

# Expert Opinion

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## Online electrochemistry/mass spectrometry in drug metabolism studies: principles and applications

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**Importance of the field:** Conventional metabolism studies in early stages of drug development include *in vitro* tests, often based on hepatic cells and cell extracts, as well as *in vivo* tests on the basis of animal models. Complementary to these existing techniques, the electrochemical study of oxidative metabolism reactions is gaining increasing attention. Electrochemistry (EC) allows the fast detection of sites labile towards oxidation in a drug molecule and can mimic the formation of potential oxidative metabolites.

**Areas covered in this review:** The present review summarizes the developments of EC-based methods to study the oxidative metabolism of drugs in the past 10 years. The implementation of different coulometric and amperometric cells is described. Furthermore, online set-ups utilizing the hyphenation of EC, liquid chromatography and electrospray ionization mass spectrometry are discussed regarding their applicability in metabolism studies. Besides mass spectrometric detection, the isolation and advanced characterization of oxidation products are reviewed. This includes structure elucidation based on NMR spectroscopy as well as the evaluation of the reactivity of metabolites towards trapping agents or proteins.

**What the reader will gain:** A major focus of the article is directed to the comparability between electrochemically predicted metabolites and those occurring *in vitro* and *in vivo*. This comprises the discussion of typical oxidative metabolism reactions and provides a guideline about how far EC is capable of generating a specific oxidative metabolite.

**Take home message:** The study of drug metabolism reactions in an electrochemical cell enables the prediction as well as the synthesis of oxidative metabolites. In particular, reactive metabolites are directly detected using online EC/electrospray ionization mass spectrometry. Thus, the electrochemical technique is a promising tool that complements existing *in vivo* and *in vitro* techniques in drug metabolism studies.

**Keywords:** electrochemistry, liquid chromatography, mass spectrometry, metabolism

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### 1. Introduction

The development of a new molecular entity (NME), bearing a specific biological activity and thus, the potential to be introduced into the pharmaceutical market as an innovative drug, is a time-consuming and costly process. In 2008, only 21 NMEs were approved by the US FDA and average worldwide research and development costs of 50 billion US dollars were estimated [1]. The development process of a new drug starts with the design and synthesis of a novel compound.

**Article highlights.**

- Oxidation reactions, catalyzed by enzymes of the CYP group, play a crucial role in the biotransformation of drugs.
- Using EC, the majority of typical oxidative metabolism reactions can be simulated.
- The oxidative metabolites are generated in an electrochemical cell and are subsequently detected by ESI-MS.
- State-of-the-art techniques are online EC/ESI-MS and EC/LC/ESI-MS set-ups, including electrochemical flow-through or thin-layer cells.
- Due to the absence of endogenous molecules, EC/ESI-MS enables the direct detection of highly reactive metabolites.
- By the addition of trapping agents (e.g., glutathione) or proteins to the effluent of the electrochemical cell, the reactivity of metabolites towards these compounds can be studied.
- EC provides a fast and efficient technique for metabolite synthesis and subsequent NMR characterization.
- The hyphenation of EC/LC/ICP-MS has been used to gain quantitative information about oxidation products.

This box summarizes key points contained in the article.

Based on a lead structure, different molecules are constructed and are evaluated regarding their biological activity as well as their physical and chemical properties. For compounds showing promising characteristics, tedious preclinical studies based on hepatic cells, cell fractions and radiolabelled ADME (adsorption, distribution, metabolism and excretion) studies in animals are a next step towards the development of a safe pharmaceutical for human use [1,2]. The majority of potential drug candidates are excluded from the further development process during metabolism studies. This is due to very different reasons: Some compounds are very fast and extensively metabolized into inactive metabolites. Hence, they lose their desired biological activity before reaching the intended site of action. Other potential drug candidates are highly reactive towards endogenous molecules such as proteins or DNA nucleotides. Adduct formation between drug compounds and DNA is often fixed by repair mechanisms in the organism, but can also result in apoptosis or the promotion of cancer. In a large number of cases, the metabolites rather than the potential drug itself are the major cause of difficulties. The precursor compound is bioactivated into a toxic metabolite, which is very often trapped by endogenous conjugation agents such as glutathione or glucuronic acid. However, under specific circumstances, for instance, a depletion of glutathione, toxic side effects are caused by these reactive metabolites [3-5].

Only a small number of original drug candidates passes the preclinical studies and can be further developed and, at best, be distributed as pharmaceutical specialty. From the viewpoint of the pharmaceutical industry, ideal metabolism studies should include the exhaustive identification of all toxic

and non-toxic metabolites. Furthermore, they should expose reactions between the drug itself and its metabolites with endogenous molecules or xenobiotics, and finally enable the exclusion of toxic drug candidates at a very early stage of the drug development process. Besides conventional *in vivo* and *in vitro* methods, a purely instrumental technique, consisting of an electrochemical cell coupled online to electrospray ionization mass spectrometry (ESI-MS), has been proposed as screening tool for potential oxidative metabolites in drug discovery and development. Oxidation reactions play a crucial role in the biotransformation of drugs. Catalyzed by enzymes of the cytochrome P450 (CYP) superfamily, oxidation reactions including *N*-dealkylation, hydroxylation or epoxidation take place [6]. Further oxidation reactions occurring *in vivo* are catalyzed by peroxidase enzymes [7] or are initiated by reactive oxygen species (ROS) such as hydroxyl radicals [8,9]. Using electrochemistry (EC), the majority of typical oxidative metabolism reactions can be simulated [10]. The oxidation products and potential oxidative metabolites are generated in an electrochemical cell and are subsequently detected by ESI-MS. Due to the absence of endogenous molecules the purely instrumental EC/ESI-MS set-up enables the direct detection of highly reactive metabolites [11]. Besides predicting potential oxidative metabolites, EC has recently gained increasing attention with respect to the synthesis of metabolites and their further characterization, including structure elucidation by NMR spectroscopy [11].

The historical development of the electrochemical metabolism simulation has been comprehensively reviewed in the references [12-16]. The focus of the present review is the development in the past 10 years. Besides instrumental developments regarding the electrochemical cell itself, the implementation of EC in different online set-ups has been realized and has shown its benefits in the metabolism simulation of several drugs. Within this review, the comparability between the electrochemically simulated metabolism reactions and those occurring *in vivo* and *in vitro* is extensively discussed. On the basis of this comparison we aim to provide a guideline, describing whether or not EC can add valuable information towards the metabolism of a particular drug.

## 2. Conventional methods: *in vitro*, *in vivo* and *in silico*

The electrochemical simulation of oxidative metabolism reactions has a high potential as complementary tool to existing methods used in drug metabolism studies. To estimate the advantages and useful applications for EC/ESI-MS-based studies, a brief overview on the existing state-of-the-art techniques is given.

In early stages of drug discovery, the disposition of a drug candidate is commonly studied *in vitro* using liver cells or subcellular fractions from animals and humans since they are readily available and the methods are relatively straightforward. Microsomes, cytosol and S9 are broadly

used subcellular fractions that are prepared by differential ultracentrifugation of liver cell homogenate. Providing a different range of enzymes and enzyme concentrations, they are applicable for different purposes [17-19]. Liver cell microsomes contain vesicles derived from the hepatocyte's endoplasmic reticulum and thus, the majority of drug-metabolizing enzymes, particularly CYPs. The liver cytosolic fraction comprises soluble Phase II enzymes and S9 fractions consist of both, reticulum-associated enzymes as well as cytosolic enzymes. Hence, S9 fractions provide lower conversion rates for CYPs than microsomes, but can offer a more comprehensive overview of the metabolism reactions ongoing in the hepatic cell.

