Electrochemical and Enzymatic *In Vitro* Studies on Reactive Drug Metabolites

Synthesis, Characterization and Avoidance

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UNIVERSITY OF GOTHENBURG

DOCTORAL THESIS

Submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in Chemistry

Electrochemical and Enzymatic *In Vitro* Studies on Reactive Drug Metabolites
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ISBN: 978-91-628-8089-7

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Printed by Intellecta Infolog AB
Göteborg, 2010

Nothing in progression can rest on its original plan.
Edmund Burke (1729-1797)

Om snöret inte håller, utan går av, är det bara att försöka med ett annat snöre.
A.A. Milne (1882-1956)

ABSTRACT

During development of pharmaceuticals, it is essential to as early as possible identify and preferably avoid formation of reactive metabolites and intermediates. Reactive metabolites may represent obstacles in the development and use of drugs, due to their potential toxicity. The formation of reactive metabolites often involves oxidation reactions and the cytochrome P450 (CYP) enzymes are the most important enzymes catalyzing oxidative phase I drug metabolism. The present studies were initiated with the aim to chemically mimic phase I drug oxidations, using electrochemistry for generation, characterization and identification of reactive drug metabolites and to avoid metabolic activation by altering the chemical structure of the parent drug.

Three complementary chemical systems were evaluated for their abilities to mimic CYP-catalyzed oxidations. All relevant oxidative reactions were mimicked by at least one of the three systems. The oxidative metabolism of the antimalarial agent amodiaquine was studied in liver microsomes and recombinant enzymes. Electrochemical oxidation was used to characterize metabolic intermediates and enabled the structural determination of an aldehyde metabolite of amodiaquine by NMR spectroscopy. In addition, the bioactivation of the neuroleptic drug haloperidol was studied. Proposed iminium species of haloperidol were observed in the on-line electrochemical oxidation setup. Subsequent trapping with cyanide, both in liver microsomes and in the electrochemical system, strengthened the proposal of iminium intermediate formation in the oxidative metabolism of haloperidol. Further, in the cyanide trapping experiments the presence of the dihydropyridinium species and the absence of the corresponding cyano adduct indicated that an unstable cyano adduct was formed. Trapping of an exocyclic iminium species with cyanide in the electrochemical experiments but not in the liver microsomal incubations implied that this intermediate, obligatory in the electrochemically mediated *N*-dealkylation, may not be formed in the CYP-catalyzed reaction.

Metabolic studies on haloperidol and trifluperidol, in comparison with their corresponding silicon analogues, were performed in liver microsomes and hepatocytes. The replacement of one single carbon atom by a silicon atom resulted in significant changes in the metabolic fate, including the absence of silapyridinium metabolites and glucuronidation on the silanol group.

In conclusion, several different examples of how electrochemistry can be applied in studies on reactive metabolites are provided in this thesis. For drug risk assessment, it is important to characterize formed reactive metabolites and if possible, alter chemical design to avoid reactivity.

Keywords: Cytochrome P450, Drug, Electrochemical oxidation, Electrochemistry, Intermediate, Metabolism, Metabolic activation, Mimicry, Reactive Metabolite, Trapping.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals I-IV. The papers are appended at the end of the thesis.

I **Mimicry of Phase I Drug Metabolism - Novel Methods for Metabolite Characterization and Synthesis**
Johansson, T., Weidolf, L., Jurva, U.
Rapid Communications in Mass Spectrometry **21**, 2323-2331 (2007)

II **Novel Metabolites of Amodiaquine Formed by CYP1A1 and CYP1B1: Structure Elucidation Using Electrochemistry, Mass Spectrometry and NMR**
Johansson, T., Jurva, U., Grönberg, G., Weidolf, L., Masimirembwa, C.
Drug Metabolism and Disposition **37**, 571-579 (2009)

III **P450-Catalyzed vs. Electrochemical Oxidation of Haloperidol Studied by Ultra-Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry**
Johansson Mali'n, T., Weidolf, L., Castagnoli, N., Jr., Jurva, U.
Accepted for publication in Rapid Communications in Mass Spectrometry.

IV **In Vitro Metabolism of Haloperidol and Sila-Haloperidol: New Metabolic Pathways Resulting from Carbon/Silicon Exchange**
Johansson, T., Weidolf, L., Popp, F., Tacke, R., Jurva, U.
Drug Metabolism and Disposition **38**, 73-83 (2010)

CONTRIBUTION REPORT

Paper I Minor contribution to the formulation of the research problem; performed all the experimental work; interpreted the results, and wrote the manuscript.

Paper II Contributed to the formulation of the research problem; performed the experimental work except for the NMR analysis; interpreted the results, and wrote the manuscript.

Paper III Formulated the research problem; performed all the experimental work; interpreted the results, and wrote the manuscript.

Paper IV Contributed to the formulation of the research problem; performed the in vitro experimental work in microsomes; interpreted the results, and wrote the manuscript.

ABBREVIATIONS

ACN	Acetonitrile
AQ	Amodiaquine
AQQI	Amodiaquine quinoneimine
CYP	Cytochrome P450
Da	Dalton
DNA	Deoxyribonucleic acid
EC	Electrochemistry/electrochemical
EC-ESI/MS	Electrochemistry on-line with electrospray ionization mass spectrometry
EC-Fenton	Electrochemically assisted Fenton
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
GSH	Glutathione
^{1}H NMR	Proton NMR
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLM	Human liver microsomes
LC	Liquid chromatography
MA	Methoxylamine
MH^+	Positively charged protonated molecule
MS	Mass spectrometry
m/z	Mass-to-charge-ratio
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridinium
N0437	2-(*N*-propyl-*N*-2-thienylethylamine-)-5-hydroxytetralin
NAC	*N*-Acetyl-L-cysteine
NAL	*N*-Acetyl-L-lysine
NAT	*N*-Acetyl-L-tyrosine
NADPH	Dihydronicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
ppm	Parts-per-million
QTOF	Quadrupole time of flight
RLM	Rat liver microsomes
rCYP	Recombinantly expressed cytochrome P450
SPE	Solid phase extraction
UDPGA	Uridine-5'-diphosphoglucuronic acid
UGT	UDP-glucuronosyltransferase

CONTENTS

1 Introduction

1.1 DRUG METABOLISM

Very simplified, the fate of an orally administered drug in the body consists of processes such as absorption, distribution, interaction with target, and eventually elimination from the body. Some drugs may be excreted in their original state but usually drugs are metabolized prior to excretion to facilitate elimination.

