EC/LC-MS may therefore provide an effective means of characterizing some of the many unknown metabolites, including reactive species, encountered in multivariate profiling studies.

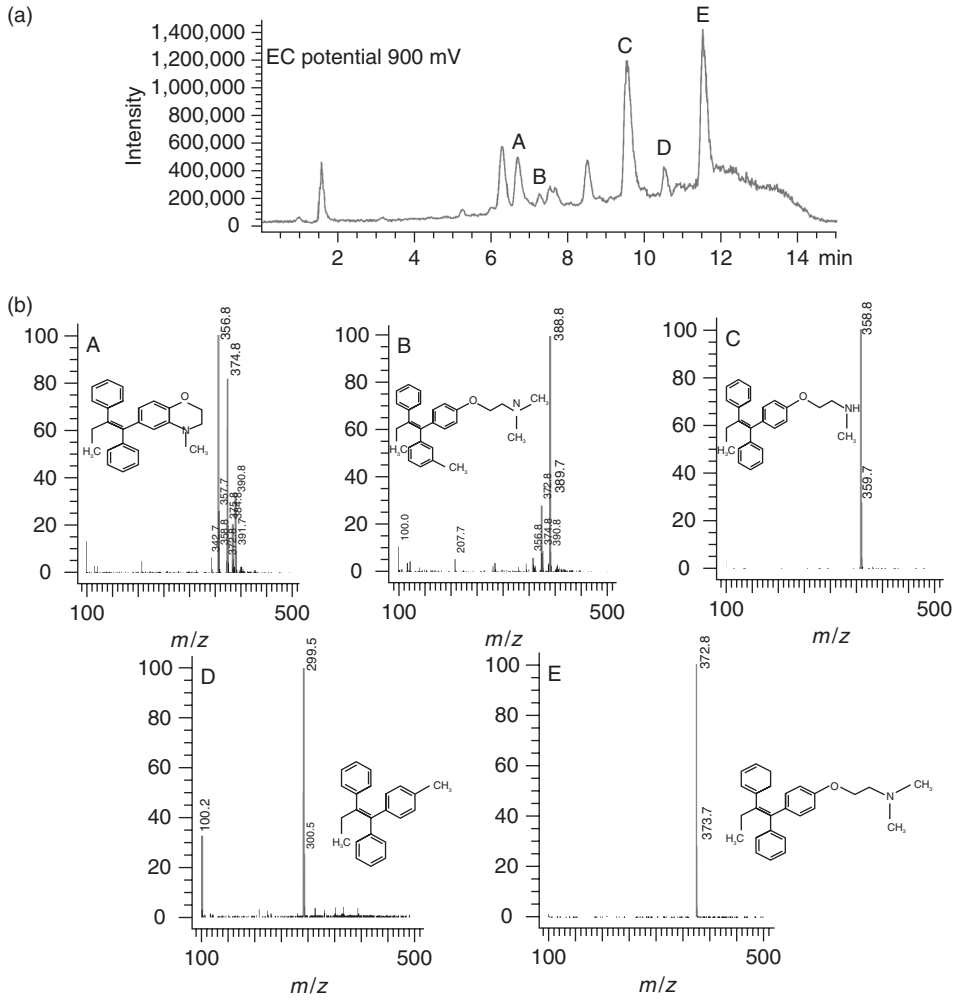

Figure 11.3 On-line oxidation of tamoxifen with precolumn EC cell at 900 mV versus Pd. Remaining conditions are as in Fig. 11.2. (a) Total ion chromatogram and (b) full-scan mass spectra of chromatographic peaks labeled A through E.

11.3.5 EC Generation and Analysis of Reactive Intermediates

Many studies suggest that redox metabolism of a wide range of chemical structures leads to the formation of reactive electrophiles that participate in a diverse array of toxic processes that typically involve covalent binding or other modifications to small and large molecules (e.g., DNA, proteins, peptides, and lipids), redox cycling, antioxidant/scavenger depletion, and other elements of oxidative stress [4–28]. The propensity of compounds to undergo redox-based metabolic activation to form reactive electrophilic species is therefore a major consideration in pharmaceutical development [29,30]. The topic of bioactivation and reactive metabolite assays is further discussed in the chapter titled ***Bioactivation and Reactive Metabolite Assays*** of this volume. Several reports have shown EC/LC-MS useful in the

study of reactive intermediate metabolites [21–35]. A variety of experimental configurations have been described that incorporate a nucleophilic trapping agent (typically, GSH), by either coinjection or infusion, with FIA, precolumn EC, or postcolumn EC. Lohmann *et al*. [36] have recently described EC simulation of the oxidative metabolism of paracetamol, amodiaquine, and clozapine. Interestingly, by using a reaction coil between the EC cell and HPLC injector, they were able to demonstrate adduct formation of the reactive metabolites with the proteins β-lactoglobulin A and human serum albumin. The formed drug–protein adducts were characterized with on-line time-of-flight MS, and the modification site was localized using FTICR-MS (Fourier transform ion cyclotron resonance-mass spectrometry). The authors describe this approach as a simple and fast method for the rapid risk assessment of covalent protein binding as well as for the synthesis of covalent drug–protein adducts in high purity and high yield. FIA and precolumn EC have been used to analyze neat drug solutions in order to investigate the susceptibility of a parent drug to EC oxidation, to characterize the reactive intermediates formed, and to study the chromatographic and MS behavior of trapped species. Postcolumn configurations have also been used to analyze biological (microsomal) preparations to investigate individual metabolite susceptibility to EC oxidation and to identify which metabolites form reactive species.