Since the enzyme group CYP consists of different isoforms, which show varying distributions in each organism, it is of particular importance to determine the specific enzyme isoforms that contribute to the metabolic pathway. These phenotyping studies are performed using cDNA-expressed individual CYPs [20]. The use of intact hepatocytes is more complex than using liver cell homogenate. The isolation can be difficult and cell damage may occur. However, intact hepatocytes contain the entire lot of drug-metabolizing enzymes and are often employed to study enzyme induction processes [21,22]. Going from single cell incubations to two-dimensional and three-dimensional methods such as liver cell culture systems, liver slices or perfused livers enables the examination of long-term toxic responses as well as the study of complex reactions mediated by the interplay among several different cell types [17,19,23].

ADME studies on animals are usually conducted throughout the entire drug discovery and drug development process. Human ADME studies, demanding far more resources and following rigorous regulatory requirements, are carried out during the late clinical Phase I to early Phase III studies [1]. Sophisticated extrapolation of pharmacokinetic behaviour from animals to humans enables the prediction of the majority of metabolites expected to be formed in humans [24]. Nevertheless, a unique metabolite, solely appearing in humans, remains a serious issue since it will slow down or even stop the drug development process years after the first major investments have been made. Hence, the FDA recommends performing human ADME studies as early as possible [25].

From the analytical point of view, radiolabelling techniques and MS are the basis of metabolite detection and identification [26]. The use of radioactively labelled drugs during *in vitro* and *in vivo* tests allows the detection of drugs and metabolites in body fluids and tissues at very low limits of detection [27,28]. Furthermore, qualitative and quantitative information about metabolites is obtained by the hyphenation of liquid chromatographic or gas chromatographic separation systems with MS techniques [29,30]. Having isolated a specific metabolite in sufficient concentration, NMR spectroscopy remains a mandatory tool for absolute identification [31].

The detection of reactive metabolites in *in vivo* and *in vitro* approaches demands particular attention. Reactive metabolites

are highly electrophilic species that undergo follow-up reactions with nucleophilic endogenous compounds. In the presence of trapping agents such as glutathione or glucuronic acid, they undergo conjugation reactions and are thereby detoxified and subsequently excreted. In the absence of these trapping agents, adduct formation with proteins may take place. Thus, during protein precipitation steps of microsomal incubation techniques, those adducts are removed from the analysis mixture and remain unidentified. In order to determine hard and soft electrophilic intermediates of the metabolism pathway, potassium cyanide, glutathione and *N*-acetylcysteine are commonly added to the incubation mixtures [32,33].

In a completely different emerging branch of metabolism research, the prediction of metabolites is performed by *in silico* methods [34-36]. These computer-based approaches can be divided into different categories. There are, for instance, expert systems that are based on the experience and knowledge arising from the numerous metabolism studies of functional groups in comparable drugs [37,38]. Other methods aim to predict the site of metabolism based on knowledge of the CYP-active site [39,40]. In the future, *in silico* metabolism studies may allow the estimation of metabolites and the metabolic rate prior to the synthesis of new molecules. Thereby, a lead structure can be optimized towards its metabolic stability, which results in a more efficient drug discovery and development process.

### 3. Electrochemical cells for EC/(LC)/ESI-MS metabolism studies

Electrochemical cells have been used in the analysis of pharmaceuticals for different purposes. Cyclic voltammetry and related techniques, such as differential pulse voltammetry or linear sweep voltammetry, are widely applied for studying reaction mechanisms of electroactive drugs [41,42]. Liquid chromatography (LC) with electrochemical detection is used for the sensitive and selective determination of redox-active species such as neurotransmitters and amino glycosides in complex matrices [43,44]. Furthermore, electrochemical cells have been implemented in LC/ESI-MS techniques in order to enhance the ionization efficiency and thereby the ion intensities of non-polar compounds [45]. EC as an instrumental tool to study oxidative metabolism reactions was first discovered by Shono *et al.* in 1980 [46]. Using an offline electrochemical batch reactor, the anodic *N*-dealkylation of diazepam and imipramine was studied and the results were compared to conventional methods based on liver cell microsomes. In 1986, Hambitzer and Heitbaum introduced the hyphenation of EC to thermospray MS for the analysis of oxidation products of non-volatile compounds [47]. It was ~ 10 years later that Iwahashi and Ishii extended the setup by an LC separation [48]. EC was coupled to LC/ESI-MS, allowing the online generation, separation and detection of oxidation products.

State-of-the-art techniques for the electrochemical mimicry of oxidative drug metabolism are online EC/ESI-MS and EC/LC/ESI-MS set-ups. In general, suitable electrochemical cells require high conversion rates, low adsorption for non-polar drugs, a large potential window and a good reproducibility. In the following, coulometric and amperometric cell types as well as electrochemical cells, integrated into the electrospray source, are described regarding their applicability in EC/LC/ESI-MS metabolism studies. Exemplary cell set-ups are shown in Figure 1. Typical conditions for the electrochemical conversion are summarized in Table 1.

### 3.1 Coulometric cells

The set-up of a typical coulometric cell (flow-through cell) is shown in Figure 1A (Model 5021, ESA Biosciences, Chelmsford, MA, USA). The core of the cell is a porous glassy carbon (GC) working electrode (WE), integrated into a typical three-electrode set-up with a Pd/H<sub>2</sub> reference electrode and a Pd counter electrode. The drug containing solution is most commonly passed through the cell by a syringe pump and the compounds are then oxidized on the large GC surface [10,49,50]. The main advantage of coulometric cells is a good conversion rate even at high flow rates. Being installed directly in front of a LC column, the electrochemical oxidation has to be performed at flow rates  $\leq 1.5$  ml/min [51]. In EC/ESI-MS systems as well as in improved EC/LC/ESI-MS ones that contain an injection valve in between EC and LC, lower flow rates of 10  $\mu$ l/min are applied [11,52]. Generally, lowering the flow rate enhances the dwelling time of the compounds in the electrochemical cell and thus the conversion rate. However, lowering the flow rate too far may result in an increased adsorption on the electrode surface. In order to achieve the maximum conversion rate for the isolation and NMR characterization of a specific metabolite, Jurva *et al.* performed the oxidation of the antimalaria agent amodiaquine in a coulometric cell at flow rates of 1  $\mu$ l/min with a supplemental make-up flow of 10  $\mu$ l/min [53]. To prevent adsorption of non-polar drugs, solvent mixtures of aqueous buffer solutions (ammonium acetate/formate) with  $\leq 90\%$  organic solvent (acetonitrile/methanol) are used [10,54]. The buffer salt acts as electrolyte and can be adjusted to the desired pH value, depending on the reaction type being simulated [10]. A further characteristic of coulometric cells is the low maintenance effort. The electrodes are usually simply cleaned by flushing with appropriate solvents. Even though adsorption can take place on the WE surface and residues might not be fully removed, the effects on the oxidation process often remain negligible due to the large WE surface area.

### 3.2 Amperometric cells

In contrast to coulometric flow-through cells, amperometric cells (thin-layer cells) are equipped with a planar WE (Figure 1B, Reactor Cell, Antec Leyden, Zoeterwoude, The Netherlands). Several thin-layer cells, commercially available