1.1.1 Phase I and phase II drug metabolism

The metabolism of drugs may be divided into phase I and phase II drug metabolism. Phase I reactions are functionalization reactions where a functional group is unveiled or introduced, e.g. by oxidation. Phase II reactions are mainly conjugation reactions where this or another functional group may be conjugated with an endogenous molecule or moiety, e.g. glucuronic acid or sulfate.[1]

1.1.2 Cytochrome P450 enzymes

The most important phase I metabolic reactions are enzyme-catalyzed oxidations. A number of different enzyme systems are responsible for such oxidations, where the most important enzymes belong to the cytochrome P450 superfamily. The cytochrome P450 enzymes (CYPs) are expressed in the highest levels in the liver and are mainly located in the endoplasmatic reticulum of the cells.[1] All cytochrome P450 enzymes are monooxygenases i.e. they cleave dioxygen to incorporate one oxygen atom into the substrate while the other oxygen atom is reduced by two electrons to give water. Dihydronicotinamide adenine dinucleotide phosphate (NADPH) usually provides the two electrons needed for this process.[2] The overall reaction is presented below:

$$\text{Substrate} + O_2 + 2e^- + 2H^+ \xrightarrow{\text{CYP}} \text{Substrate(O)} + H_2O$$

The CYP enzymes have broad substrate specificity and a large range of biotransformations are catalyzed by these enzymes. The most important and commonly encountered cytochrome P450-catalyzed oxidations are hydroxylations, epoxidations, heteroatom oxidations and dealkylations, dehalogenations and alcohol oxidations. Like hemoglobin, the CYP enzymes are hemoproteins containing a common heme moiety, iron protoporphyrin IX. Thus the reactive site of all CYP

enzymes contains this iron protoporphyrin IX (Figure 1.1) where the iron is coordinated to four pyrrol nitrogen atoms with cysteine thiolate as the fifth ligand, leaving the sixth coordination site to bind and activate molecular oxygen.[1,2]

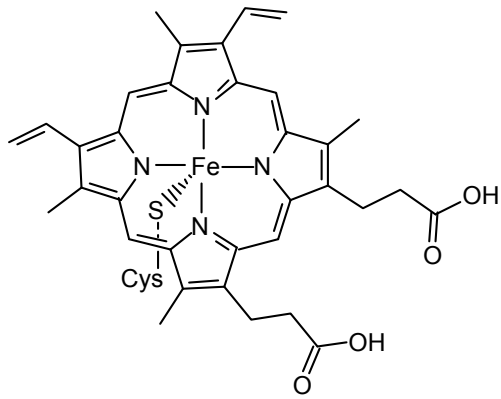


Figure 1.1. Iron protoporphyrin IX with cysteine thiolate as the fifth ligand.

The catalytic cycle of cytochrome P450 is given in Figure 1.2. The resting form of the enzyme is a six-coordinate ferric state (**1**) with water as the exchangeable sixth ligand. The catalytic cycle is initiated when the substrate (R–H) enters the active site and the water molecule is displaced. A five-coordinate ferric state (**2**) is formed, which is reduced by one electron to a ferrous state (**3**). Molecular oxygen reacts with **3**, forming an oxyferrous complex **4a**, that due to the strong electronegativity of O_2 rearranges by resonance to **4b**. The following one electron reduction is supposed to give a negatively charged iron(III)-peroxo complex (**5**). Protonation of **5** results in a hydroperoxide complex (**6**). A second protonation, followed by heterolytic cleavage of the oxygen–oxygen bond, generates water and a reactive iron-oxo species (**7**). This intermediate is often referred to as an oxoferryl porphyrin radical and is generally considered to be the electrophilic species acting as the active oxidant in most cytochrome P450-catalyzed oxidations. The intermediate **7** transfers an oxygen atom to the substrate, giving the product R(O)H. When this oxidized substrate dissociates, the ferric state **1** is regenerated with one molecule of water as ligand, and the catalytic cycle of cytochrome P450 is complete.[1-3]