Figure 11.4 provides a basic example of precolumn EC 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 [5]. MS data indicate that EC oxidation of APAP in the presence of GSH (coinjected) resulted in two separate peaks corresponding to monoglutathionyl conjugates and one peak indicative of a diglutathionyl conjugate. These results are comparable to those reported nearly two decades ago by Getek and colleagues [31] using coulometric EC cells with thermospray MS. Several other "model" compounds have been studied using these techniques, including 4-aminophenol, BHT (butylated hydroxytoluene), estradiol and metabolites, phenacetin

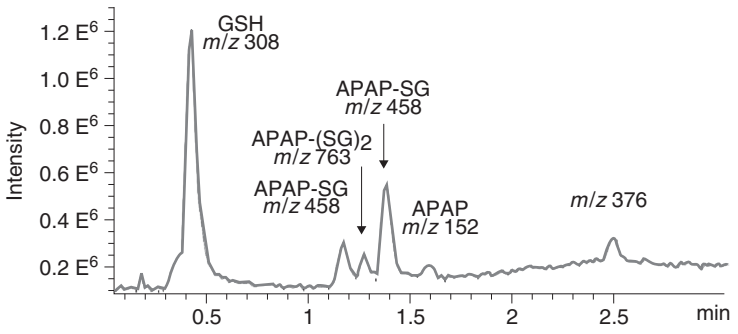

Figure 11.4 Precolumn oxidation of APAP with GSH as trapping agent. 10 μL of 20 μg/mL APAP, 1 mM GSH mixture with precolumn ESA Model 5021 cell at 500 mV versus Pd. Binary gradient elution from 1% to 80% acetonitrile (ACN) in 5 min, 20 mM ammonium acetate as supporting electrolyte. Shiseido C18 MG 3 μm 50 × 4.6 mm i.d. column, 1 mL/min, 200 μL/min split to MS. MS conditions are as in Fig. 11.3, except scan range that is 80–1000 *m/z*.

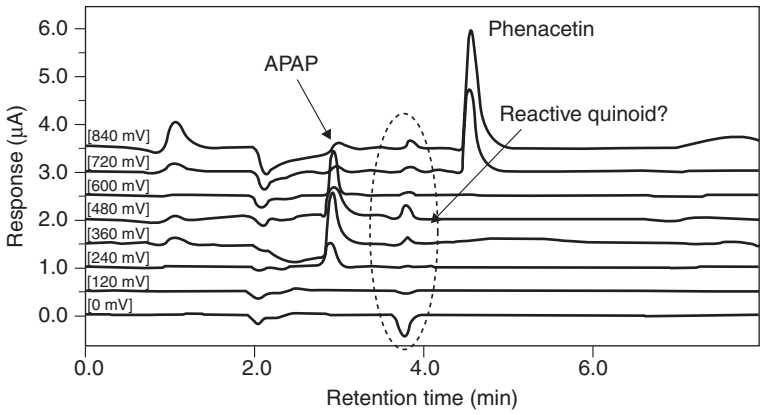

Figure 11.5 Precolumn oxidation of phenacetin. Conditions are as in Fig. 11.2, except LC detector that was an eight-channel EC-Array (CoulArray, ESA Biosciences, Inc., Chelmsford, MA, USA) with Model 6210 EC cell potentials of 0–840 mV versus Pd in 120-mV increments.

[21], and clozapine [33]. While the products expected from the known systems are generally formed and can be interrogated to reveal structural information, the EC oxidation sometimes results in a wide array of oxidation products not always seen in biological systems. It has been recognized that nonmicrosomal and nonenzymatic redox reactions may play a role in toxic processes [37], and further study is required to assess the significance of these observations. These data do, however, highlight the usefulness of EC/LC-MS as a rapid and efficient means of generating trapped products of reactive intermediates for structural characterization by LC-MS/MS from both parent drug and *in vitro* metabolites.

Figure 11.5 illustrates the precolumn oxidation of a phenacetin solution with LC-EC-Array detection (discussed in more detail in the following section). A peak, which eluted at 3.8 min, shows a characteristic voltammetric profile (i.e., reduction followed by oxidation) of a quinone species. On the basis of EC-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. Thus, an additional advantage to the use of on-line EC/LC-MS includes the ability to examine short-lived species that, although potentially relevant, may not as readily be observed in biological systems. Many other pharmaceutically relevant applications of EC-MS have been described and are beyond the scope of this discussion. These applications include EC derivatization to enhance MS ionization [38], mass tagging [39], and protein cleavage techniques, several of them are discussed in a recent focus issue on EC combined with MS [1].

11.3.6 EC "Mimicry" of Metabolism

There are many examples, as above, which have demonstrated that the EC oxidation products for a given compound often coincide with metabolites observed in biological assays [31–33,40–42]. These and other EC oxidation products may also be observed in purposeful degradation or stability studies. It must be recognized, however, that

enzyme-catalyzed, chemical, and EC oxidations are often very complex and involve different phenomena. Studies conducted by Jurva and colleagues [43,44] provide very useful insight into the mechanistic aspects of EC and enzymatic oxidative reaction pathways, particularly in the context of cytochrome P450 (CYP450) metabolism. They have shown that EC oxidation using porous carbon WE generally leads to the formation of similar products in those enzyme-catalyzed reactions that are supposed to proceed through a mechanism initiated by a one-electron transfer oxidation. Examples of CYP450 reactions mimicked by carbon-based WE include dehydrogenation, N-deacetylation, N-dealkylation, S-oxidation, and aromatic O-dealkylation. Reactions initiated by H-atom abstraction, such as aliphatic C-oxidation and hydroxylation of aromatic rings without electron-donating groups, are, however, generally not mimicked by EC oxidation. There are several examples of alternative or modified WE materials and reaction conditions to affect specific reactions. This includes the immobilization of enzymes [45] and biomimetic redox indicators [46] on solid electrodes and solution-phase conditions such as those described for EC-assisted Fenton reactions [47]. Johansson *et al*. [48] and Lohmann and Karst [49] have recently studied the extent to which EC oxidation, electrochemically assisted Fenton chemistry, and synthetic metalloporphines can be used in combination to mimic a large range of CYP450-catalyzed oxidations. While these approaches are extremely useful for many applications, EC is generally considered to be a complement to, rather than a substitute for, a given *in vitro* assay. A similar argument applies to chemical oxidative degradation studies, which are discussed in a later section.