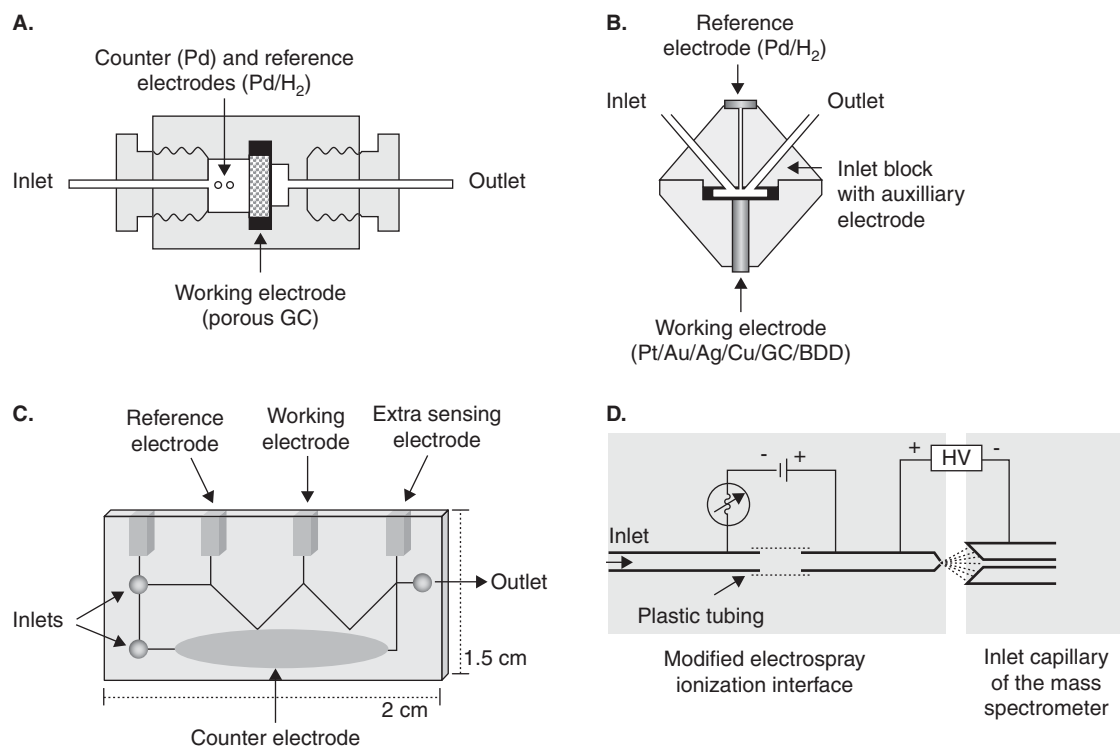
as well as homemade ones, have been coupled online to MS for studying oxidation processes of biomolecules [55-61]. The significant lower surface area requires flow rates at a maximum of 10  $\mu$ l/min. A striking advantage of thin-layer cells is the possibility to use different WE materials. Besides GC, platinum and boron-doped diamond (BDD) electrodes are the most promising alternatives [61,62]. Both show in general less adsorption for non-polar drugs and provide an enlarged potential window. BDD may be used up to a potential of 2,500 mV due to its high overpotential for the anodic generation of oxygen [63]. The planar WE surface demands less organic content in the solvent mixture and even if adsorption residues are observed, they can be manually polished from the surface. In a recently published article, the thin-layer geometry has been transferred onto a microchip (Figure 1C) and successfully used for the simulation of the oxidative metabolism of amodiaquine [64]. The application of microchips provides the advantage that the chip design, regarding the geometry and electrode materials, can be adjusted towards individual needs. Further development might include an on-chip LC separation and the direct coupling of the chip to MS [65].

### 3.3 In-source electrochemical cells

The cell types described earlier are coupled to ESI-MS or LC/ESI-MS by transfer capillaries. Since the electrospray interface itself can be seen as an electrochemical cell, transferring neutral compounds into charged analytes, electrochemical units have been designed which are part of the electrospray interface itself. The major research work in the use of electrochemical cells integrated into the interface of ESI-MS has been accomplished by the groups of Van Berkel [66-69], Cole [70] and Brajter-Toth [71-73]. The in-source generation of oxidation products specifically enables the observation of short-living reactive species such as the formation of radical cations. A typical set-up from the group of Brajter-Toth is shown in Figure 1D. The interface needle of an electrospray mass spectrometer is modified so that compounds are oxidized prior the electrospray process. The set-up has been utilized to study the oxidation processes of uric acid, dopamine and purine bases [71-73]. Van Berkel and co-workers employed thin-layer cells as well as flow-through cells for their interface and used the system to enhance the MS detection of biomolecules [66-69]. Further set-ups and the general application of the electrospray interface as an electrochemical cell were subject to an excellent recent review by Prudent and Girault [74].

## 4. Comparison: *in vitro* methods, electrochemical simulation

The EC/ESI-MS-based metabolism studies aim at the simulation of the most relevant metabolic oxidation reactions occurring *in vivo*. These include enzyme-catalyzed reactions as



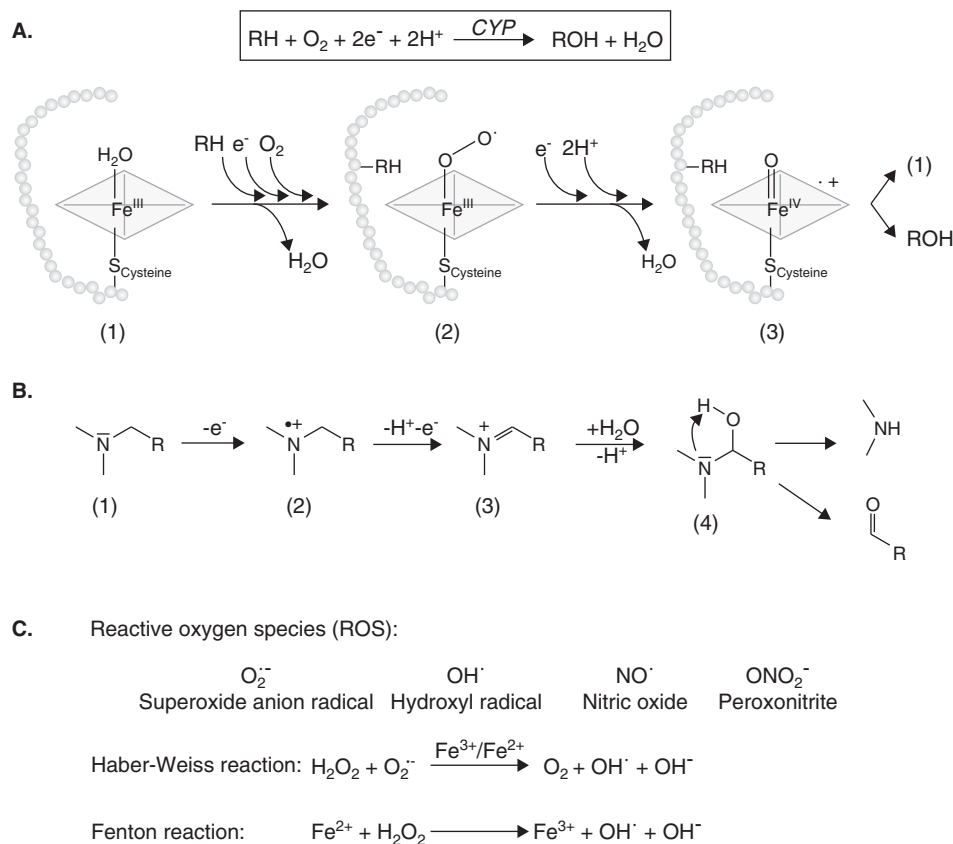
**Figure 1. Electrochemical cells implemented for the simulation of oxidative metabolism reactions. A.** Coulometric flow-through cell [10-12,50-53]. **B.** Amperometric thin-layer cell [61,62]. **C.** Thin-layer cell geometry integrated into a microchip [64]. **D.** Electrochemical cell integrated into an ESI interface [71].

BDD: Boron-doped diamond; ESI: Electrospray ionization; GC: Glassy carbon; HV: High voltage.