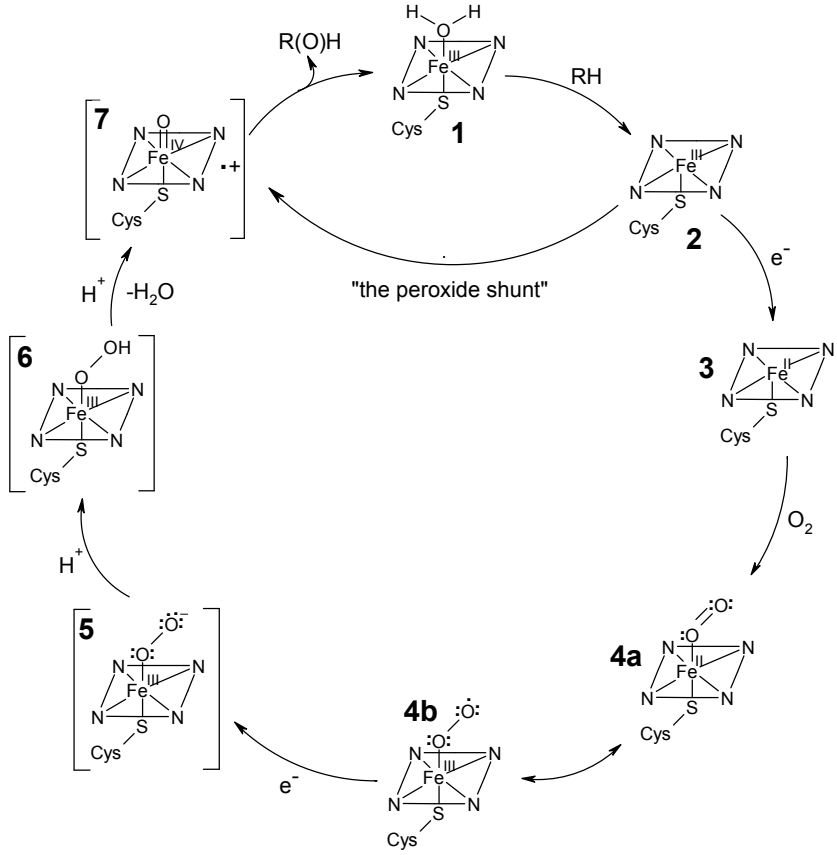


Figure 1.2. The catalytic cycle of cytochrome P450.

Oxygenating compounds, like hydrogen peroxide and alkylhydroperoxides, may short-cut the catalytic cycle of cytochrome P450 by reacting with **2** in Figure 1.2 to give the oxoferryl porphyrin radical (**7**) directly. This mechanism, known as the peroxide shunt, is included in Figure 1.2.[2]

Two different mechanisms have been proposed to be involved in the reactivity of cytochrome P450, namely single electron transfer (SET) and hydrogen atom transfer (HAT).[4,5] Proposed SET and HAT mechanisms for oxidation leading to *N*-dealkylation are presented in comparison with the electrochemically mediated *N*-dealkylation in section **1.2.3**.

1.1.3 Reactive drug metabolites

In general, drug metabolism is a route for detoxification which generates more hydrophilic drug metabolites to facilitate excretion. Despite this, reactive metabolites

or intermediates may be formed either by phase I or phase II drug metabolizing enzymes. Reactive phase I drug metabolites formed by cytochrome P450 are often electrophilic species, such as epoxides and quinoneimines, or radical species.[6] A phase II metabolite forming a reactive intermediate may be exemplified by sulfate conjugation of a hydroxylamine group, followed by elimination of SO_4^{2-} to generate a highly reactive nitrenium species.[7] The metabolism of drugs and chemicals to reactive species is also commonly referred to as metabolic activation or bioactivation.[8]

Covalent binding of reactive species to biological macromolecules, such as proteins, DNA or enzymes, may cause drug-induced toxicity.[9] Reactive metabolites are often short-lived and seldom directly detected or isolated, and hence, different reagents are commonly used for trapping reactive metabolites. In general, more stable and detectable conjugates are formed, from which the reactive metabolite may be identified. Trapping reagents used in metabolism studies are e.g. glutathione (GSH), cyanide, *N*-acetylcysteine (NAC), *N*-acetyllysine (NAL), *N*-acetyltyrosine (NAT), cysteine and methoxylamine (MA).[6]

1.2 MIMICRY OF PHASE I DRUG METABOLISM

During drug development, metabolic fates of compounds are very important to explore and confirm, not least from a safety perspective. Different chemical systems may be used to facilitate this process and to complement the *in vitro* and *in vivo* work. Mimicry of oxidative drug metabolism has recently been reviewed.[10]

1.2.1 Metalloporphyrins

Several synthetic metalloorganic complexes are similar to the active site of cytochrome P450 and may be used as models or mimics for the non enzyme bound form of the active site. The entire catalytic cycle of cytochrome P450 is difficult to mimic chemically. However, oxidants may short-cut the catalytic cycle via the peroxide shunt, as described earlier. By adding oxidants, like hydrogen peroxide or hypochlorite (ClO^-), to metalloorganic complexes similar to the CYP active site, a reactive species may be formed. This generated reactive species, similar to the oxoferryl porphyrin radical **7**, can insert oxygen into substrates.[2]

A typical system consists of a porphyrin/porphine complex with a metal ion, usually manganese or iron, together with an oxygen donating compound such as NaClO or hydrogen peroxide. This system is able to insert oxygen with good efficacy through epoxidations, hydroxylations, *N*-oxidations and/or *S*-oxidations on a test compound.[11] A water-soluble iron (III) porphyrin is exemplified in Figure 1.3.

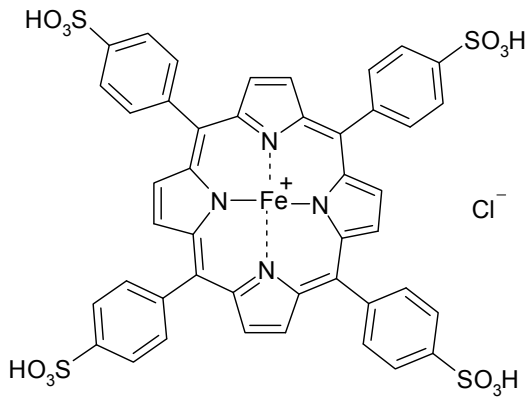


Figure 1.3. Iron(III)meso-tetra(4-sulfonatophenyl)porphine chloride.

1.2.2 Electrochemically assisted Fenton reaction

The classical Fenton reaction is described in Figure 1.4. This is a simplified version of a much more complicated mechanism, which is still a controversy.[12] As Fe^{2+} donates an electron to hydrogen peroxide, a hydroxyl radical is produced. This hydroxyl radical is highly electrophilic and adds readily to double bonds and aromatic rings. In addition, the hydroxyl radical can abstract a hydrogen atom from various organic compounds. The end products of both mechanisms are often hydroxylations, epoxidations and dehydrogenations.[13]

In the reaction, Fe^{2+} loses an electron and is thus by definition oxidized to Fe^{3+}, inactivating further reaction. In a chemical Fenton system, Fe^{2+} is regenerated by the addition of a reducing agent such as ascorbic acid.[14] In an electrochemically assisted Fenton (EC Fenton) system, the regeneration of Fe^{2+} is achieved by reduction of Fe^{3+} at the electrode surface.[15]

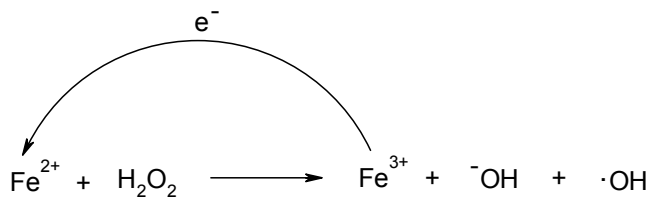


Figure 1.4. The principle of the Fenton reaction. Regeneration of Fe^{2+} from Fe^{3+} is done by chemical reduction with L-(+)-ascorbic acid, or by electrochemical reduction at the working electrode.

