Electrochemistry on-line with mass spectrometry
Jurva, Johan Ulrik

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2004

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 2

Comparison between electrochemistry/mass spectrometry and cytochrome P450 catalyzed oxidation reactions

In these studies we have investigated to what extent electrochemistry on–line with electrospray mass spectrometry can be used to mimic cytochrome P450 catalyzed oxidations. Comparisons on a mechanistic level have been made for most reactions in an effort to explain why certain reactions can, and some can not, be mimicked by electrochemical oxidations. The EC/MS/MS system provided successful mimics in cases where the P450 catalyzed reactions are supposed to proceed via a mechanism initiated by a one electron oxidation, such as N–dealkylation, S–oxidation, P–oxidation, alcohol oxidation and dehydrogenation. The P450 catalyzed reactions thought to be initiated via direct hydrogen atom abstraction, such as O–dealkylation and hydroxylation of unsubstituted arenes generally had a too high oxidation potential to be electrochemically oxidized below the oxidation potential limit of water, and were not mimicked by the EC/MS/MS system. Even though the EC/MS/MS system is not able to mimic all oxidations performed by cytochrome P450, valuable information can be obtained concerning the sensitivity of the substrate towards oxidation and the regions on the molecule where oxidations are likely to take place. For small–scale electrochemical synthesis of metabolites, starting from the drug, the EC/MS/MS system should be very useful for quick optimization of the electrochemical conditions. The simplicity of the system, and the ease and speed with which it can be applied to a large number of compounds, make it a useful tool in drug metabolism research.

2.1 Introduction

Drug metabolism is normally divided into phase I, II and III metabolism. Phase I metabolism involves primarily functionalization, including oxidation, reduction, hydrolysis and isomerization. Phase II metabolism is often related to conjugation, including glucuronidation, sulfation, glutathione conjugation, methylation, acetylation and condensation. The term phase III metabolism is used to describe transport processes, such as biliary and renal excretion. The most important phase I metabolic pathways are enzyme
catalyzed oxidations. Several enzymes are involved and the single most important enzyme system is cytochrome P450.

There is a large body of knowledge on electrochemical oxidations of organic compounds\(^1\) and the aim of our studies was to investigate the potential of an electrochemical system to mimic phase I oxidative metabolism. Results presented by Shono et. al suggest electrochemical oxidation as a mild synthetic route to \(N\)-dealkylated drug metabolites and it has been demonstrated that there is analogy between cytochrome P450 catalyzed \(N\)-dealkylation and electrochemical oxidation of amines and amides.\(^2-4\). Watanabe et al. have shown that the rates of sulfoxide formation for a number of \(para\)-substituted thioanisoles by a reconstructed system with purified cytochrome P450 were correlated with the electrochemical oxidation potentials.\(^5\) They observed the same correlation for the oxidation of the corresponding sulfoxides to sulfones. A correlation between the cytochrome P450 oxidation rates and the electrochemical oxidation potentials in a series of \(N,N\)-dimethyl-anilines has been reported by Guengerich and Macdonald.\(^6,7\)

In 1986, Hambitzer et al. studied the redox reactions of \(N,N\)-dimethyl-aniline in an electrochemical cell on–line with thermospray mass spectrometry.\(^8\) In the same year Getek used electrochemistry on–line with thermospray mass spectrometry to study the oxidation of acetaminophen.\(^9\) With the addition of glutathione or cysteine, these authors also were able to mimic phase II metabolism by the conjugation of glutathione and cysteine with the oxidation products of acetaminophen.\(^10\) The early developments in electrochemistry/mass spectrometry (EC/MS) for the simulation of metabolism have been reviewed by Volk et al.\(^11\) Despite the potential of the technique, only a few papers have been published on EC/MS in the field of drug metabolism.\(^12-18\) There could be several reasons for this.

First, insufficient quality of instrumentation may have prevented routine use. Thermospray mass spectrometry requires tight control of the vaporizer temperature, which depends on solvent composition and condition of the vaporizer. As a result, long–term stability and ease of use are difficult to achieve. High flow rates used in thermospray lead to a short residence time of samples in the electrochemical cell, and may give rise to low conversion efficiency. Electrospray MS provides high sensitivity and reliability at the low flow rates required for high electrochemical reaction efficiency.

Second, developments in the field of molecular biology have provided time efficient alternatives to \textit{in vivo} studies. Liver microsomes and organ slices are used widely for the study of enzymatic oxidation reactions, and individual cytochrome P450 enzymes are available for detailed \textit{in vitro} reaction studies.\(^19\)

Third, the study of metabolism of drugs and new chemical entities was previously done in the later stages of drug development when the number of drug candidates had been reduced such that the time and cost involved in a real \textit{in vivo} metabolism study was not considered to be prohibitive. Now, drug metabolism is considered much earlier in drug
discovery requiring simple tests of a large number of compounds and, with the developments in instrumentation, a reevaluation of EC/MS may be appropriate.