**Table 1. Commonly used electrochemical conditions for the mimicry of oxidative metabolism reactions in an online EC/(LC)/ESI-MS system.**

Parameter	Set-up/typical adjustment	Influence/dependency on
Cell type	Flow-through cell	High conversion rates; suited for synthesis, isolation, advanced characterization of metabolites; commercially available
	Thin-layer cell	Low adsorption on WE surface; adjustable electrode material; enlarged potential window; commercially available
	In source oxidation	Particularly suited for detection of short-living species; homemade systems
Potential	0 – 2,500 mV versus Pd/H <sub>2</sub>	Depending on electrode material, electrode configuration, type of oxidation reaction
Flow rate	10 – 500 μl/min	Lowering flow rates generally enhances conversion rates; large volume 'coulometric' flow-through cells tolerate high flow rates
Organic content	0 – 90% acetonitrile/methanol	Depending on polarity of the drug, WE structure and material; flow-through electrodes and non-polar drugs require high organic contents
Buffer/electrolyte system	Ammonium acetate/formate buffer	Sufficient electrolyte concentration required; pH values from 3 to 10, depending on the reaction type; influence on pH-dependent reference electrodes

EC: Electrochemistry; ESI-MS: Electrospray ionization mass spectrometry; LC: Liquid chromatography; WE: Working electrode.



**Figure 2. A.** CYP-mediated substrate (RH) oxidation. The active iron species, located in a haeme complex in the centre of the protein, is shown in different transition states of the catalytic cycle [75,76]. **B.** Electrochemical amine oxidation, following a one-electron oxidation pathway [80]. **C.** Reactive oxygen species (ROS) occurring *in vivo* [9] and reaction schemes for iron-mediated formation of hydroxyl radicals *in vivo* (Haber-Weiss reaction) [83] and (electro)-chemically assisted Fenton reaction [52].

well as radical-mediated oxidation processes. Comparing *in vivo* and electrochemically driven reaction mechanisms reveals the potential applications, but also limitations of the electrochemical technique.

#### 4.1 Enzyme-catalyzed substrate oxidation

The majority of oxidation reactions in the organism are CYP-mediated. CYP-catalyzed oxidation reactions primarily include hydroxylation, *N*-dealkylation, epoxidation, heteroatom (*N*, *S*, *P*) oxidation and dehydrogenation [75,76]. Figure 2A briefly shows an extract of the catalytic cycle of CYP-mediated oxidation reactions [76]. The resting state of the CYP enzyme consists of a porphyrin complex, bearing  $\text{Fe}^{3+}$  as central atom (1). The catalytic selectivity is based on the interaction of the substrate (RH) with the amino acids of the protein [77]. During the catalytic cycle, RH is kept in close proximity to the haeme complex by the protein (2). After a reduction step via electrons transferred by NADPH-P450 reductase, molecular oxygen is bound to the haeme complex (2). A second electron transfer, followed by a hydrogenation step and the loss of water, results in the

active iron complex (3) that finally incorporates oxygen into the substrate. The intermediate species (2,3) shown in Figure 2 are still under debate and different iron-oxygen species are discussed to be part of the oxidation process [78]. Besides CYP, flavin containing monooxygenase (FMO), peroxidase, monoamine oxidase, aldehyde oxidase and dehydrogenases are involved in enzyme-catalyzed oxidation processes. The FMO oxidation reaction, for example, follows the same stoichiometry than CYP-mediated oxidations (Figure 2A) but the substrates are generally restricted to soft nucleophiles (*N*, *S*, *P* oxidation) [79].

#### 4.2 Electrochemical one-electron oxidation

The extent to which EC can simulate the oxidation reactions that take place *in vivo* has extensively been studied and discussed by Bruins and Jurva *et al.* [10,80-82]. The mechanism for the electrochemical oxidation of an amine is shown in Figure 2B [80]. This mechanism is initiated by the abstraction of an electron from the nitrogen atom and results in the formation of a secondary amine and an aldehyde. As a general rule, it can be stated that oxidation reactions

proceed via a one-electron oxidation pathway when using electrochemical conditions within the potential limits of water. This includes the hydroxylation of activated aromatics, *N*-dealkylation of amines, *S*- and *P*-oxidation, the oxidation of alcohols to aldehydes and dehydrogenation reactions. In comparison, CYP-mediated oxidation reactions can not only be initiated by the abstraction of an electron but also by a deprotonation step. Therefore, the dealkylation of ethers, the oxidation of alcohols or aldehydes to carboxylic acids and epoxidation reactions, which are also CYP-catalyzed reactions occurring *in vivo*, are not observed under electrochemical conditions [80]. Furthermore, it has to be taken into account that under electrochemical conditions, the reaction takes place at all oxidative labile sites in the molecule, whereas in enzyme-catalyzed reactions, the site of oxidation is sterically influenced by drug–protein interactions.

### 4.3 Oxidation via hydroxyl radicals

Besides enzyme-catalyzed oxidation reactions, oxidation processes in the organism can derive from ROS such as the superoxide anion radical, hydroxyl radicals or nitric oxide (Figure 2C) [9]. ROS are endogenous species arising from various cellular processes in the organism. For example, macrophages are known to produce numerous radicals, including superoxide and nitric oxide. A well-known reaction for the generation of hydroxyl radicals *in vivo* is the Haber–Weiss process, in which Fe<sup>3+</sup> species react with superoxide anions (Figure 2C) [83]. Electrochemically, the formation of hydroxyl radicals can be achieved using the Fenton reaction [52], where hydrogen peroxide is reduced by Fe<sup>2+</sup> under the formation of hydroxyl radicals and hydroxyl anions. Subsequently, Fe<sup>3+</sup> is recycled to Fe<sup>2+</sup>, either chemically by the addition of ascorbic acid or electrochemically under reductive conditions (Figure 2C). The electrochemically assisted Fenton system has successfully been applied to perform hydroxylations in aromatic position or at double bonds [52]. A hydroxylation in aliphatic position has been achieved using an electrochemical cell equipped with a platinum WE while applying a potential of 2,000 mV versus Pd/H<sub>2</sub> [61]. Under these conditions, hydroxyl radicals are supposed to take part in the oxidation mechanism. Even more suited to generate hydroxyl radicals by EC are BDD WEs [63]. Utilizing BDD and potentials of 2,500 mV, the oxidation of DNA nucleotides has been studied and oxidation products such as 8-oxo-guanosine, well-known as biomarkers for oxidative damage *in vivo*, have been generated [62].

In Table 2, oxidative metabolism reactions that have been simulated by EC/(LC)/ESI-MS so far are summarized together with the electrochemical conditions used for oxidation. Each reaction is demonstrated for an exemplary compound in Figure 3. It is important to note that this table is still under construction. The study of further compounds under varying conditions regarding, for example, the electrode material and the potential range may still reveal novel oxidation pathways.

## 5. Recent applications and coupling techniques in electrochemistry metabolism studies

The direct comparison of enzyme-catalyzed oxidation reactions in the organism and the types of oxidation reactions that can be simulated by EC reveals the high potential of EC in metabolism studies. EC can be used to predict oxidative labile sites in a molecule, which are very likely to be metabolized *in vivo*. Furthermore, metabolites of interest can be synthesized by EC and can be isolated for an advanced identification and characterization. Regarding the different purposes, optimized instrumental set-ups have been developed and evaluated towards their applicability for several drug molecules.

### 5.1 EC/ESI-MS for the generation of mass voltammograms

The direct hyphenation of an electrochemical cell to ESI-MS (Figure 4A) can basically be achieved by two different set-ups. External electrochemical cells (thin-layer or flow-through cells, Figures 1A and 1B) can be coupled to ESI-MS by short transfer capillaries. Alternatively, in source electrochemical cells, integrated into the interface, can be used (Figure 1D). The oxidation behaviour of a drug can very fast and comprehensively be studied by applying a potential ramp in the electrochemical cell. Along the potential ramp, emerging oxidation products are monitored online by MS. At increasing potential, a decrease of the ion trace correlating to the mass-to-charge ratio (*m/z*) of the drug can be observed while the ion traces of oxidation products are increasing. If the formed products are unknown, analyzing the results based on extracted ion traces can be time-consuming and unexpected oxidation products might remain unidentified. For a simplified and comprehensive detection of the entire lot of oxidation products, EC/ESI-MS results can be plotted as so-called three-dimensional (3-D) mass voltammograms (Figure 5). Mass voltammograms were published by Lohmann *et al.* for the drugs amiodarone [84] and toremifene [85]. Van Berkel *et al.* studied the oxidation of reserpine and generated 3-D mass voltammograms by using thin-layer and flow-through cells integrated into the ESI interface [66]. Since EC/ESI-MS data can be acquired within a couple of minutes, the respective methods are well-suited for the optimization of electrochemical conditions. The intensities of oxidation products can be maximized by varying the potential, the buffer, the organic content or the flow rate. However, it has to be taken into account that changes in the flow rate and the solvent composition always have an impact on the ionization efficiency in the ESI interface, too.