1.2.3 Electrochemical oxidation

Electrochemistry (EC) is a field within chemistry where electron transfer reactions that occur at the interface between electrodes and solutions are studied. EC is an alternative technique that may be used for inducing oxidations in order to simulate oxidative phase I drug metabolism. Recently, the use of electrochemistry coupled to mass spectrometry in drug metabolism and protein research has been reviewed.[16] Figure 1.5 presents mechanisms for cytochrome P450-catalyzed versus electrochemical oxidation resulting in *N*-dealkylation. The mechanism proposed for the CYP-catalyzed *N*-dealkylation proceeds either via single electron transfers or via a hydrogen atom transfer to a common radical intermediate, which after oxygen rebound collapses to the final products.[3,4,17] The corresponding electrochemical mechanism consists of an initial electron abstraction, followed by deprotonation and the subsequent abstraction of another electron results in an iminium intermediate. Following hydrolysis, the intermediate decomposes into the end products.[18]

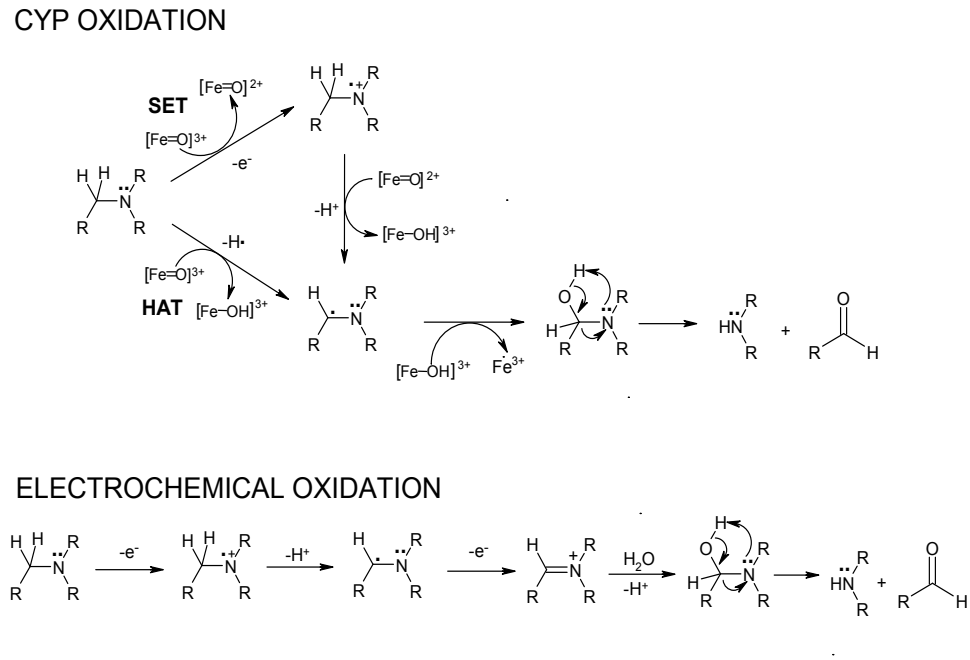


Figure 1.5. Comparison of general mechanisms for CYP-catalyzed vs. electrochemical oxidation resulting in *N*-dealkylation.

1.2.4 EC for generating reactive metabolites

The electrochemical technique is also useful for studying reactive drug metabolites. Since reactive metabolites often are short-lived, an on-line setup may be used for a direct detection where the electrochemical cell is coupled on-line with a mass spectrometer. Reactive metabolites may also be generated electrochemically, caught by

a trapping reagent and the formed adduct may be characterized, e.g. by liquid chromatography/mass spectrometry (LC/MS) or nuclear magnetic resonance (NMR).

Although the number of studies aimed to generate reactive metabolites by electrochemical oxidation has escalated during recent years, there are also examples of early studies. The electrochemical oxidation of acetaminophen (also called paracetamol, see Figure 1.6) to its *N*-acetyl-*p*-benzoquinoneimine has been investigated in numerous studies. Getek et al. used an on-line system where glutathione or cysteine was added to trap the quinoneimine before entering the mass spectrometer.[19] In another study, the electrochemically generated *N*-acetyl-*p*-benzoquinoneimine was trapped by glutathione and *N*-acetylcysteine. The investigation also indicated that glutathione was slightly more reactive towards the quinoneimine than *N*-acetylcysteine.[20] Acetaminophen has also been used as a test compound to develop an electrochemical method for studying reactive phase I drug metabolites. The acetaminophen quinoneimine was trapped by glutathione, *N*-acetylcysteine and *N*-acetyltyrosine, and the reaction rate with glutathione was studied by cyclic voltammetry. The method was evaluated with a set of compounds known to form quinones, quinone methides, quinoneimines, imine methides and nitrenium ions. These reactive metabolites were trapped with glutathione and one of the resulting conjugates, the glutathione conjugate of clozapine, was structurally determined by NMR spectroscopy.[21] Previously, clozapine has been shown to be electrochemically oxidized to the reactive nitrenium species, and the subsequent trapping resulted in several isomeric glutathione conjugates.[22]