11.4 EC-ARRAY TECHNIQUES

11.4.1 General Considerations

The general concepts of EC-Array are described in detail elsewhere [7,50]. Briefly, this technique employs up to 16 series EC cells (Fig. 11.6) with porous graphitic carbon-based WE, similar in concept to those described in the previous section. Each cell is typically poised at a different fixed potential, thereby spanning a wide potential window to allow detection of a broad scope of redox-active analytes during the transit of a single injected aliquot through the cells. Efficient electrolysis obtained with high surface area coulometric WE allows for selective detection and resolution of coeluting analytes, on the basis of differences in their relative ease of oxidation and/or reduction. Stated differently, each analyte in a coeluting band will demonstrate signal dominance

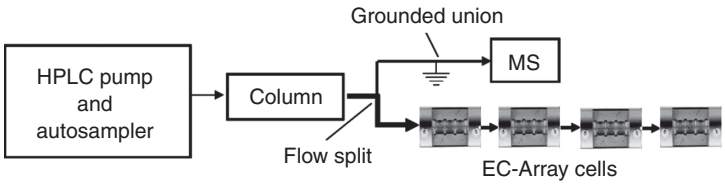

Figure 11.6 Representative configuration of LC with postcolumn flow passively split to EC-Array and MS. Model 6210 EC cells were controlled with a 16-channel CoulArray (ESA Biosciences, Inc., Chelmsford, MA, USA). *Source*: Reprinted from Ref. 75 by permission of Elsevier, Inc.

on a different WE based on their relative ease of oxidation or reduction. In general, EC reactions are typically observed according to the following general rank order (by relative ease of oxidation): *o*, *p*-quinol and *o*, *p*-aminophenol > tertiary amine <*m*-quinol ≈ phenol ≈ arylamine > secondary amine ≈ thiol > thioether ≠ primary amines and aliphatic alcohols. HDVs for each redox-active metabolite are obtained from the response across adjacent EC-Array sensors. These data are a reflection of the kinetic and thermodynamic components of electron-transfer reactions. Since chemical structure is a critical determinant of an analyte's redox behavior, the intrinsic generation of an HDV with EC-Array provides qualitative information for each species.

11.4.2 Applications Using Flow Injection Analysis

The use of FIA with a single EC cell in series with MS was described above to study compound susceptibility to oxidation. This requires multiple injections for each compound. An alternative approach is to use EC-Array (without MS) to generate voltammetric data from a single injection. This has the advantage of providing higher throughput but lacks the structure–activity and product information provided by MS. MS is not used downstream of EC-Array since the products that are selectively formed at upstream electrodes may further react at downstream electrodes, thus preventing their detection by MS. Many laboratories use EC-Array to generate voltammetric data to study oxidative stability, largely based on the pioneering work of Lombardo and Campus [51]. Chemical oxidation, a common mode of degradation for active compounds and drug products [3], is a significant concern at all stages of drug discovery and development. The relative tendency for compounds to undergo chemical oxidative degradation via electron-transfer mechanisms is closely related to their EC redox potentials [3]. As described in the previous section, oxidation reactions are often complex phenomena with a variety of mechanisms not necessarily modeled by a given EC technique. EC is typically used as part of a suite of techniques (e.g., oxygen/radical initiator, photolytic) for comprehensive study.

Representative data from FIA with 16-channel EC-Array detection is shown in Fig. 11.7. Using the described conditions, voltammetric data obtained for several model

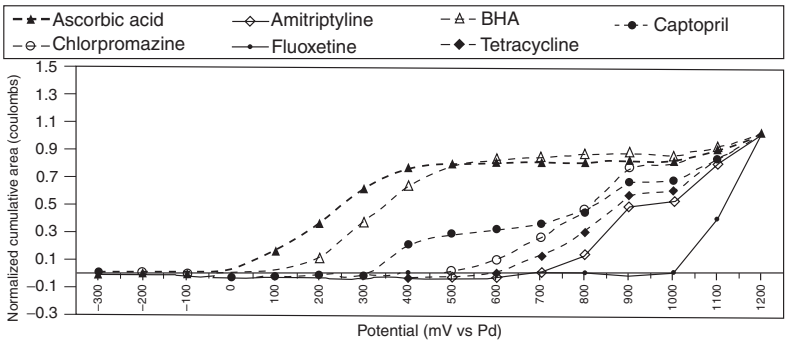

Figure 11.7 Voltammetric plots for seven model compounds representing normalized cumulative peak area. Dashed lines correspond to those compounds reported as oxidatively unstable; solid lines are indicative of oxidative stability.

compounds demonstrated that the most stable compounds oxidized at the highest potentials, while the least stable compounds oxidized at the lowest potentials [52]. While FIA was suitable for this pilot study, the approach was found to be inadequate for high throughput library-stage screening, particularly when considering the possible presence of electroactive impurities. Therefore, Lombardo and Campus [51] developed chromatographic methodology that used a short (3 cm) C18 column and isocratic elution in a strong (90% organic) eluent. This together with the inherent selectivity of EC-Array and the appropriate choice of signal threshold has allowed the generation of voltammetric data with a typical throughput of eight 96-well plates per week per instrument. This work, now applied to more than 30,000 compounds, was used to develop alerts for oxidatively unstable compounds, thus reducing the potential for later-stage issues. Furthermore, there are several additional potential uses of this approach, which are currently under investigation. These uses include the ability to assess the potential for degradation on library storage, to develop predictive structure–stability relationships, and to examine excipient compatibility. Webster *et al*. [53] have recently described the use of this technique in antioxidant selection for pharmaceutical formulations.