### 5.2 EC/LC/ESI-MS

Mass voltammograms may provide a very fast overview on oxidative labile sites in a drug. However, isomeric compounds such as aromatic drug metabolites, bearing hydroxyl groups in

Table 2. Typical oxidative metabolism reactions simulated by EC.

Metabolism reactions simulated by EC	EC conditions (WE, potential, pH)	Exemplary compounds/literature
Aliphatic hydroxylation	Pt, 2,000 mV, pH 3; Fenton reaction	Tetrazepam [61], testosterone [10]
Benzylic hydroxylation	Fenton reaction	Metoprolol [10]
Dealkylation of amines	Pt/GC, 300 – 2,000 mV, pH 3 – 10; Fenton reaction	Tetrazepam [61], metoprolol [10], toremifene [85], amodiaquine [11], clozapine [86], lidocaine [10]
Dealkylation of ethers	GC, 700 – 1,500 mV, pH 3 – 7	Metoprolol [10], toremifene [85], S-methylthiopurine [80], arylpropionamide-derived SARM [98]
Aromatic hydroxylation	GC 200 – 1,500 mV, pH 3 – 7; Fenton reaction	Toremifene [85], metoprolol, mephenytoin [10], dopamine agonist N0437 and N0923, diclofenac [89], boscalide [88]
N-oxidation	GC 300 – 1,500 mV, pH 7 – 10; Fenton reaction	Lidocaine [10], amodiaquine [11]
S-oxidation	GC 200 – 500 mV, pH 3	S-Methylthiopurine [80], promethazin [87]
P-oxidation	600 mV, pH 3	Parathion [80]
Alcohol oxidation	1,500 mV, pH 3; Fenton reaction	Metoprolol [10], 2-(hydroxymethyl) pyridine [80]
Dehydrogenation	Pt/GC, 200 – 2,000 mV, pH 3 – 7	Amodiaquine, amsacrine, mitoxantrone [11], toremifene [85], clozapine [86], paracetamol [50], trimethoprim [12], troglitazone [91], $\alpha$ -tocopherol [51]

Conditions used for oxidation regarding WE material, potential versus Pd/H<sub>2</sub> and pH range are presented in the second column. It has to be considered that the conditions do not only depend on the type of reaction, but also on the drug compound itself.

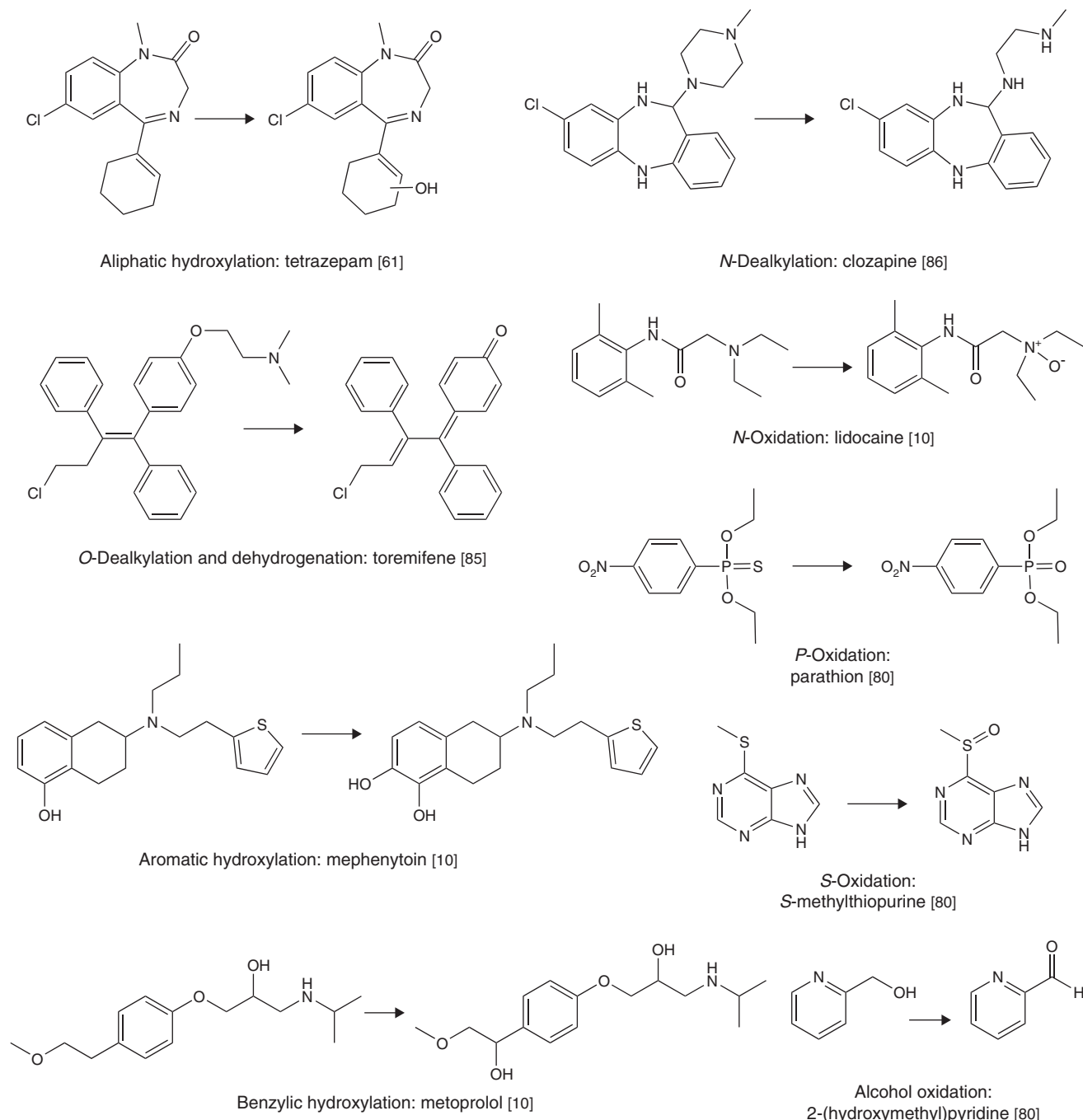
EC: Electrochemistry; GC: Glassy carbon; SARM: Selective androgenic receptor modulator; WE: Working electrode.

different positions, cannot be distinguished from each other. Extending the EC/ESI-MS set-up by LC allows the separation and detection of such isomeric compounds. Furthermore, based on LC, information about the polarity of metabolites can be gained. Using reversed-phase LC conditions, hydroxylation products, for example, usually elute earlier than the drug itself, whereas quinones show an enhanced retention. In online EC/LC/ESI-MS set-ups, the electrochemical cell can be placed directly in front of the column (Figure 4B) [48,50,86,87]. In this case, coulometric cells have to be employed, since the cell has to tolerate high flow rates. Moreover, it has to be considered that in this set-up, the electrochemical conditions regarding the buffer salt and the organic solvent content cannot be optimized independently as they are determined by the LC separation. A low content of organic solvent, for example, may result in peak tailing caused by adsorption effects on the electrode surface. In order to overcome these disadvantages, an improved EC/LC/ESI-MS set-up has been developed, in which EC and LC conditions are independent from each other. As shown in Figure 4C, the effluent of the electrochemical cell is collected in an injection loop mounted into a six-port valve. By switching the valve, oxidation products are flushed onto the column. This set-up was first used by Jurva *et al.* for online EC/LC/ESI-MS Fenton reactions [52] and has shown its applicability in a number of studies [11,62,85,88]. Since the improved set-up allows the use of low flow rates for electrochemical conversion, thin-layer cells can now be used in online EC/LC/ESI-MS studies [61]. This has recently been shown for the drug tetrazepam. *In vivo*, tetrazepam is hydroxylated upon oxidative metabolism in five different positions at the cyclohexenyl ring (Figure 3). The formation of these five

isomeric oxidation products was simulated in an electrochemical thin-layer cell, followed by online LC separation and ESI-MS/MS detection [61].