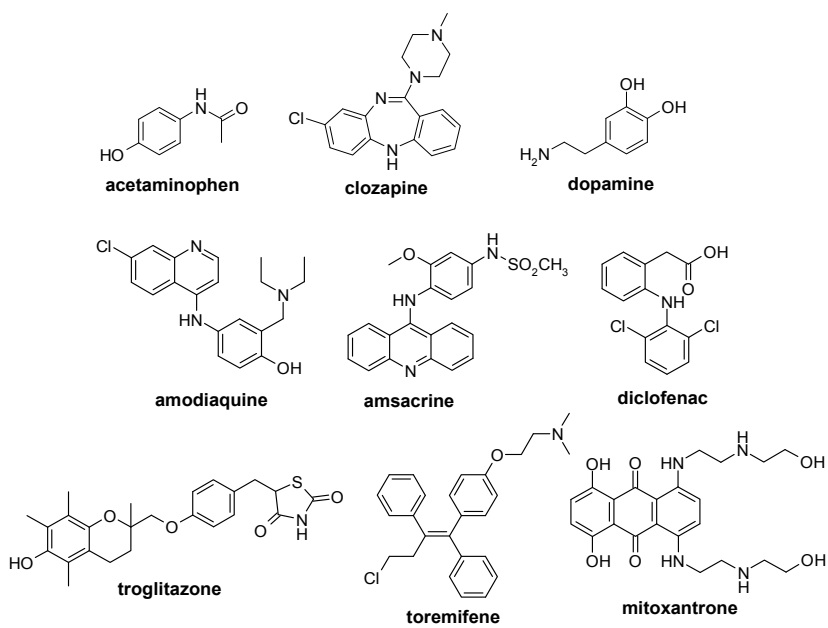


Figure 1.6. Examples of drugs shown to be electrochemically oxidized to reactive metabolites or intermediates.

Trapping of electrochemically generated quinone species has also been studied. The *ortho*-quinone of dopamine has been successfully trapped with glutathione and *N*-acetylcysteine. Cyclic voltammetry was used to elucidate the stability of the conjugates, identified by mass spectrometry and NMR spectroscopy.[23,24] In addition, *N*-acetyldopamine and *N*-β-alanyldopamine has been electrochemically oxidized to their quinones, and trapped by the nucleophiles *N*-acetylcysteine and thiourea.[25]

Different on-line methods have also been developed and two setups have mainly been used: electrochemistry coupled on-line with mass spectrometry (EC/MS)[26,27] and electrochemistry coupled on-line with liquid chromatography and mass spectrometry (EC/LC/MS). In the EC/MS setting, the mixture of oxidation products leaving the electrochemical cell is simultaneously monitored, while in the EC/LC/MS mode a separation takes place before entering the mass spectrometer. EC/LC/MS has recently been used for the detection of the quinoneimine of amodiaquine, the quinonediimine of amsacrine and the quinone of mitoxantrone.[28]

Previously, the electrochemical oxidation of amodiaquine to its quinoneimine has been used to exemplify dehydrogenation. The trapping of the quinoneimine with glutathione and cysteine resulted in four glutathionyl conjugates and four cysteinyl conjugates. The trapping with cysteine were repeated on a preparative scale and the major conjugates were characterized by NMR spectroscopy.[29] Another electrochemical trapping study was performed on the two hydroxylated metabolites of diclofenac, 4'-OH-diclofenac and 5-OH-diclofenac, which may be further metabolized to the corresponding quinoneimines. Electrochemical oxidation and subsequent trapping by glutathione resulted in mono-, di- and triglutathionyl conjugates.[30]

Madsen et al. have performed an electrochemical study for discriminating between three proposed pathways for the formation of troglitazone reactive metabolites. The electrochemically generated reactive metabolite was trapped by *N*-acetylcysteine and glutathione. The NMR data on the *N*-acetylcysteine conjugate lead to the proposal that the *ortho*-quinone methide is the major reactive metabolite of troglitazone [31]. Another group oxidized toremifene to its quinone methide and the trapping with glutathione was done in the absence and presence of the enzyme glutathione-*S*-transferase. One of the two formed glutathionyl conjugates was only observed when glutathione-*S*-transferase was present.[20]

Recently, an electrochemical system for generation and detection of covalent protein modification by reactive drug metabolites has been used. The test compounds were oxidized to their reactive species, the quinoneimines of amodiaquine and acetaminophen, and the nitrenium ion of clozapine. Adduct formation was shown for these reactive species with the protein β-lactoglobulin A and for human serum albumin, protein modification was observed after reaction with the nitrenium ion of clozapine.[32]

1.3 AVOIDING REACTIVE METABOLITE FORMATION

Several strategies to avoid formation of reactive, potentially toxic metabolites by structural alterations have been proposed. One approach is replacement of metabolically susceptible functionalities, where the moiety of the drug molecule causing the structural alert is replaced by a moiety which is generally resistant to metabolism or is metabolized to a stable metabolite. There are for instance several examples where aniline substituents have been replaced by other aromatic rings, e.g. toluenes. Another strategy is to block the site of initial metabolism that subsequently leads to reactive metabolite formation. Alternative metabolic soft spots may also be introduced in the drug molecule to achieve a metabolic shift away from the reactive metabolite formation. Another possibility is the introduction of substituents that minimizes the bioactivation leading to reactive metabolites, by affecting the electronic properties and/or steric hindrance.[8] In reality, the different strategies are often combined. This may be exemplified by an aryloxy-substituted drug candidate, where the phenyl group was replaced by a 5-trifluoromethyl pyridine group, in order to reduce the metabolic activation potential.[33]

2
Aims of the Study

The overall aim of the work presented in this thesis was to generate knowledge regarding metabolic activation of drugs, with emphasis on reactive species and/or intermediates. The specific aims were:

1. To mimic oxidative drug metabolism with different chemical systems.

2. To characterize reactive metabolites/intermediates with different trapping reagents.

3. To scale up electrochemical generation of a metabolite for identification by NMR spectroscopy.

4. To compare oxidative mechanisms of CYP and electrochemistry.

5. To avoid metabolic activation by altering the chemical composition of the drug.

3 Methods and Techniques

Detailed descriptions of the methods used in this thesis are included in enclosed publications (Paper I-IV). This part covers selected methodological and technical considerations.