11.4.3 Quantitative Bioanalysis of Complex Matrices

The more traditional use of EC-Array is with LC separation for multicomponent quantitative and qualitative analyses. The primary advantages of this technique include two-dimensional (i.e., chromatographic and voltammetric) resolution, femtomole sensitivity, and data-dependent acquisition (autoranging), which facilitates use with gradient elution and provides a 10^5 dynamic response range [50,54]. LC-EC-Array has been widely used for routine analysis of redox-active substances with primary *in vivo* application to clinical chemistry [50,55,56], neurochemistry [57–59], and redox biochemistry [60–66]. Many pharmaceutical laboratories have adopted EC-Array for LC analysis of degradants and metabolites (i.e., related substances). A majority (estimated >85%) [67] of pharmacologically active small molecules possess one or more "EC-active" moieties (see above). The sensitivity (typically 10- to 1000-fold greater than UV–vis absorbance detection) [50,54], selectivity, and qualitative information obtained can provide significant advantages for a variety of applications. There are many examples of the use of EC-Array, specifically for bioavailability and pharmacokinetic studies [68–74]. The coupling of EC-Array as a parallel detector with MS is a logical extension of these approaches and is discussed below.

11.4.4 Metabolomics—Use of EC-Array in Parallel with MS

A major aspect of our recent work has been to couple EC-Array as a parallel detector with MS for metabolomic studies [75]. The comprehensive study of small molecules in living systems, that is, metabolomics, has several challenges, including analyte number and diversity, range of concentrations, and complexity of sample matrices. A variety of techniques exist that are each effective in studying certain classes of molecules within well-delineated concentration ranges; however, no one technique can overcome all the field's inherent challenges. For instance, NMR is able to detect any molecule that contains an active nuclide (e.g., ^{1}H and ^{13}C); however, its detection is limited to microgram quantities and does not apply to certain functional groups such as amines and sulfates. MS is very versatile but relies on the ionizability of the compound. EC techniques,

specifically EC-Array, are extremely sensitive detectors of redox-active compounds that, in many instances, are not observed with MS. The parallel use of EC and MS provides the capability to obtain more information from a given metabolite and to extend the number of metabolites and range of chemical classes that can be effectively studied.

A representative chromatogram of rat urine analyzed by parallel EC-Array-MS is shown in Fig. 11.8. As expected, the MS base peak chromatogram shows relatively few, directly visible, metabolite peaks. Mass spectral data from full-scan exploratory studies are typically processed by extracting discrete signals each defined by a particular retention time and m/z and using algorithms to help distinguish analytical