### 5.3 Reactive metabolites

One major advantage of the electrochemical technique compared to conventional *in vitro* techniques is the direct detection of reactive metabolites. In cell matrices, reactive metabolites, like the highly electrophilic quinones, undergo subsequent reactions with endogenous compounds. Enzyme-catalyzed Phase II metabolism reactions may result in the formation of glutathione or glucuronic acid adducts. Furthermore, electrophilic intermediates may bind to macromolecules such as proteins, lipids or DNA. Thus, they are often not detected in the analysis of body fluids or cell extracts. In purely instrumental EC/(LC)/ESI-MS measurements, no reaction partner is present, thus, reactive metabolites can directly be detected. Furthermore, dehydrogenation reactions of phenolic compounds, leading to the formation of reactive quinones, quinone imines and quinone methides can readily be mimicked by EC. This has been shown for numerous compounds, including paracetamol, amodiaquine, clozapine, diclofenac and troglitazone (Figure 6) [11,50,89-91]. Troglitazone has been used in the treatment of type II diabetes, but was withdrawn from the market due to its hepatotoxicity. In 2008, Madsen *et al.* studied troglitazone by EC/ESI-MS [91]. Even though they could not directly detect a reactive metabolite, they identified an adduct formed after oxidation of troglitazone in the presence of glutathione or *N*-acetylcysteine. Based on NMR studies, they postulated that adduct formation proceeds via a reactive *o*-quinone imide metabolite of troglitazone. This was verified by Tahara *et al.* in 2009, who identified the

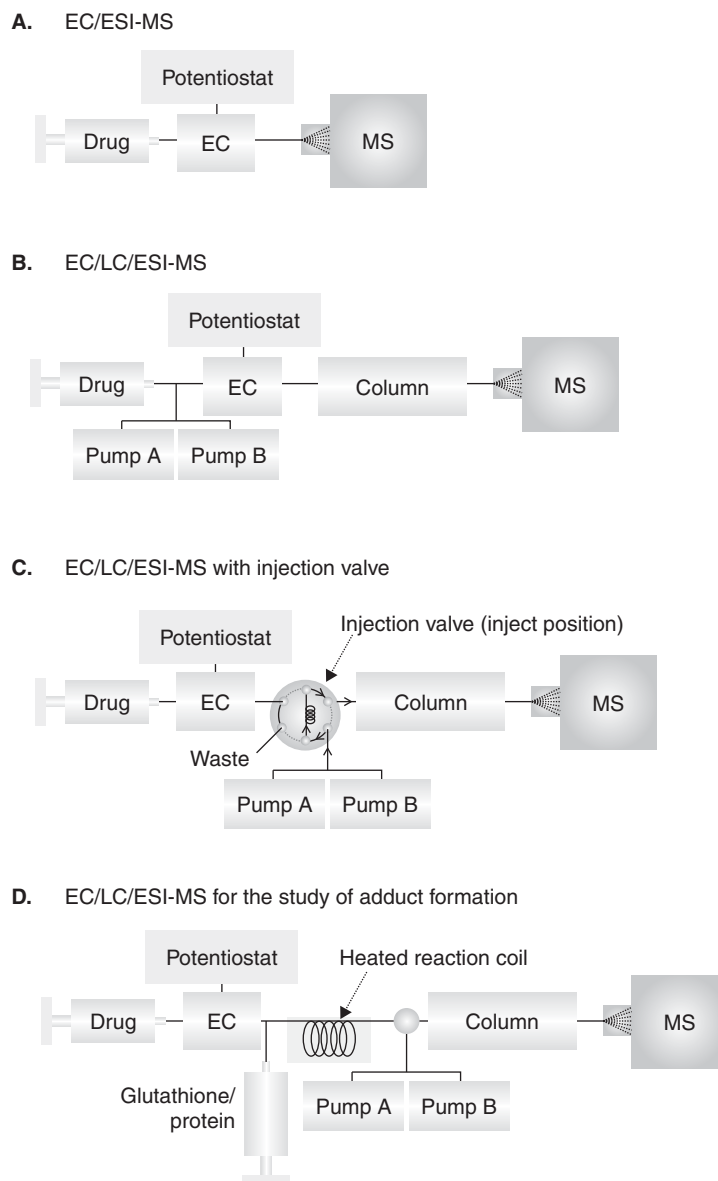


**Figure 3. Electrochemical simulation of typical oxidative metabolism reactions.** Each reaction is shown for an exemplary compound. Electrochemical conditions and further examples are listed in Table 2.

reactive *o*-quinone imide after electrochemical oxidation of troglitazone in non-aqueous media [92].

As described for troglitazone, reactive metabolites can not only be generated by EC, but also further characterized in terms of their reactivity towards endogenous compounds and trapping agents such as glutathione or potassium cyanide. As long as the oxidation potential for the formation of a

certain reactive metabolite does not exceed the potential limit for the degradation of the trapping agent, the oxidation can be performed in the presence of this agent. At high oxidation potentials, however, the agent has to be added to the effluent of the electrochemical cell. This has been realized in the online set-up shown in Figure 4D. A second reagent (e.g., glutathione, potassium cyanide, proteins) can be

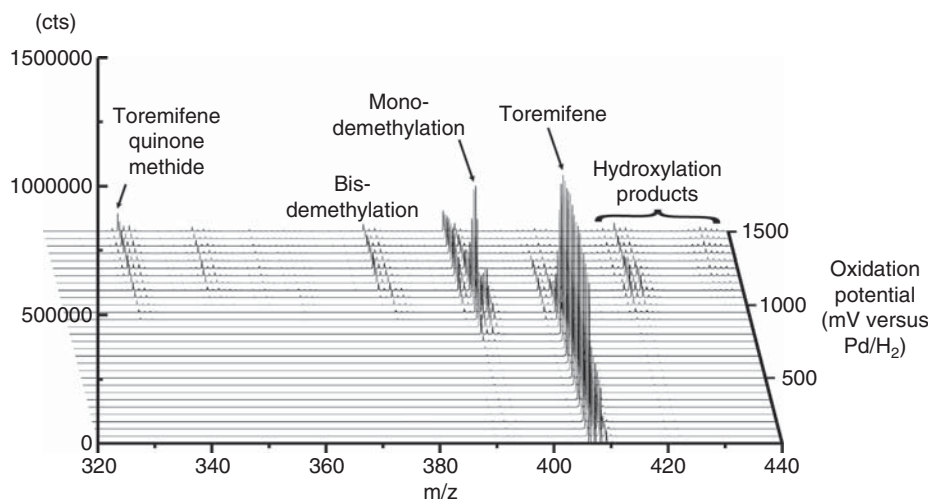


**Figure 4. Different EC/(LC)/ESI-MS online set-ups employed for the study of oxidative drug metabolism reactions.**

EC: Electrochemistry; ESI-MS: Electrospray ionization mass spectrometry; LC: Liquid chromatography.

introduced after the electrochemical cell. In a tempered reaction coil, the oxidation products can undergo subsequent reactions with the reagent. This set-up has been used to study the adduct formation of reactive metabolites of toremifene (Figure 3) with glutathione. Aiming at the mimicry of Phase II metabolism reactions, a mixture of glutathione and glutathione-S-transferase were added via the second syringe and the enzyme-catalyzed formation of glutathione adducts was observed [85]. Furthermore, the set-up shown in Figure 4D was applied to study adduct formation between reactive metabolites and proteins. Adducts of  $\beta$ -lactoglobulin A and human serum albumin with reactive metabolites

of amodiaquine, clozapine and paracetamol (Figure 6), respectively, were generated by EC and identified by high resolution ESI-MS [93]. A comparable method, though having a different intention, was introduced by Girault *et al.* [94] and Van Berkel *et al.* [95]. Utilizing the adduct formation between electrochemically generated quinones and free thiol groups of cysteine moieties, they aimed at the quantitative determination of cysteine groups per protein. For these experiments, they used electrochemical cells, integrated into the ESI interface of a mass spectrometer. The electroactive 'tagging reagent' (hydroquinone) was delivered in solution, whereas the protein was sampled on the surface of a GC electrode.