3.1 *IN VITRO* METABOLISM STUDIES

Several different *in vitro* systems are available to study drug metabolism. The most commonly used are recombinant enzymes, liver microsomes, liver S9 preparations, hepatocytes and liver slices. *In vitro* systems used for the metabolic studies in the present work were: rat or human liver microsomes (RLM or HLM respectively; Papers I-IV), recombinant CYPs (rCYPs; Paper II) and rat, dog and human hepatocytes (Paper IV). Studies with rat and human liver microsomes supplemented with uridine-5'-diphosphoglucuronic acid (UDPGA) were also performed (Paper IV). In addition, studies with 3-methylcholanthrene-induced rat liver microsomes were conducted (Paper II).

3.1.1 Microsomal incubations

The incubation mixture consisted of RLM/HLM/rCYPs, substrate and NADPH in potassium phosphate buffer (pH 7.4). Trapping experiments were performed in the presence of GSH, NAC, NAL, NAT, cysteine, MA or potassium cyanide. Control experiments were performed in the absence of NADPH and in the absence of trapping reagents. Blank samples without substrate were also prepared. Incubations were initiated by addition of NADPH after 5 min of pre-incubation at 37 °C, and terminated after 30 or 60 min by addition of ice-cold acetonitrile. The supernatants were, after centrifugation, analyzed using LC-electrospray ionization (ESI)-MS.

3.1.2 Hepatocyte incubations

The incubation mixture consisted of rat, dog or human hepatocytes and substrate in hepatocyte incubation medium. The hepatocyte incubation medium contained L-glutamine and HEPES, diluted with William's E medium. The pH of the incubation mixture was 7.4. The incubations were initiated by addition of the pre-warmed substrate solution after 10 min pre-incubation at 37 °C. Blank samples without substrate were also prepared. For the time zero sample, the hepatocyte incubations were treated with the quenching solution prior to the addition of substrate solution.

The incubations were terminated after 120 min by addition of three volumes of ice-cold stop solution; acetonitrile containing formic acid and a volume marker. The samples were kept at -20 °C for at least 20 min, and then centrifuged at 2750 *g* at 4 °C for 20 min. The supernatants were diluted 1:1 with water and the samples were kept at -20 °C until analysis by LC-ESI/MS.

3.2 STUDIES OF CHEMICAL REACTIVITY

Studies with different trapping reagents were performed, both in the metabolic incubations and in the electrochemical system. The trapping experiments were conducted with the aim to trap and thereby enable identification of short-lived, unstable intermediates in metabolic pathways. The reactivity towards different trapping reagents may also be a measure of the chemical reactivity of formed metabolites. The majority of reactive metabolites formed by CYP metabolism are soft electrophilic species. These soft electrophiles can be trapped by soft nucleophiles, such as GSH or NAC. Hard electrophiles may on the other hand be trapped by hard nucleophiles, such as cyanide. Methoxylamine may be used to trap aldehydes.

3.3 ELECTROCHEMICAL STUDIES

In general, the substrate solution was infused through the electrochemical cell via a syringe pump. A make-up flow, consisting of organic solvent and aqueous solution, was added before the electrochemical cell. For the aqueous part of the make-up flow, different electrolytes were used to obtain a suitable pH. The electrochemical cell was controlled by a potentiostat, which was programmed at a fixed potential between 0-1500 mV or to perform a potential scan from 0-1500-0 mV (scan rate 5 mV/s).

3.3.1 The on-line EC-ESI/MS system

The outlet of the electrochemical cell was connected to a mass spectrometer equipped with an ESI interface (see Figure 3.1). Full scan spectra were acquired continuously.

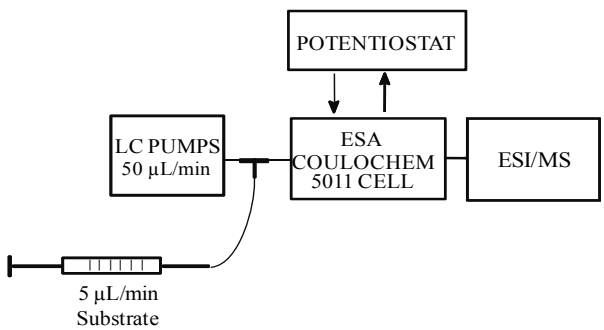


Figure 3.1. Schematic overview of the on-line electrochemical system, directly coupled to the mass spectrometer.

3.3.2 Collection for analysis by LC-ESI/MS

For identification of the electrochemically generated oxidation products, ESI/MS was usually not sufficient. In this alternative setup, the sample was collected in a vial after the electrochemical cell for subsequent analysis by LC/MS (see Figure 3.2). For a complete structural determination of an oxidation product, analysis by NMR spectroscopy may be performed (see Paper II).

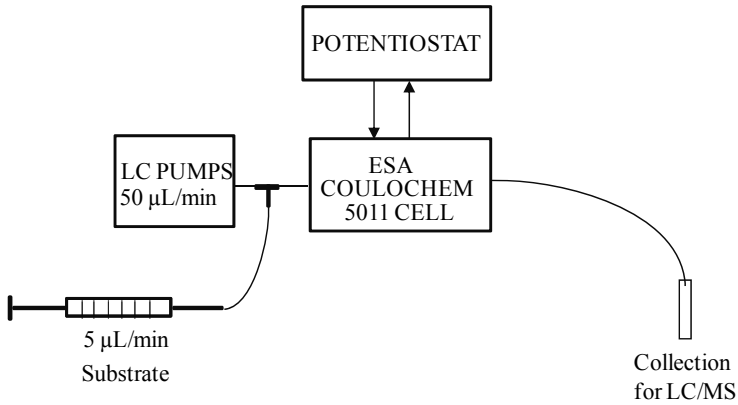


Figure 3.2. Schematic overview of the electrochemical system with sample collection after the cell.