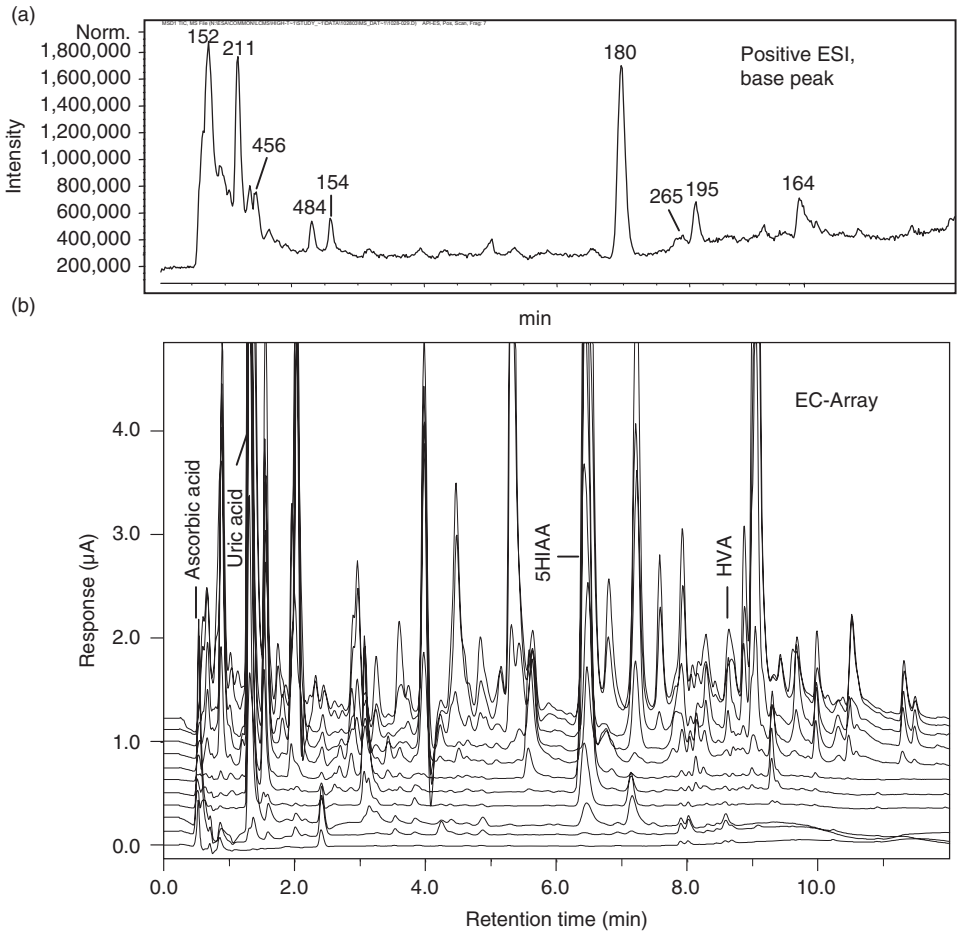

Figure 11.8 (a) MS base peak chromatogram labeled with base peak *m/z*, and (b) EC-Array multichannel chromatogram (11 of 16 channels shown for clarity). Analytical conditions: 20 μL of 10-fold diluted urine. Gradient elution 1–100% aqueous acetonitrile with 10 mM ammonium formate and 50 mM formic acid; flow rate 1.5 mL/min; Shiseido C18, 3 mm, 75 mm × 4.6 mm i.d. column; and 4:1 passive postcolumn flow split to EC-Array/MS. EC-Array potentials 0–1050 mV in increments of 70 mV. ESI positive mode, capillary voltage 3500 V, fragmentor voltage 70 V scan range *m/z* 50–850, and scan speed 1.2 s per cycle. *Source*: Reprinted from Ref. 75 by permission of Elsevier, Inc.

signal from background noise [76]. The resulting data, often consisting of hundreds of discrete signals, are then typically processed by chemometric techniques such as principle component analysis (PCA). Chromatographic variability, ionization suppression, adduct formation and in-source oxidation with MS, and electrode adsorptive and other non-Faradaic processes with EC are important complicating factors to consider in these multivariate analyses. Also, as evidenced in Fig. 11.8, a significant challenge in metabolomics studies is to identify the many unknown peaks that are often observed in a typical biological sample. Our data show that the concurrent acquisition of EC-Array and MS data for each metabolite peak helps to address these potential issues. For example, the observation of a particular redox-active metabolite peak allows the analyst to conduct a more informed and targeted interrogation of the corresponding MS data. Furthermore, specific MS data are useful to normalize for retention time variability observed with both MS and EC-Array data. In addition, our results indicate that many redox-active urinary metabolites exist as solution-phase neutral species under a variety of reversed-phase chromatographic conditions; for example, peaks annotated in Fig. 11.8 are ascorbic acid (AA), uric acid (UA), 5-hydroxyindoleacetic acid (5HIAA), and homovanillic acid (HVA). Of these metabolites, only UA was detected from extracted ion chromatograms (above baseline noise) as its protonated or adducted molecule [e.g., $M + X$ ($X = H^+$, Na^+, K^+, $NH_4{}^+$)]. The combined detection scope of MS and EC-Array thus provided higher coverage in a single analysis of the wide dynamic range and broad chemical diversity of urinary metabolites.

In a model study of APAP-induced hepatotoxicity, results from PCA of EC-Array data showed consistent differentiation between experimental groups of animals receiving high dose APAP (200 or 300 mg/kg, 0–8 h collection), low dose (20 mg/kg) APAP, high dose (200 mg/kg) acetylsalicylic acid, and controls. Figure 11.9 shows some of the individual redox-active components that, as indicated from PCA, largely contributed to the observed differentiation. This includes both endogenous (E1–E4) and xenobiotic (M1, M3, and M4) metabolites. These data further demonstrate the complementary nature of EC and MS detection. For example, MS data, along with prior knowledge of a parent compound's analytical behavior and informed prediction of biotransformations, was used to distinguish xenobiotic and endogenous metabolites. This allowed more direct study of changes in endogenous metabolite profiles by PCA. Also, EC-Array data showed clear evidence of an APAP metabolite that was not detected by MS (peak M1). Furthermore, MS data indicated that the peak M3 consisted of two major components having *m/z* 313 and 232. These *m/z* values are consistent with the commonly observed O-sulfated metabolite and the less frequently observed mercapturic acid metabolite of APAP, respectively. The similarity in voltammetric response between parental APAP and the peak corresponding to *m/z* 232 suggests that the easily oxidized 4-amidophenol group is intact. These data are consistent with the commonly observed ring thioether metabolites associated with high dose APAP [77]. Since APAP-mercapturate is a recognized marker of the so-called toxic pathway of APAP, the described approach of exploratory multivariate analysis and targeted interrogation of EC and MS data were an efficient and effective way to determine relevant changes associated with high dose APAP toxicity and to provide insight into the chemical identification of potential biomarkers. These results demonstrate that the combined use of EC-Array and MS may provide significant advantages over either individual technique in addressing the complex nature of metabolomic studies.