The introduction of combinatorial chemistry has resulted in a dramatic increase in the number of new chemical entities. To limit the cost of drug development, it is vital to try to eliminate as many compounds as possible at the early stages of the discovery route. One important consideration in high-throughput screening is metabolic stability, and, in case of instability, identification of the main metabolite. EC/MS, because it is a purely instrumental method, may have advantages over, or be complementary to, the existing methods of screening, i.e., in vitro studies with recombinantly expressed enzymes or organ fractions e.g. microsomes, hepatocytes and liver slices.

Cytochrome P450 enzymes are present in virtually every mammalian tissue and organ, and they play an important role in the oxidation of endogenous substances as well as a tremendous range of drugs and xenobiotics.\textsuperscript{20-23} The cytochrome P450 enzymes catalyze diverse types of reactions, such as hydroxylation of aliphatic and aromatic compounds, dealkylation and oxygenation of heteroatom containing compounds, oxidation of alcohols and aldehydes etc.\textsuperscript{24,25} The mechanisms for most of the enzymatic reactions are still debated and different mechanistic pathways have been suggested for many of the reactions shown in this article. All of the mechanisms shown here are based on the assumption that the active oxygen intermediate of Cytochrome P450 is the electrophilic oxoferryl porphyrin cation radical shown in Figure 2.1. For simplicity of presentation, this species is presented as [Fe=O]\textsuperscript{3+} in the figures.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{cytochrome_P450.PNG}
\caption{The electrophilic oxoferryl porphyrin cation radical, proposed reactive species of cytochrome P450.}
\end{figure}

In the experiments described below, we have tried to mimic these different enzymatic oxidations in an EC/MS system. Comparisons on the mechanistic level are made for
several reactions in an effort to explain why certain reactions can, and some can not, be mimicked by electrochemical oxidations.

2.2 Experimental

Chemicals

N–0923 is the S(–)enantiomer of the dopamine agonist N–0437 [2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin] and has been synthesized earlier.26 Amphetamine sulfate and mephenytoin were generous gifts from the Department of Pharmaceutical Analysis, University of Groningen, the Netherlands. Xanthohumol was a generous gift from Dr. J. F. Stevens at the Institute of Plant Biochemistry, Halle, Germany and Dr. C.L. Miranda at Oregon State University, Corvallis, Oregon, USA. The following chemicals were obtained commercially: 2–acetamidofluorene, Sigma; benzo[a]pyrene, 97 %, Aldrich; ammonia, 25 % solution in water, p.a., MERCK; ammonium acetate, p.a., MERCK; coumarin, Aldrich; dibutylsulfide, 96 %, Aldrich; dibutylsulfoxide, 96 %, Aldrich; diclofenac, Ciba–Geigy B.V.; diphenylsulfide, 98 %, Aldrich; diphenylsulfoxide, 96 %, Aldrich; N,N–diethyl nicotineamide, 97 %, Janssen; N,N–dimethylaniline, 99 %, Acros; 7–ethoxycoumarine, >99%, Fluka; glacial acetic acid, p.a., MERCK; hexylamine, 99 %, Fluka; 2–(hydroxymethyl)–pyridine (2–pyridylcarbinol), 98 %, Aldrich; p–hydroxyphenethylamine (tyramine), 97 %, Acros; lauric acid, 99.5 %, Aldrich; lidocaine, >99%, Inter Pharm; 2,3–lutidine, 99 %, Aldrich; 7–methoxycoumarine, 98 %, Aldrich; 6–methylmercaptopurine, Sigma; p–nitrophenol, spectrophotometric grade, Sigma; paracetamol (acetaminophen), ICN Biochemicals Inc.; parathion, Riedel de Haën; phenethylamine, 99 %, Janssen; 2–picoline, 98 %, Aldrich; picolinic acid, 99 %, Aldrich; 2–picolylchloride hydrochloride, 98 %, Aldrich; pyridine, 99 %, Acros; Pyridine–2–aldehyde, 99 %, Aldrich (2–pyridinecarboxaldehyde); thioanisole, 99 %, Janssen. The water used in the experiments was purified in a Maxima Ultrapure water system (ELGA, High Wycombe, Bucks, U.K.) and was sonicated for about 15 minutes before use.
Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions

Instrumentation

A schematic overview of the EC/MS system is given in Figure 2.2. A Series 200 micro LC pump (Perkin Elmer, Norwalk, CT, USA) delivered a flow of 50 µl/min. The analytes were injected with a 0.5 ml injection loop and passed through an ESA Coulochem 5011 analytical cell (ESA Inc., Bedford, Massachusetts, USA) connected to a home-made potentiostat. A MacLab system with Chart 3.5.7 software (AD Instruments, Castle Hill, NSW, Australia) was used to control the potentiostat and to apply the desired potential over the electrochemical cell. The ESA working electrode is porous graphite and all reported cell potentials were recorded versus a palladium reference electrode. A program was made in Chart to ramp the potential from 0 to +1500 mV in steps of 10 mV during 5 minutes. The cell current was continuously registered by Chart. Full scan spectra or product ion spectra, were taken continuously with an API 3000 triple quadrupole mass spectrometer (MDS–Sciex, Concord, Ontario, Canada) equipped with a TurboIonSpray interface. The MS was operated at such a low orifice voltage that “up front” collision induced dissociation did not take place. For certain compounds, such as benzo[a]pyrene and the sulfides discussed in the results and discussion section, no ions originating from the sample could be detected with electrospray. For these compounds, an APCI (Atmospheric Pressure Chemical Ionization) interface was used. The oxidation products of the compounds described above were all easily detected. The delay between the electrochemical cell and the mass spectrometer was determined as follows. At a continuous flow of analyte, a potential step from 0 to +1000 mV was performed. The time between the potential step and the appearance of the oxidation product at the mass spectrometer was measured by selected ion monitoring of the main oxidation product. With the delay between the electrochemical cell and the mass spectrometer determined to be 45 s, spectra
can be shown for any given potential and the signal from the different oxidation products can be extracted from the full scan data file and plotted against the potential.