**Figure 5. Mass voltammogram of toremifene generated by EC/ESI-MS (Figure 4A).** Mass voltammogram adapted from [85]. The mass spectra are plotted against the potential applied in the electrochemical cell. In case of toremifene, oxidation products are detected at a potential of >800 mV versus Pd/H<sub>2</sub>.

EC: Electrochemistry; ESI-MS: Electrospray ionization mass spectrometry.

#### 5.4 Metabolite characterization: EC with dedicated detection systems

Besides EC/ESI-MS for metabolite generation and identification, EC with subsequent NMR structure elucidation has gained increasing interest in the past 3 years. When an unknown metabolite is detected in *in vivo* or *in vitro* studies, the amount of emerging metabolite is often hardly sufficient for NMR characterization. In comparison to the often laborious and time-consuming organic synthesis of metabolites, EC provides a fast and efficient technique for metabolite generation. The successful electrochemical preparation, followed by NMR characterization of metabolites, was shown by Tahara *et al.* for troglitazone [96] and by Jurva *et al.* for amodiaquine [53,97], clozapine [12] and diclofenac [89]. In case of amodiaquine, the reactive metabolite amodiaquine quinone imine was formed at an oxidation potential of 600 mV in a coulometric flow-through cell. The quinone imine species was isolated and studied by infrared spectroscopy (IR) [97]. Increasing the oxidation potential up to 1,500 mV resulted in the formation of a quinone imine aldehyde [53]. This species was collected and reduced at 200 mV to the respective amodiaquine aldehyde (Figure 6). The structure was confirmed by NMR measurements. Comparative studies with CYP1A1 and CYP1B1 revealed that this metabolite is also formed *in vitro*. Lohmann *et al.* used the coulometric flow-through cell to synthesize a metabolite of a possible sports drug (arylpropionamide-derived selective androgenic receptor modulator, SARM; Figure 6) [98]. After NMR-based structure elucidation, the metabolite was included into routine doping control procedures by LC/ESI-MS/MS and served as reference compound for several doping control specimens. In addition to NMR and IR,

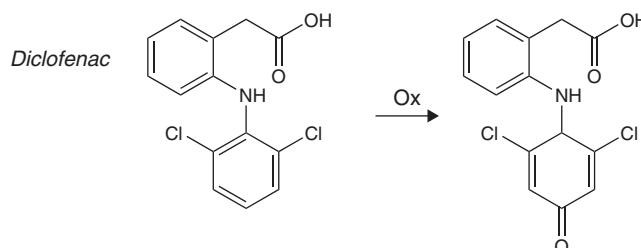
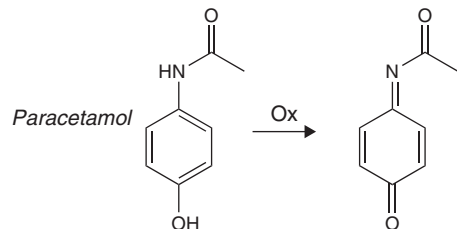
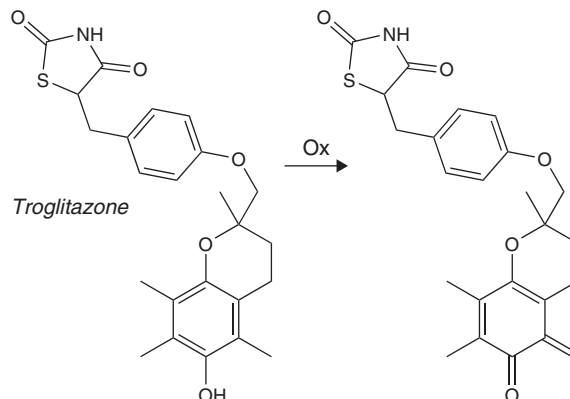
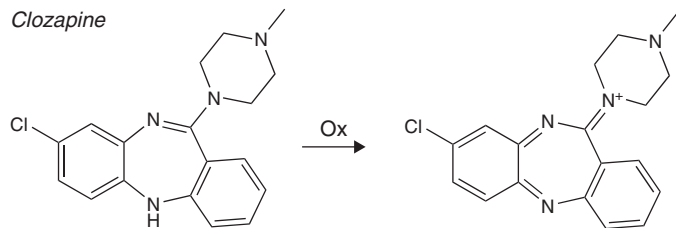
spectroscopic detection was performed with a circular dichroism detector, coupled online to EC/LC. The set-up was applied for the analysis of diastereomeric oxidation products of the chiral compound  $\alpha$ -tocopherol (Figure 6) [51].

The combinations of EC with ESI-MS, ESI-MS/MS and NMR are powerful tools to obtain qualitative information about the nature of a metabolite. Besides that, quantitative information is necessary to evaluate the relevance of a specific metabolite. Since the ionization efficiency in ESI-MS measurements strongly depends on the properties of each compound, quantitative information can only be achieved by using adequate reference materials. For drug compounds bearing heteroatoms such as iodine or platinum, inductively coupled plasma-MS (ICP-MS) detection is an excellent alternative. Compounds are atomized and ionized in a plasma source and subsequently detected by MS. ICP-MS provides low limits of detection for iodine-, bromine-, sulfur-, metal- and metalloid-containing compounds [99,100]. Since the ionization efficiency solely depends on the specific element, reference compounds are readily available. The hyphenation of EC/LC/ICP-MS has been used to gain quantitative information about the products formed upon oxidation of the iodine-containing antiarrhythmic drug amiodarone. In comparison, the metabolic conversion of amiodarone in incubation mixtures with rat liver microsomes was studied by LC/ICP-MS [84]. Since molecular information is lost under plasma conditions, complementary measurements based on EC/LC/ESI-MS have been inevitable for metabolite identification and revealed *N*-dealkylation as the main metabolism reaction for amiodarone (Figure 6).