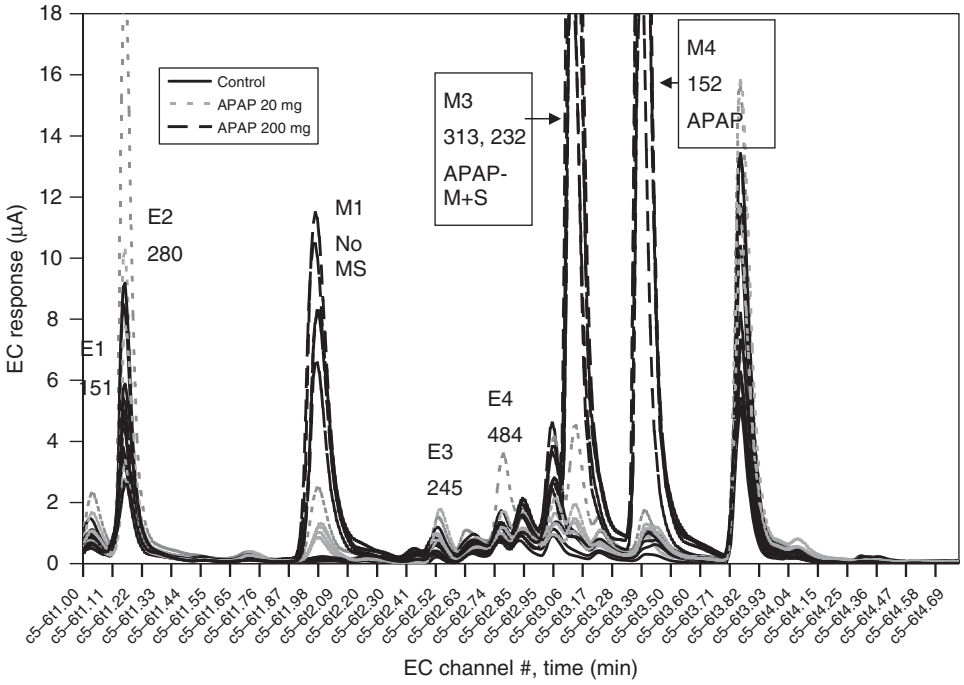

Figure 11.9 Overlay of EC-Array data from urine of rats administered (i.p.) vehicle 20 mg/kg or APAP 300 mg/kg ($n = 5$ in each group). Peaks labeled E indicate endogenous metabolites, while peaks labeled M indicate drug metabolites. Base peak *m/z* ratios as determined from corresponding MS data are shown. APAP-M + S indicates coelution of sulfate and mercapturate metabolites of APAP. *Source*: Reprinted from Ref. 75 by permission of Elsevier, Inc.

11.5 CONCLUSION

EC cells may be combined with LC-MS in a variety of experimental configurations that provide analytical utility and insight into drug discovery and development. This includes their use as reaction devices in series with MS for studies relevant to oxidative metabolism, degradation, and reactive species formation. EC-Array in parallel with MS provides the capability to increase the detection scope of LC-based multivariate profiling with high sensitivity, selectivity, wide dynamic range, and complementary qualitative data. The usefulness of these hyphenated techniques and the data generated for drug discovery and development are largely dependent on the challenges of a particular program and should be enhanced by their informed strategic implementation.

REFERENCES

1. Van Berkel GJ. Focus issue on electrochemistry combined with mass spectrometry. J Am Soc Mass Spectrom 2004;15:1691–1692.
2. Permentier H, Bruins A, Bischoff R. Electrochemistry-mass spectrometry in drug metabolism and protein research. Mini Rev Med Chem 2008;8:46–56.
3. Waterman KC, Adami RC, Alsante KM, *et al*. Stabilization of pharmaceuticals to oxidative degradation. Pharm Dev Technol 2002;7:1–32.

4. Hill BA, Kleiner HE, Ryan EA, *et al*. Identification of multi-S-substituted conjugates of hydroquinone by HPLC-coulometric electrode array analysis and mass spectroscopy. Chem Res Toxicol 1993;6:459–469.
5. Eyer P. Reactions of oxidatively activated arylamines with thiols: reaction mechanisms and biologic implications. An overview. Environ Health Perspect 1994;102(Suppl 6):123–132.
6. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. New York: Oxford University Press; 1999.
7. Acworth IN, Gamache PH. The coulometric electrode array for use in HPLC analysis: part 1: theory. Am Lab; 1996;5:33–37.
8. Riis B. Comparison of results from different laboratories in measuring 8-oxo-2′-deoxyguanosine in synthetic oligonucleotides. Free Radic Res 2002;36:649–659.
9. Gonzalez de la Huebra MJ, Bordin G, Rodriguez AR. Comparative study of coulometric and amperometric detection for the determination of macrolides in human urine using high-performance liquid chromatography. Anal Bioanal Chem 2003;375:1031–1037.
10. Sabbioni C, Saracino MA, Mandrioli R, *et al*. Simultaneous liquid chromatographic analysis of catecholamines and 4-hydroxy-3-methoxyphenylethylene glycol in human plasma. Comparison of amperometric and coulometric detection. J Chromatogr A 2004;1032:65–71.
11. Nagels LJ, Mush G, Massart DL. Rapid-scan hydrodynamic voltammetry and cyclic voltammetry of pharmaceuticals in flow injection analysis conditions. J Pharm Biomed Anal 1989;7:1479–1483.
12. Lund H, Baizer MM. Organic electrochemistry, an introduction and guide. New York: Marcel Dekker; 1991.
13. Chen JG, Woltman SJ, Weber SG. Electrochemical detection of biomolecules in liquid chromatography and capillary electrophoresis. Adv Chromatogr 1996;36:273–313.
14. Kissinger PT, Heineman WR Laboratory techniques in electroanalytical chemistry. New York: Marcel Dekker; 1996.
15. Lacourse WR. Electrochemical detectors: functional group analysis. Enantiomer 2001;6:141–152.
16. Rocklin RD. Working-electrode materials. LC-GC 1984;2:588–594.
17. Bowers ML. A new analytical cell for carbohydrate analysis with a maintenance-free reference electrode. J Pharm Biomed Anal 1991;9:1133–1137.
18. Catarino RI, Conceicao AC, Garcia MB, *et al*. Flow amperometric determination of pharmaceuticals with on-line electrode surface renewal. J Pharm Biomed Anal 2003;33:571–580.
19. Liu S, Griffiths WJ, Sjovall J. On-column electrochemical reactions accompanying the electrospray process. Anal Chem 2003;75:1022–1030.
20. Zhou F, Van Berkel GJ. Electrochemistry combined online with electrospray mass spectrometry. Anal Chem 1995;67:3643–3649.
21. Gamache P, Smith R, McCarthy R, *et al*. ADME/Tox profiling using coulometric electrochemistry and electrospray ionization mass spectrometry. Spectroscopy 2003;18:14–21.
22. Desta Z, Ward BA, Soukhova NV, *et al*. Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system *in vitro* : prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 2004;310:1062–1075.
23. Kieser B, Impey G, Meyer DF, *et al*. Metabolite profiling utilizing an in-line electrochemical system for LC/MS. Nashville (TN): American Society for Mass Spectrometry; 2004.
24. Gamache P, Solomon M, Acworth IN, *et al*. Rapid on-line electrochemical synthesis of pharmaceutical degradants and metabolites for profiling, identification and quantitation. Chicago (IL): Pittcon; 2004.
25. Tahara K, Yano Y, Kanagawa K, *et al*. Successful preparation of metabolite of troglitazone by in-flow electrochemical reaction on coulometric electrode. Chem Pharm Bull 2007;55:1207–1212.