**Electrochemical reaction conditions**
All compounds were tested in the following aqueous supporting electrolyte solutions: acetic acid, ammonium acetate, and ammonia. The supporting electrolytes were used in 1, 10 and 100 mM concentrations. To enhance solubility of the analyzed compounds and the oxidation products, 20 % acetonitrile was added to all electrolyte solutions. To keep benzo[a]pyrene in solution, 90 % acetonitrile was added. Generally, a high electrolyte concentration gave a better electrochemical conversion of the sample but also gave increased background ion signals and decreased the sample ion signal. Most oxidations were found to be pH dependent. Some amines, for example, are not oxidized at all in 100 mM acetic acid, while they are readily oxidized in neutral or basic solutions.

### 2.3 Results and discussion

**Table 2.1** Aliphatic hydroxylation, aromatic hydroxylation and epoxidation

<table>
<thead>
<tr>
<th>Enzyme catalyzed oxidation</th>
<th>Electrochemical oxidation products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a)</strong> Aliphatic hydroxylation</td>
<td>Ex. CYP4A11 catalyzed 12-hydroxylation of lauric acid</td>
</tr>
<tr>
<td>Ex. CYP4A11 catalyzed 12-hydroxylation of lauric acid</td>
<td><img src="image1" alt="Reaction" /></td>
</tr>
<tr>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>No electrochemical reaction</td>
</tr>
<tr>
<td><strong>b)</strong> Aromatic hydroxylation (1)</td>
<td>Ex. N-0923, aromatic hydroxylation in rat liver experiment</td>
</tr>
<tr>
<td><img src="image3" alt="Chemical Structure" /></td>
<td><img src="image4" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><img src="image5" alt="Chemical Structure" /></td>
<td><img src="image6" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><strong>c)</strong> Aromatic hydroxylation (2)</td>
<td>Ex. CYP2C19 catalyzed 4-hydroxylation of mephenytoin</td>
</tr>
<tr>
<td><img src="image7" alt="Chemical Structure" /></td>
<td><img src="image8" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><img src="image9" alt="Chemical Structure" /></td>
<td><img src="image10" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><strong>d)</strong> Epoxidation</td>
<td>Ex. 4,5-epoxidation of benzo[a]pyrene</td>
</tr>
<tr>
<td><img src="image11" alt="Chemical Structure" /></td>
<td><img src="image12" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><img src="image13" alt="Chemical Structure" /></td>
<td><img src="image14" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>
Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions

**Aliphatic hydroxylation**

The CYP4A11 catalyzed 12–hydroxylation of lauric acid is described in Table 2.1a. No oxidation was observed in the electrochemical cell. The electrochemical oxidation potentials for aliphatic hydrocarbons are generally very high and no electrochemical reactions on aliphatic sidechains have been observed for any of the compounds tested throughout these experiments. Hydroxylation of aliphatic C–H bonds is chemically very difficult, and the mechanism for cytochrome P450 catalyzed hydroxylations of this kind is still debated.

**Aromatic hydroxylation**

The metabolism of the dopamine agonist N–0923 has been studied intensively. Hydroxylation ortho to the phenolic group to give the corresponding catechol metabolite, shown in Table 2.2.1b, is a major metabolic pathway reported from experiments with in vitro rat liver perfusion. Electrochemical oxidation of the phenol function yields a catechol or a p–hydroquinone at about +200 mV in 1 mM acetic acid. The products are immediately oxidized further to the corresponding quinones. Information about the ortho, meta, para distribution can not be obtained without the application of further techniques such as NMR and HPLC–separation.

The CYP2C19 catalyzed hydroxylation of mephenytoin is given in Table 2.1c. No electrochemical reaction was observed on the aromatic ring.

A range of other compounds has been tested for aromatic hydroxylation in the EC/MS system, including coumarin, p–nitrophenol, diclofenac, xanthohumol, phenethylamine, p–hydroxy–phenethylamine (tyramine) and N,N–dimethylaniline. The conclusion from these experiments is that, in order to be oxidized electrochemically within the potential limits of water, the aromatic ring has to be activated by an electron donating group, such as a hydroxyl or amino group. For an aromatic ring without an electron donating group, the starting material is considerably more difficult to oxidize than the product. Benzene is for example oxidized at a potential about 1.3 V more anodic than phenol. As a consequence, the oxidation can not be stopped at the phenol stage, but will proceed to the benzoquinone.