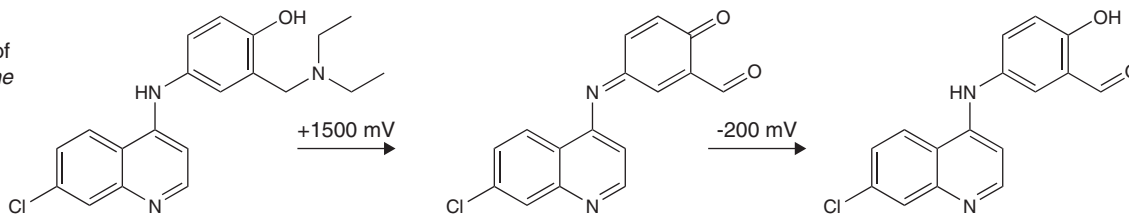
Aside from these instrumental detection systems, biological systems have been utilized to evaluate the biological activity of

Formation of reactive metabolites

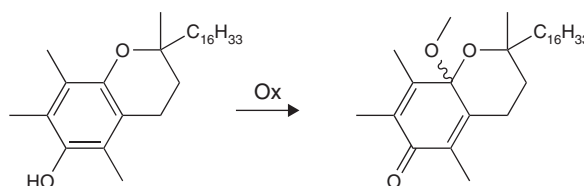
Clozapine



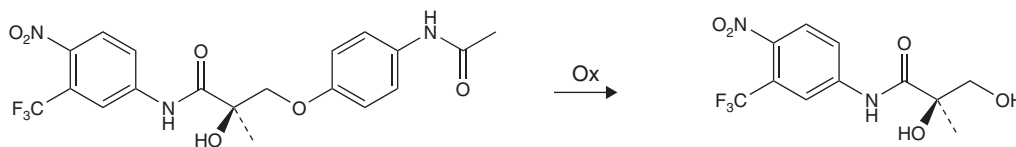
Synthesis of amodiaquine aldehyde



Diastereomeric metabolites of *α*-tocopherol



EC synthesis of a metabolite of an arylpropionamide-derived SARM (sports drug)



N-Dealkylation of amiodarone

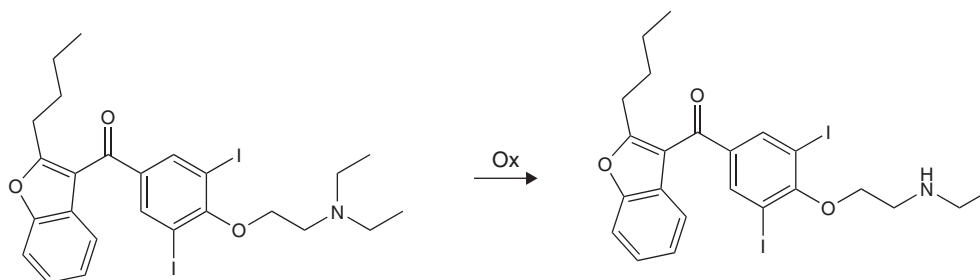


Figure 6. Structures of drugs and metabolites discussed in Section 5.

Ox: Electrochemical oxidation.

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electrochemically generated metabolites. Paci *et al.* isolated metabolites of ifosphamide and cyclophosphamide after anodic oxidation in methanol. Both drugs are prodrugs and used in cancer chemotherapy. They are activated upon CYP-mediated metabolism and act as alkylating, antineoplastic agents. The cytotoxicity of the electrochemically formed metabolites 4-methoxy-ifosphamide and 4-methoxy-cyclophosphamide was studied *in vitro* against carcinoma cell lines and were found to be significantly higher than for the unoxidized drugs [101].

## 6. Conclusion

The electrochemical oxidation of a drug leads to the formation of oxidation products that are very often equal to those occurring upon enzyme-catalyzed oxidative metabolism *in vivo*. This finding was for the first time reported in 1981 for paracetamol and has since been proven for numerous compounds. In the past 10 years, the use of EC coupled online to ESI-MS has made tremendous progress. Coulometric flow-through cells with porous GC electrodes and thin-layer cells with novel electrode materials such as BDD have been connected to ESI-MS. Besides that, modified ESI interfaces have been developed, allowing the drug oxidation directly in the interface needle and thus, the detection of short-lived species. Fundamental work has been done by Bruins and Jurva *et al.* who evaluated the extent to which oxidative metabolism reactions occurring *in vivo* can be simulated by EC [10,80]. By now, EC is capable of mimicking the majority of oxidative metabolism reactions, including *N*-dealkylation, dehydrogenation, aromatic and aliphatic hydroxylation as well as heteroatom oxidations. In order to simulate the oxidative metabolism of drugs, different online systems have been developed. The hyphenation of EC/ESI-MS enables the generation of so-called 3-D mass voltammograms, which provide a very fast overview on oxidative labile sites in a drug molecule. The extension to an EC/LC/ESI-MS system allows online generation and separation of isomeric oxidation products. By the addition of trapping agents such as glutathione to the effluent of the electrochemical cell, the reactivity of metabolites towards endogenous compounds can be studied. Furthermore, EC has been combined with NMR studies for structure elucidation of EC generated metabolites. Aiming at the quantitation of metabolites, an EC/LC/ICP-MS online system has been developed, allowing the determination of many heteroatom-containing drug metabolites.

## 7. Expert opinion

State-of-the-art metabolism studies consist of various sophisticated methods, in the majority on the basis of cells and cell extracts or animal models in combination with powerful analytical techniques. These methods target the elucidation of metabolism pathways, ideally before drug candidates enter clinical studies. In contrast to conventional drug tests,

the electrochemical metabolism simulation is a purely instrumental technique and oxidative metabolites are generated by physical and chemical processes. Hence, it is unquestionable that EC will not replace existing *in vitro* and *in vivo* methods. Nevertheless, the research done in the past 10 years has revealed the high potential of EC as complementary technique in drug development processes. Furthermore, the major benefit that EC can add to metabolism studies results from the absence of biological matrices. EC enables the generation of metabolites, in particular reactive metabolites, and their direct detection or isolation and advanced characterization. Since reactive metabolites are one of the main causes for drug-induced hepatotoxicity, their identification is of particular importance, especially when considering the fact that the FDA recommends the performing of human ADME studies as early as feasible during the drug development process.

The main precondition for the simulation of metabolism reactions by EC is that typical metabolism reactions can be mimicked in an electrochemical cell. Despite a few exceptions, this has been achieved and proven by studying a large number of drug compounds under varying electrochemical conditions. Even though the majority of electrochemical metabolism studies is in good agreement with metabolites formed *in vitro*, it always has to be taken into account that different mechanisms are involved in the electrochemical and the CYP-catalyzed oxidation reactions. Furthermore, the steric control, given by interactions between protein and substrate in enzymatically catalyzed reactions, does not apply for the electrochemical conversion. Electrochemically, the oxidation takes place at oxidative labile sites in a drug molecule, including sites that are also likely to be modified upon metabolism *in vivo*. However, in a number of cases EC predicts additional or less metabolites to those occurring *in vivo*.

Up to now, EC-based studies of the oxidative metabolism have been performed in a limited number of university and industry research laboratories. The successful application of EC for an increasing number of drug metabolism studies might enhance its implementation as screening tool for oxidative metabolites. Even more important for a broader implementation of EC/(LC)/ESI-MS in the pharmaceutical industry is the development of robust and automated measurement systems. The discussed online techniques have the potential to be expanded to automated systems. However, the electrochemical cells often require cleaning or activation procedures, caused by adsorption processes on the WE surfaces. Besides these technical issues, the range of metabolic processes that can be studied limits the applicability of the electrochemical technique. Solely based on EC, only oxidative metabolism reactions can be studied. The study of subsequent conjugation reactions (Phase II metabolism) or adduct formation with proteins requires the extension of the online set-up by reaction coils or subsequent offline bath experiments.

In the future, the synthesis and isolation of metabolites by EC is expected to have an increasing impact. In the research field of EC metabolism simulation, recent publications had a major focus on metabolite synthesis and NMR characterization. Additionally, electrochemically synthesized metabolites have been examined in terms of their reactivity or biological activity. Compared to organic synthesis of metabolites, electrochemical synthesis is mostly straightforward and can often be performed within a couple of hours. The matrices usually consist of aqueous solutions with volatile buffer salts and organic solvents, which facilitate the isolation. Furthermore, offline EC for the synthesis of a specific metabolite requires less resources than complex online EC/(LC)/ESI-MS set-ups. However, it has to be considered that the electrochemical conversion always depends on the drug and

the type of oxidation reaction. Thus, for some metabolites, the achieved conversion rates are still not sufficient for an isolation.

In summary, the electrochemical technique can add highly valuable information to existing *in vitro* and *in vivo* methods. The most important aspects of EC/(LC)/ESI-MS methods are the fast evaluation of the oxidative stability of drug candidates, the identification of reactive metabolites and the use of EC for rapid syntheses of metabolites, followed by an advanced characterization.

### Declaration of interest

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