26. Tahara K, Nishikawa T, Hattori Y, *et al*. Production of a reactive metabolite of troglitazone by electrochemical oxidation performed in nonaqueous medium. J Pharm Biomed Anal 2009;50:1030–1036.
27. Madsen K, Olsen J, Skonberg C, *et al*. Development and evaluation of an electrochemical method for studying reactive phase-I metabolites: correlation to *in vitro* drug metabolism. Chem Res Toxicol 2007;20:821–831.
28. Dieckhaus CM, Thompson CD, Roller SG, *et al*. Mechanisms of idiosyncratic drug reactions: the case of felbamate. Chem Biol Interact 2002;142:99–117.
29. Evans DC, Baillie TA. Minimizing the potential for metabolic activation as an integral part of drug design. Curr Opin Drug Discov Devel 2005;8:44–50.
30. Evans DC, Watt AP, Nicoll-Griffith DA, *et al*. Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. Chem Res Toxicol 2004;17(1):3–16. Erratum in: Chem Res Toxicol 2005;18:1777.
31. Getek TA, Korfmacher WA, McRae TA, *et al*. Utility of solution electrochemistry mass spectrometry for investigating the formation and detection of biologically important conjugates of acetaminophen. J Chromatogr 1989;474:245–256.
32. Deng H, Van Berkel GJ. A thin-layer electrochemical flow cell coupled online with electrospray-mass spectrometry for the study of biological redox reactions. Electroanalysis 1999;11:857–865.
33. van Leeuwen SM, Blankert B, Kauffmann JM, *et al*. Prediction of clozapine metabolism by on-line electrochemistry/liquid chromatography/mass spectrometry. Anal Bioanal Chem 2005;382:742–750.
34. Lohmann W, Karst U. Simulation of the detoxification of paracetamol using on-line electrochemistry/liquid chromatography/mass spectrometry. Anal Bioanal Chem 2006;386:1701–1708.
35. Lohmann W, Karst U. Generation and identification of reactive metabolites by electrochemistry and immobilized enzymes coupled on-line to liquid chromatography/mass spectrometry. Anal Chem 2007;79:6831–6839.
36. Lohmann W, Hayen H, Karst U. Covalent protein modification by reactive drug metabolites using online electrochemistry/liquid chromatography/mass spectrometry. Anal Chem 2008;80:9714–9719.
37. Smith MT. Mechanisms of troglitazone hepatotoxicity. Chem Res Toxicol 2003; 16:679–687.
38. Chen H, Zhang Y, Mutlib A, *et al*. Application of on-line electrochemical derivatization coupled with high-performance liquid chromatography electrospray ionization mass spectrometry for detection and quantitation of (p-chlorophenyl) aniline in biological samples. Anal Chem 2006;78:2413–2421.
39. Dayon L, Roussel C, Girault H. Probing cysteine reactivity in proteins by mass spectrometric EC-tagging. J Proteome Res 2006;5:793–800.
40. Volk KJ, Lee MS, Yost RA, *et al*. Electrochemistry/thermospray/tandem mass spectrometry in the study of biooxidation of purines. Anal Chem 1988;60:720–722.
41. Volk KJ, Yost RA, Brajter-Toth A. On-line electrochemistry/thermospray/tandem mass spectrometry as a new approach to the study of redox reactions: the oxidation of uric acid. Anal Chem 1989;61:1709–1717.
42. Jurva U, Wikstrom HV, Bruins AP. *In vitro* mimicry of metabolic oxidation reactions by electrochemistry/mass spectrometry. Rapid Commun Mass Spectrom 2000;14:529–533.
43. Jurva U, Wikstrom HV, Weidolf L, *et al*. Comparison between electrochemistry/mass spectrometry and cytochrome P450 catalyzed oxidation reactions. Rapid Commun Mass Spectrom 2003;17:800–810.
44. Jurva U, Bissel P, Isin E, *et al*. Model electrochemical-mass spectrometric studies of the cytochrome P450-catalyzed oxidations of cyclic tertiary allylamines. J Am Chem Soc 2005;127:12368–12377.