2-Phenylethylamine is an example of a compound containing an aromatic ring without activating groups. 2-p-Hydroxy-phenylethylamine is activated by a hydroxyl group in the para-position. The electrochemical oxidation of 2-phenylethylamine gave no hydroxylation products, but 2-p-hydroxy-phenylethylamine was oxidized to the quinone at 250 mV. Since the intermediate catechol is more easily oxidized than the starting phenol, further oxidation to the quinone can not be prevented.
The mechanism for the cytochrome P450 catalyzed oxidation of π–bonded systems is still debated, and different compounds will most likely react via different mechanistic pathways. Guengerich and co–workers have suggested the mechanism described in Figure 2.3 for π–bonded systems. The π–electrons of a C–C double bond form an intermediate with the [Fe=O]$^{3+}$ form of cytochrome P450. Three reaction steps are possible for the intermediate. i) A single electron transfer to yield a radical cation of the π–system. ii) A radicaloid addition to the π–system to produce a σ–bonded radical complex. iii) An electron addition process that leads directly to a σ–complex. The nature of the π–system and the physical features of the P450 active site will dictate the specific pathway taken. In the first case, the suggested radical cation intermediate is deprotonated to yield the corresponding radical. Insertion of the “OH radical” from the formed [Fe–OH]$^{3+}$ (oxygen rebound) gives the hydroxylated compound. P450 catalyzed hydroxylation of the aromatic rings of anilines and phenols most likely proceed via this mechanism. Another fate of the radical cation is the formation of a σ–complex with the iron–oxygen species. This complex will react further and give hydroxylated products, epoxides etc. Nonenzymatic opening of arene oxides is probably a common reaction pathway for the formation of hydroxylated aromatic compounds.
Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions

The suggested mechanism for electrochemical oxidation of phenols is given in Figure 2.4.\textsuperscript{36} Compared to the cytochrome P450 catalyzed oxidation, it is initiated in the same way as pathway i) in Figure 2.3. Compounds that are easily electrochemically oxidized probably follow this pathway in the cytochrome P450 catalyzed oxidation, while compounds with high oxidation potentials are more likely to proceed via one of the other pathways upon oxidation by cytochrome P450. In an electrochemical system, the nature of the \( \pi \)-system, the physical features of the working electrode and the surrounding electrolyte, will determine the fate of the compound. Different substituents on the aromatic ring often result in different products. Phenols are generally oxidized to quinones as described in Figure 2.4 while other substituents on the aromatic ring will give a different outcome of the reaction. In the electrochemical oxidation of \( N,N \)-dimethyl-aniline, formation of dimers was favored over further oxidation and reaction with water. This can be explained by the high stability of the intermediate radical of \( N,N \)-dimethyl-aniline.\textsuperscript{39}

In conclusion, the hydroxylation of aromatic compounds, as performed by cytochrome P450, shows some mechanistic resemblance to electrochemical oxidation. However, because of the lack of an active iron–oxygen species in the electrochemical system, the radical intermediate obtained after the initial oxidation and deprotonation steps will react further in various ways that will be determined by the substituents. In cases where hydroxylation takes place, such as for phenols, the products generally have a lower oxidation potential than the starting compound, and will be further oxidized.

**Epoxidation**

The cytochrome P450 catalyzed oxidation of benzo[a]pyrene yields an epoxide in the 4,5–position as described in Table 2.1d. The main electrochemical oxidation products were presumably a mixture of 1,6–, 3,6–, and 6,12–benzo[a]pyrene quinones and no epoxides were formed.\textsuperscript{40} Xu, Lu and Cole analyzed benzo[a]pyrene in an on-line electrochemical cell that generates electrochemical intermediates and products in situ at the tip of the
electrospray capillary needle. In addition to benzo[a]pyrene quinones, they also observed an ion at m/z 267 as a major oxidation product, corresponding to an intermediate 6-oxobenzo[a]pyrene cation. In our experiments, the ion at m/z 267 was present only in trace amounts. The lower yield of this oxidation product in our experiments is probably due to a higher water content (10% compared to 0.2% by Xu) and a longer delay time between the electrochemical cell and the mass spectrometer, that favored hydrolysis of the intermediate. The enzymatic 4,5-epoxidation of benzo[a]pyrene was thus not mimicked by the EC/MS system. It should be noted that the metabolism of polyaromatic hydrocarbons is usually very complex and is not limited to epoxidation. The carcinogenic character of many polyaromatic hydrocarbons is associated with the formation of radical cations through one electron oxidation by cytochrome P450. The end products of such oxidations are often quinones and the electrochemical oxidation of benzo[a]pyrene is probably very similar to this process.

Benzo[a]pyrene is not easily ionized in solution, and is therefore difficult to analyze with electrospray mass spectrometry. The main electrochemical oxidation products, the benzo[a]pyrene quinones, are readily protonated in aqueous solutions, and can thus be detected with electrospray. This experiment is a good example of how electrochemical oxidation can be used to enhance the signals of compounds that are otherwise difficult to detect with electrospray mass spectrometry. The S-oxidation of dibutylsulfide to dibutylsulfoxide described later is another example of a compound that is impossible to detect before oxidation, but has a detectable oxidation product.