3.3.3 Electrochemical trapping experiments

For trapping after the electrochemical cell, the oxidized sample was collected in a glass vial containing a solution of one of the trapping reagents, NAC, NAL, NAT, cysteine, GSH, MA or KCN. For trapping of short-lived intermediates, the trapping reagent had to be present in the electrochemical cell during oxidation. The substrate and the trapping reagent were infused through the electrochemical cell via two different syringe pumps, with a make-up flow added before the electrochemical cell (see Figure 3.3). A fixed potential of the electrochemical cell was maintained during the experiment. The samples were collected in vials, after the electrochemical cell. All collected samples were either analyzed immediately or stored at -18 °C until analysis.

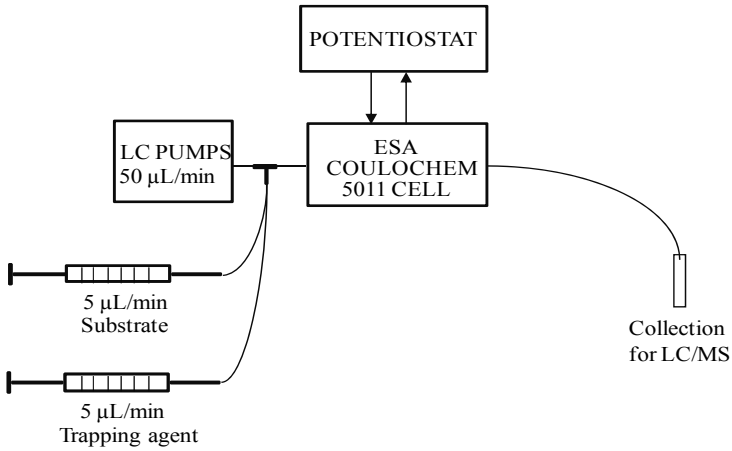


Figure 3.3. Schematic overview of the electrochemical system with trapping inside the cell.

3.4 ELECTROCHEMICALLY ASSISTED FENTON SYSTEM

A reaction mixture with the substrate, EDTA/$FeCl_3$, water, acetonitrile (ACN) and hydrogen peroxide was infused through the electrochemical cell with a syringe pump (see Figure 3.4). The hydrogen peroxide was added shortly before the start of the infusion. The electrochemical cell was controlled by a potentiostat, which was programmed at a fixed potential of -500 mV. The samples were collected in vials, after the electrochemical cell. All collected samples were either analyzed immediately by LC-MS or stored at -18°C until analysis.

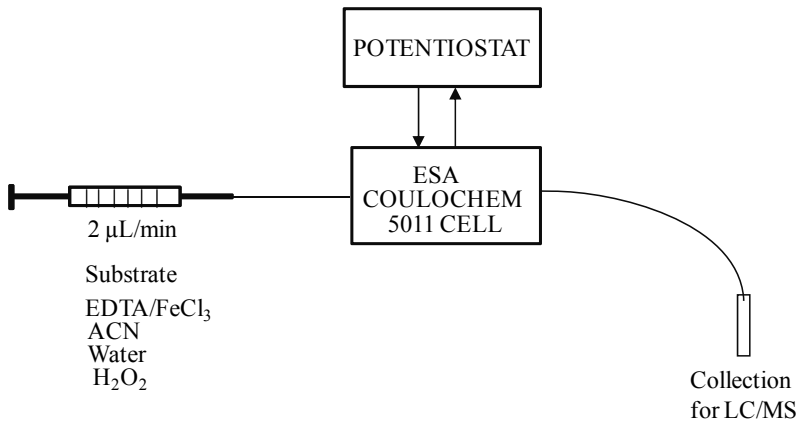


Figure 3.4. Schematic overview of the electrochemically assisted Fenton system.

3.5 PORPHYRIN STUDIES

Each reaction mixture consisted of the substrate, acetonitrile, formic acid, an iron porphyrin and hydrogen peroxide, in a glass tube. The reaction was initiated by the addition of hydrogen peroxide. The reaction mixture was stirred for 30 min at 50 °C. The samples were either analyzed immediately by LC-MS or stored at -18 °C until analysis.

3.6 GENERAL COMMENTS

In these studies, relative amounts of metabolites or oxidation products are based on integrated peak areas of extracted ion chromatograms. Because the response in the mass spectrometer may vary between different metabolites and oxidation products, the reported areas should be regarded as approximate determinations. LC-MS was used for identification of metabolites and oxidation products. For MH^+ assignments, accurate mass measurements were applied, in comparison with the theoretical exact mass. In general, interpretation of product ion spectra was utilized for structural information.

4 Mimicry of Oxidative Drug Metabolism

4.1 METHODS FOR MIMICKING PHASE I DRUG METABOLISM (PAPER I)

The aim of this study was to evaluate chemical methods for mimicking oxidative drug metabolism, that together would cover as many relevant phase I metabolic reactions as possible. Three chemical oxidation systems were used: the electrochemical oxidation system (EC), the porphyrin system and the electrochemically assisted Fenton system (EC-Fenton).

4.1.1 Mimicry of metoprolol metabolism

Metoprolol is a well-known β-receptor blocking agent, used in the treatment of various cardiovascular diseases. The fact that its metabolism is thoroughly investigated and several metabolite standards were available made metoprolol a suitable test compound for developing and/or optimizing the oxidation systems.

For development of a suitable porphyrin system, several parameters were investigated, e.g. different organic solvents, percentage of organic solvent, acidic/neutral/basic aqueous solution, substrate concentration, temperature, reaction time, different oxidizing agents and different metalloorganic complexes. The conditions established for optimal yield of the metoprolol metabolites were later used for the diverse set of test compounds.