45. Joseph S, Rusling JF, Lvov YM, *et al*. An amperometric biosensor with human CYP3A4 as a novel drug screening tool. Biochem Pharmacol 2003;65:1817–1826.
46. Shiryaeva IM, Collman JP, Boulatov R, *et al*. Nonideal electrochemical behavior of biomimetic iron porphyrins: interfacial potential distribution across multilayer films. Anal Chem 2003;75:494–502.
47. Jurva U, Wikstrom HV, Bruins AP. Electrochemically assisted Fenton reaction: reaction of hydroxyl radicals with xenobiotics followed by on-line analysis with high-performance liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2002; 16:1934–1940.
48. Johansson T, Weidolf L, Jurva U. Mimicry of phase I drug metabolism-novel methods for metabolite characterization and synthesis. Rapid Commun Mass Spectrom 2007; 21:2323–2331.
49. Lohmann W, Karst U. Biomimetic modeling of oxidative drug metabolism. Anal Bioanal Chem 2008;391:79–96.
50. Gamache P, Freeto SM, Acworth IN. Coulometric array HPLC analysis of lipid soluble vitamins and antioxidants. American Clinical Laboratory; 1999;18:18–19.
51. Lombardo F, Campos G. How do we study oxidative chemical stability in discovery? Some ideas, trials, and outcomes. In: Borchardt RT, Kerns EH, Lipinski CA, *et al*., editors. Pharmaceutical profiling in drug discovery for lead selection. Arlington (VA): AAPS Press; 2004. pp 183–194.
52. Gamache P, McCarthy R, Waraska J, *et al*. Pharmaceutical oxidative stability profiling with high-throughput voltammetry. Am Lab 2003;35:21–25.
53. Webster GK, Craig RA, Pommerening CA, *et al*. Confirmation of pharmaceutical antioxidant selection by dissolved oxygen monitoring. Orlando (Fl): Pittcon; 2010.
54. Ferruzzi MG, Sander LC, Rock CL, *et al*. Carotenoid determination in biological microsamples using liquid chromatography with a coulometric electrochemical array detector. Anal Biochem 1998;256:74–81.
55. Cheng MH, Lipsey AI, Lee J, *et al*. Automated analysis of urinary VMA, HVA, and 5-HIAA by gradient HPLC using an array of eight coulometric electrochemical detectors. Lab Rob Autom 1991;4:297–303.
56. Gamache PH, Kingery ML, Acworth IN. Urinary metanephrine and normetanephrine determined without extraction by using liquid chromatography and coulometric array detection. Clin Chem 1993;39:1825–1830.
57. Volicer L, Langlais PJ, Matson WR, *et al*. Serotoninergic system in dementia of the Alzheimer type. Abnormal forms of 5-hydroxytryptophan and serotonin in cerebrospinal fluid. Arch Neurol 1985;42:1158–1161.
58. Beal MF, Matson WR, Storey E, *et al*. Kynurenic acid concentrations are reduced in Huntington's disease cerebral cortex. J Neurol Sci 1992;108:80–87.
59. LeWitt PA, Galloway MP, Matson W, *et al*. Markers of dopamine metabolism in Parkinson's disease. The Parkinson study group. Neurology 1992;42:2111–2117.
60. Sofic E, Lange KW, Jellinger K, *et al*. Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. Neurosci Lett 1992;142:128–130.
61. Hensley K, Maidt ML, Pye QN, *et al*. Quantitation of protein-bound 3-nitrotyrosine and 3,4-dihydroxyphenylalanine by high-performance liquid chromatography with electrochemical array detection. Anal Biochem 1997;251:187–195.
62. Collins AR, Gedik CM, Olmedilla B, *et al*. Oxidative DNA damage measured in human lymphocytes: large differences between sexes and between countries, and correlations with heart disease mortality rates. FASEB J 1998;12:1397–1400.
63. Acworth IN, Bogdanov MB, McCabe DR, *et al*. Estimation of hydroxyl free radical levels *in vivo* based on liquid chromatography with electrochemical detection. Methods Enzymol 1999;300:297–313.

64. Hensley K, Williamson KS, Floyd RA. Measurement of 3-nitrotyrosine and 5-nitro-gamma-tocopherol by high-performance liquid chromatography with electrochemical detection. Free Radic Biol Med 2000;28:520–528.
65. Yanagawa K, Takeda H, Egashira T, *et al*. Changes in antioxidative mechanisms in elderly patients with non-insulin-dependent diabetes mellitus. Investigation of the redox dynamics of alpha-tocopherol in erythrocyte membranes. Gerontology 2001;47:150–157.
66. Christen S, Jiang Q, Shigenaga MK, *et al*. Analysis of plasma tocopherols alpha, gamma, and 5-nitro-gamma in rats with inflammation by HPLC coulometric detection. J Lipid Res 2002;43:1978–1985.
67. Jane I, McKinnon A, Flanagan RJ. High-performance liquid chromatographic analysis of basic drugs on silica columns using non-aqueous ionic eluents. II. Application of UV, fluorescence and electrochemical oxidation detection. J Chromatogr 1985;323:191–225.
68. Achilli G, Cellerino GP, Melzi d'Eril GV, *et al*. Determination of illicit drugs and related substances by high-performance liquid chromatography with an electrochemical coulometric-array detector. J Chromatogr A 1996;729:273–277.
69. Chow HH, Cai Y, Alberts DS, *et al*. Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. Cancer Epidemiol Biomarkers Prev 2001;10:53–58.
70. Graefe EU, Wittig J, Mueller S, *et al*. Pharmacokinetics and bioavailability of quercetin glycosides in humans. J Clin Pharmacol 2001;41:492–499.
71. Roy S, Venojarvi M, Khanna S, *et al*. Simultaneous detection of tocopherols and tocotrienols in biological samples using HPLC-coulometric electrode array. Methods Enzymol 2002;352:326–332.
72. Penalvo JL, Nurmi T, Haajanen K, *et al*. Determination of lignans in human plasma by liquid chromatography with coulometric electrode array detection. Anal Biochem 2004;332:384–393.
73. Zhang XZ, Gan YR, Zhao FN. Determination of salbutamol in human plasma and urine by high-performance liquid chromatography with a coulometric electrode array system. J Chromatogr Sci 2004;42:263–267.
74. Wittemer SM, Ploch M, Windeck T, *et al*. Bioavailability and pharmacokinetics of caffeoylquinic acids and flavonoids after oral administration of Artichoke leaf extracts in humans. Phytomedicine 2005;12:28–38.
75. Gamache PH, Meyer DF, Granger MC, *et al*. Metabolomic applications of electrochemistry/mass spectrometry. J Am Soc Mass Spectrom 2004;15:1717–1726.
76. Plumb R, Granger J, Stumpf C, *et al*. Metabonomic analysis of mouse urine by liquid-chromatography-time of flight mass spectrometry (LC-TOFMS): detection of strain, diurnal and gender differences. Analyst 2003;128:819–823.
77. Nelson SD. Molecular mechanisms of the hepatotoxicity caused by acetaminophen. Semin Liver Dis 1990;10:267–278.