Dealkylation and oxygenation of substrates containing a heteroatom: Introduction

![Diagram](image)

**Figure 2.5** The mechanism of cytochrome P450-catalyzed heteroatom oxidation and dealkylation proposed by Guengerich and Macdonald. X=NR, S, or O.
In Figure 2.5 the proposed mechanism for cytochrome P450–catalyzed heteroatom oxidation and dealkylation is shown.\textsuperscript{20,38,51} Depending on the nature of the heteroatom, the starting compound (A) can either undergo a one electron oxidation to give a heteroatom centered cation radical (B), or loose an $\alpha$–hydrogen atom to give a carbon based radical (C). The same neutral radical (C) is also obtained by deprotonation of the heteroatom centered cation radical (B). Oxygen rebound to the radical (C) gives a compound hydroxylated at the $\alpha$–carbon (E). Nonenzymatically, this hydroxylated compound decomposes to the final products, the dealkylated heteroatom–containing substrate and an aldehyde. If the substrate for example is a tertiary amine, the final products will be the secondary amine and the corresponding aldehyde. The heteroatom centered cation radical (B) can also react directly with the $[\text{Fe}=\text{O}]^{2+}$ complex to give an oxygenated product (D). Substrates without $\alpha$–hydrogens will obviously follow this route since the pathway A–$\rightarrow$B–$\rightarrow$C is not possible.

In addition to the presence or absence of $\alpha$–hydrogens, the outcome of the enzymatic reaction is dependent on the stability of the radical cation (B in Figure 2.5). For alkylamines, dealkylation is the main pathway in the electrochemical oxidation as well as in the enzymatic oxidation. The cation radical is better stabilized by sulfur and phosphorus, than by nitrogen. As a consequence, the main products from cytochrome P450 catalyzed oxidation, as well as from electrochemical oxidation, will be sulfoxides and phosphine oxides. If the heteroatom is oxygen, cytochrome P450 is not able to perform a one electron oxidation, due to the poor ability of oxygen to carry the positive charge. The reaction is therefore supposed to proceed via a direct hydrogen abstraction from the $\alpha$–carbon (pathway A–$\rightarrow$C–$\rightarrow$E in Figure 2.5). A summary of our experiments on the dealkylations and oxygenations of heteroatom containing compounds is given in Table 2.2.

### Table 2.2 Dealkylation and oxygenation of heteroatom containing compounds

<table>
<thead>
<tr>
<th>Enzyme catalyzed oxidation</th>
<th>Electrochemical oxidation products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a)</strong> N-dealkylation of aliphatic amines containing $\alpha$-hydrogens</td>
<td></td>
</tr>
<tr>
<td>Ex. CYP3A4 catalyzed N-deethylation of Lidocaine</td>
<td><img src="lidocaine_deethylation.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="lidocaine_deethylation.png" alt="Image" /></td>
<td><img src="lidocaine_hydroxylation.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>b)</strong> N-oxidation of nitrogen containing compounds without hydrogens at the $\alpha$-carbon.</td>
<td></td>
</tr>
<tr>
<td>Ex. N-hydroxylation of 2-acetamidofluorene</td>
<td><img src="fluorene_hydroxylation.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="fluorene_hydroxylation.png" alt="Image" /></td>
<td><img src="fluorene_hydroxylation.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions

<table>
<thead>
<tr>
<th>Enzyme catalyzed oxidation</th>
<th>Electrochemical oxidation products</th>
</tr>
</thead>
<tbody>
<tr>
<td>c) Sulfur dealkylation</td>
<td></td>
</tr>
<tr>
<td>Ex. S-demethylation of S-methylthiopurine</td>
<td><img src="image" alt="Sulfur dealkylation" /></td>
</tr>
<tr>
<td>mw 166</td>
<td>mw 152</td>
</tr>
<tr>
<td>d) Sulfur oxidation</td>
<td></td>
</tr>
<tr>
<td>Ex. S-oxidation of sulfides to sulfoxides</td>
<td><img src="image" alt="Sulfur oxidation" /></td>
</tr>
<tr>
<td>mw 146</td>
<td>mw 162</td>
</tr>
<tr>
<td>e) Phosphor oxidation</td>
<td></td>
</tr>
<tr>
<td>Ex. Phosphothionate oxidation of parathione</td>
<td><img src="image" alt="Phosphor oxidation" /></td>
</tr>
<tr>
<td>mw 291</td>
<td>mw 275</td>
</tr>
<tr>
<td>f) Oxygen dealkylation</td>
<td></td>
</tr>
<tr>
<td>Ex O-deethylation of 7-ethoxycoumarine</td>
<td><img src="image" alt="Oxygen dealkylation" /></td>
</tr>
<tr>
<td>mw 190</td>
<td>mw 162</td>
</tr>
</tbody>
</table>

No electrochemical reaction

**N–dealkylation**

The CYP3A4 catalyzed N–deethylation of the local anesthetic lidocaine is described in Table 2.2a.\(^5\) The reaction was readily mimicked by the EC/MS system.

If the heteroatom (X) in Figure 2.5 is a nitrogen with neighboring α–hydrogens, the main pathway is supposed to be A→B→C→E. The proposed mechanism for electrochemical oxidation of aliphatic amines is described in Figure 2.6.\(^5\)

![Figure 2.6](image)

**Figure 2.6** Electrochemical oxidation of aliphatic amines.
Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions

The first two steps are the same for both mechanisms. A one electron oxidation gives an aminium cation radical, which, upon deprotonation, gives an α-carbon centered radical. In the P450 catalyzed mechanism, the electron and the proton are donated to the iron–oxygen intermediate of the enzyme and the hydroxyl group is introduced on the α-carbon by subsequent reaction with the formed [Fe–OH]^{3+} species (radical recombination). In the electrochemical oxidation, the electron is donated to the working electrode, and the proton is lost to any compound acting as a base in solution. Since there is no active iron–oxygen intermediate present in the electrochemical system, the neutral radical (III) is further oxidized at the working electrode to the iminium ion (IV). Finally, hydrolysis of the iminium ion (IV) will give the dealkylated amine and the corresponding aldehyde.

As discussed earlier, the stability of the initial radical cation is very important for the outcome of the enzyme catalyzed oxidation. The same is true for the electrochemical oxidation mechanism. In addition to the data presented in Table 2.2, several nitrogen containing compounds have been tested for dealkylation in the EC/MS system. For aliphatic, tertiary amines the oxidation potential for the formation of dialkylamines decreases as the size of the alkyl chain increases. In 10 mM ammonium acetate (pH 7), a series of trialkylamines was oxidized to the corresponding secondary amines as follows: triethylamine (650 mV), tripropylamine (330 mV), tributylamine (270 mV) and tripentylamine (170 mV).

N–oxides

2–Acetamidofluorene can be oxidized enzymatically at the amide nitrogen, as described in Table 2.2b. Electrochemical oxidation of 2–acetamidofluorene in 10 mM ammonium acetate gave a product at m/z 240 at 315 mV, corresponding to a gain of oxygen. The MS/MS fragmentation pattern suggests a hydroxylation at the aromatic ring system and not at the amide nitrogen. The N–oxidation was thus not mimicked by the EC/MS system. It should be noted that hydroxylation at the aromatic ring system is also a common metabolic reaction for 2–acetamidofluorene. Pyridine and 2,3–lutidine were also studied as model compounds for a possible electrochemical formation of N–oxides, but no electrochemical reaction was observed.

In the enzymatic mechanism, described in Figure 2.5, the radical cation intermediate (B) can react directly with the [Fe=O]^{2+} complex to give the N–oxide (D) instead of donating a proton from the α–carbon. In the electrochemical system, there is no such active iron–oxygen intermediate available, and as a consequence, no N–oxidation takes place.
Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions

Sulfur, S–dealkylation and sulfoxide formation
The major products from cytochrome P450 catalyzed oxidations, as well as from electrochemical oxidations of sulfur containing compounds, are sulfoxides. However, in some cases cytochrome P450 gives the dealkylated product as well as the sulfoxide. One example is the S–demethylation of S–methylthiopurine, described in Table 2.2c. In 0.1 M acetic acid, the sulfur was electrochemically oxidized to the sulfoxide at 850 mV and dealkylation was not observed. Three other sulfides, dibutyl sulfide (Table 2.2d), diphenyl sulfide and thioanisole, were also oxidized in the electrochemical system. They were all oxidized to the corresponding sulfoxides at +250 to +450 mV in 0.1 M acetic acid. The position of the oxygen at the sulfur was confirmed by comparison of the MS/MS fragmentation pattern of the electrochemically generated sulfoxides with purchased standards of dibutyl sulfoxide and diphenyl sulfoxide. At increased potentials, the sulfoxides were further oxidized to sulfones. An acidic electrolyte clearly favored the sulfoxide formation.

In most cases, the electrochemical oxidation provides a good mimic of the enzymatic oxidation. A correlation between the rates of sulfoxide formation for a number of para–substituted thioanisoles and the electrochemical oxidation potentials has been reported by Watanabe et al.

S–Methylthiopurine is easily protonated, but the other sulfides do not give [M+H]+ ions in solution, and are thus not detectable with an electrospray interface. Electrochemical oxidation of the sulfides gives sulfoxides that are easily protonated and can be detected by electrospray MS. For these experiments, both an electrospray interface and an atmospheric pressure chemical ionization (APCI) interface have been used. When APCI was used, the sulfides could be detected before oxidation, but the oxidized products gave signals with >80 times higher intensity. For analysis of sulfides, on–line electrochemical oxidation might thus be a way to reach lower detection limits for electrospray as well as for APCI.

Phosphor, phosphine oxide formation
The P450 catalyzed phosphothionate oxidation of parathion, a widely used pesticide, is described in Table 2.2e. Electrochemical oxidation gave the same oxidation product at a potential of 600 mV in 0.1 M acetic acid. Compounds containing phosphorous, generally have an even lower oxidation potential than sulfur containing compounds, and cytochrome P450 catalyzed oxidation as well as electrochemical oxidation almost exclusively result in phosphine oxides.
Oxygen, dealkylation of ethers

Table 2.2f shows the \(O\)–deethylation of 7–ethoxycoumarin, a general test substrate for various P450 enzymes, e.g., human CYP1, CYP2 and CYP3 families.\(^{56}\) The \(O\)–deethylation could not be mimicked in the electrochemical cell. 7–Methoxycoumarine was also analyzed, but no demethylation products were observed.

Due to the electronegative character of oxygen, dealkylation of ethers by cytochrome P450 is thought to proceed via a direct hydrogen abstraction from the \(\alpha\)–carbon (pathway A→C→E in Figure 2.5).\(^{20}\) No electrochemical oxidation was observed for any of the ethers tested here. The explanation for this is most likely the same as the reason for the direct hydrogen abstraction by cytochrome P450. The oxidation potential of ethers is too high for cytochrome P450 to extract an electron, and too high for electrochemical oxidation in aqueous solutions. It should be noted that some aromatic ethers have relatively low oxidation potential and are probably oxidized by cytochrome P450 via a one electron mechanism.\(^{42,57}\)

Other types of cytochrome P450 catalyzed oxidations

Aliphatic and aromatic hydroxylations and dealkylation/oxygenation of heteroatom containing compounds are the most common reactions catalyzed by cytochrome P450. There are however other types of cytochrome P450 catalyzed oxidations, and some of these are described in Table 2.3.

**Table 2.3 Other types of cytochrome P450 catalyzed oxidations**

<table>
<thead>
<tr>
<th>Enzyme catalyzed oxidation</th>
<th>Electrochemical oxidation products</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Oxidative deamination of primary amines</td>
<td></td>
</tr>
<tr>
<td>Ex. Oxidative deamination of amphetamine</td>
<td></td>
</tr>
<tr>
<td><img src="attachment" alt="Molecule" /> (\text{mw 135}) → <img src="attachment" alt="Molecule" /> (\text{mw 134})</td>
<td><img src="attachment" alt="Molecule" /> (\text{mw 133})</td>
</tr>
<tr>
<td>b) Alcohol and aldehyde oxidation</td>
<td></td>
</tr>
<tr>
<td><img src="attachment" alt="Molecule" /> (\text{mw 109}) → <img src="attachment" alt="Molecule" /> (\text{mw 107}) → <img src="attachment" alt="Molecule" /> (\text{mw 123})</td>
<td><img src="attachment" alt="Molecule" /> (\text{mw 107})</td>
</tr>
<tr>
<td>c) Oxidative dehalogenation</td>
<td></td>
</tr>
<tr>
<td><img src="attachment" alt="Molecule" /> (\text{mw 127}) → <img src="attachment" alt="Molecule" /> (\text{mw 107})</td>
<td><img src="attachment" alt="Molecule" /> (\text{mw 93})</td>
</tr>
</tbody>
</table>
Oxidative deamination, oxidative dehalogenation and alcohol/aldehyde oxidation are basically oxygen transfer processes with insertion of a hydroxyl group at the $\alpha$–carbon, as described earlier. The structure of the substrate and the physical features of the P450 active site will dictate the specific mechanistic pathway taken. The hydroxylated products are unstable and will nonenzymatically lose ammonia, HX or water to give the final products. The mechanisms for alcohol oxidation and dehydrogenation will be discussed in more detail.

**Oxidative deamination**

Amphetamine is oxidized by cytochrome P450 to the ketone, as described in Table 2.3a. Electrochemical oxidation of amphetamine in 0.1 M NH$_4$OH gave about 5% of a product at m/z 134 at 850 mV which was presumed to correspond to the imino structure presented in Table 2.3a. In 0.1 M NH$_4$OH, hexylamine gave trace amounts of a compound at m/z 98 at high oxidation potentials, believed to originate from the corresponding nitrile.

The mechanism described for the electrochemical oxidation of primary amines is basically the same as the mechanism described for the oxidation of tertiary amines described in Figure 2.6. Presumably, hydrolysis of the intermediate imines (Figure 2.6 IV) gives ammonia and the corresponding aldehydes or ketones in the case of $\alpha$–branched primary amines. Under basic conditions, further deprotonation and oxidation to nitriles has been reported. The oxidation potentials are much higher for primary than for tertiary amines, and under the conditions used in these experiments, no oxidation was observed in acidic or neutral solutions. By changing the electrolyte and/or the electrode material, a better mimic of the enzymatic oxidation might be obtained, but under the conditions used in these experiments, no formation of ketones or aldehydes was observed for any of the amines examined.

**Alcohol and aldehyde oxidation**

Cytochrome P450 is known to catalyze the oxidation of alcohols to aldehydes that can be further oxidized to the corresponding carboxylic acids as illustrated in Table 2.3b. The electrochemical oxidation of 2–(hydroxymethyl)–pyridine gave about 3% of pyridine–2–
aldehyde at 750 mV in 0.1 M NH₄OH. The identity of the product was confirmed by comparison of the MS/MS fragmentation pattern with a purchased pyridine–2–aldehyde standard. Electrochemical oxidation of aldehydes to carboxylic acids was not observed in our experiments but has been reported to occur under different electrochemical reaction conditions.³⁶

![Figure 2.7](image)

**Figure 2.7** a) Cytochrome P450 catalyzed oxidation of benzylic alcohols to aldehydes via gem-diol as suggested by Vaz and Coon. R=H or CH₃. b) Electrochemical oxidation of benzyl alcohol to benzaldehyde.

Vaz and Coon have suggested the mechanism presented in Figure 2.7a for the oxidation of benzylic alcohols by cytochrome P450 2B4 and 2E1.⁶⁰ There are alternative pathways possible from the radical to the aldehyde, but the intermediate gem–diol seems to be the most likely route. The mechanism for electrochemical oxidation of benzyl alcohol in Figure 2.7b is initiated in the same way as the enzymatic oxidation, but the aldehyde is probably formed by one electron oxidation of the radical followed by deprotonation.³⁶

The oxidation of aldehydes by cytochrome P450 normally yields the corresponding carboxylic acid, either by hydrogen abstraction followed by “oxygen rebound” as described earlier, or by a mechanism involving nucleophilic attack by the iron–peroxo–porphyrin intermediate. This mechanism has been suggested for the P450 2B4 catalyzed oxidation of cyclohexane carboxaldehyde.²⁵

In conclusion, the P450 catalyzed oxidation of alcohols to aldehydes was mimicked by the EC/MS system but further oxidation to the carboxylic acid was not observed.

**Oxidative dehalogeneration**

The oxidation of 2-chloromethylpyridine (2–picolinechloride) shown in Table 2.3c was the only halogenated compound tested in the electrochemical system. The oxidation product
was identified as 2–picoline by comparison of MS/MS fragmentation pattern with a 2–picoline standard. The dehalogenation was thus not mimicked by the EC/MS system. Further investigation is necessary to be able to draw any conclusions about halogenated compounds in general.

**Dehydrogenation**

Acetaminophen is oxidized by several human cytochrome P450 enzymes, including CYP 2E1, 1A2, 2A6, 3A4 and 2D6, to its toxic metabolite $N$–acetyl–$p$–benzoquinoneimine.\(^6^1\) The dehydrogenation of acetaminophen in Table 2.3d was readily mimicked by the EC/MS system.

![Chemical Structures](image)

**Figure 2.8** a) Cytochrome P450 catalyzed dehydrogenation of acetaminophen proposed by Koymans et al.\(^6^2\) b) Electrochemical oxidation of acetaminophen.

The mechanism proposed by Koymans et al. is presented in Figure 2.8a.\(^6^2\) The formation of the radical intermediate is believed to occur via electron transfer followed by proton abstraction. Another one electron oxidation followed by a second proton abstraction yields the final product. The mechanism for the electrochemical oxidation in Figure 2.8b is practically the same as the suggested enzymatic mechanism.
2.4 Conclusions

This study presents an overview of the different oxidations catalyzed by cytochrome P450 and how they correlate to electrochemical oxidations. The EC/MS system provided successful mimics in cases where the P450 catalyzed reactions are supposed to proceed via a mechanism initiated by a one electron oxidation, such as N–dealkylation, S–oxidation, P–oxidation, alcohol oxidation and dehydrogenation. The P450 catalyzed reactions thought to be initiated via direct hydrogen atom abstraction, such as O–dealkylation and hydroxylation of unsubstituted aromatic rings generally had a too high oxidation potential to be electrochemically oxidized within the potential limits of water, and were not mimicked by the EC/MS system. An electrochemical system, like the one described here, obviously has a number of drawbacks. Some reactions, as for example the important aliphatic hydroxylations, are not mimicked and the stereochemistry induced by different cytochrome P450 enzymes can not be obtained without modifications of the electrode surfaces. Despite these limitations, valuable information can be obtained concerning the sensitivity of the substrate towards oxidation and the regions of the molecule where oxidations are likely to take place. In order to draw any conclusions for specific classes of compounds, more specific work is needed. Analogy between cytochrome P450 oxidation rates and electrochemical oxidation potentials for oxidations proceeding via initial one electron oxidation has been reported by the group of Guengerich and others. In a large chemical library with similar compounds, comparison of the oxidation potentials might provide useful information about the relative metabolic stability.

Electrochemistry can be very useful for small–scale synthesis of metabolites, starting from a drug. The on–line EC/MS system is suitable for optimization of the electrochemical conditions. A potential sweep with the EC/MS system, as described in this chapter, will give a quick answer regarding what potentials should be used for optimum yield of the desired product. The simplicity of the system, and the ease and speed with which it can be applied to a large number of compounds, make it a useful tool in drug metabolism research.

Acknowledgement

The authors wish to thank Ruben de Kanter for helpful discussions about cytochrome P450 metabolism.

2.5 References

Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions

Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions


38. Montellano PR, *Oxygen activation and reactivity*, In ref. 22.


Chapter 2 Comparison between EC/MS and cytochrome P450 catalyzed oxidation reactions

56. Waxman DJ, Chang TKH, *Use of 7-Ethoxycoumarin to monitor the Human CYP1, CYP2, and CYP3 Families*, p. 175-179 in ref. 19.