The metabolism of metoprolol was studied in HLM and RLM and is summarized in Figure 4.1. The observed biotransformations were benzylic hydroxylation, aromatic hydroxylation, *O*-dealkylation, *N*-dealkylation, oxidation to benzaldehyde and oxidation to carboxylic acid. The benzylic hydroxylation was mimicked by the electrochemical system, the EC-Fenton system and the porphyrin system. The aromatic hydroxylation was mimicked by the EC-Fenton system and the porphyrin system. The *O*-dealkylation was mimicked by the electrochemical system (in low yields), the EC-Fenton system and the porphyrin system. The *N*-dealkylation was mimicked by the electrochemical system, the EC-Fenton system and the porphyrin system, although the electrochemical system generated considerable higher yields of the *N*-dealkylated product compared to the other two systems. Oxidation to the

benzaldehyde was mimicked by the electrochemical system, the EC-Fenton system and the porphyrin system. The oxidation to the carboxylic acid was mimicked by the porphyrin system.

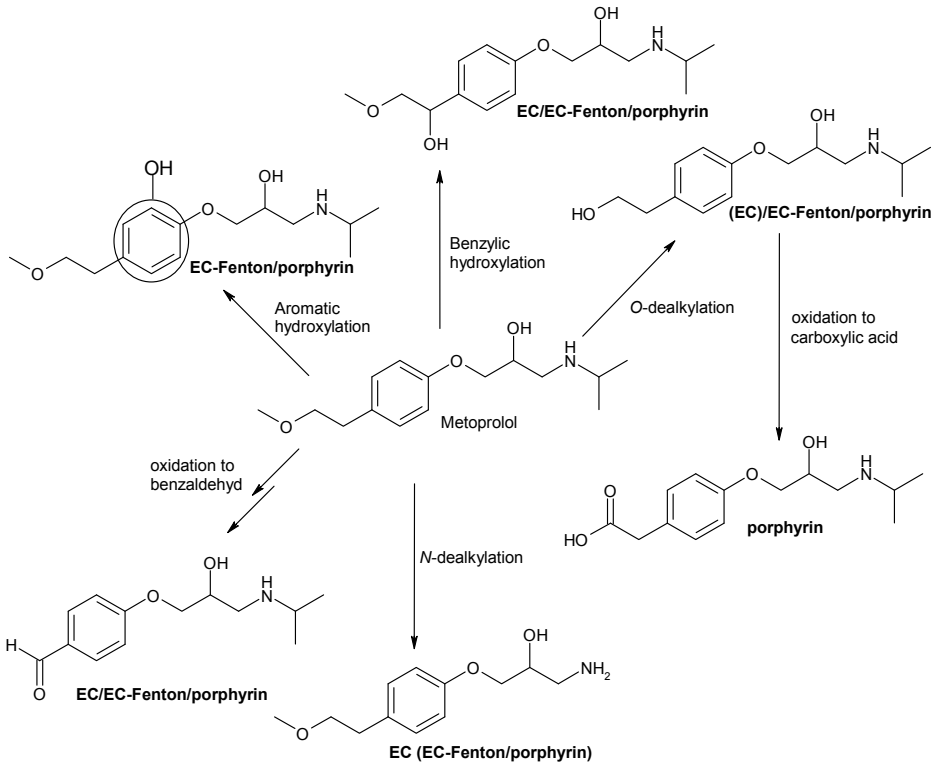


Figure 4.1. Observed metabolites of metoprolol in liver microsomes and means of mimicry.

4.1.2 Method evaluation using a set of test compounds

The metabolism of metoprolol included several important cytochrome P450-catalyzed oxidations and metoprolol was a suitable test compound for the three chemical systems. However, in order to examine the ability of the three systems to mimic cytochrome P450 oxidations, a set of compounds, containing functional groups susceptible to the most important metabolic oxidations catalyzed by CYP, were chosen to extend the study. In addition to metoprolol, the set of test compounds (see Figure 4.2) consisted of testosterone, 2-(*N*-propyl-*N*-2-thienylethylamine-)-5-hydroxytetralin (N0437), mephenytoin, lidocaine, 7-ethoxycoumarin, S-methylthiopurine and amodiaquine. The results from these experiments are summarized in Table 4.1.

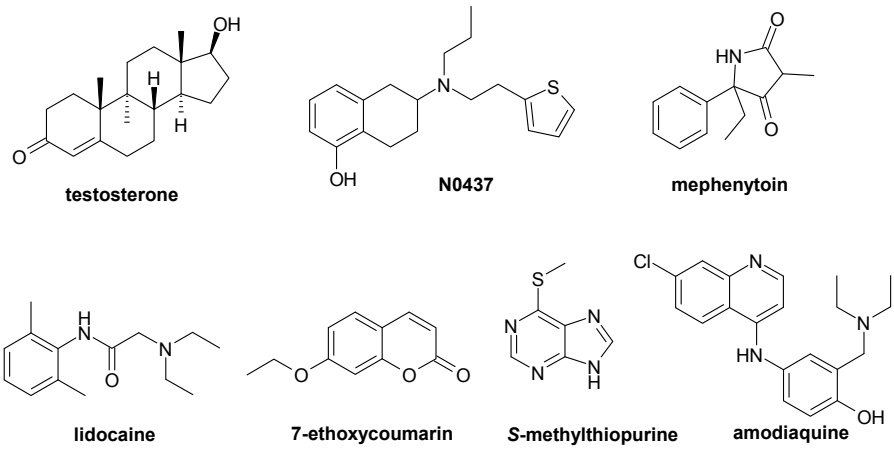
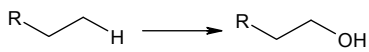
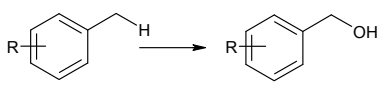
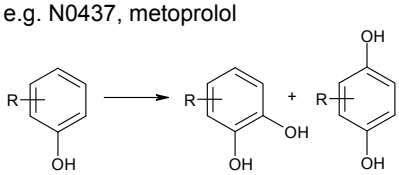
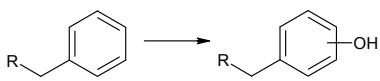
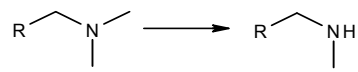
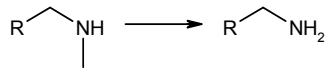
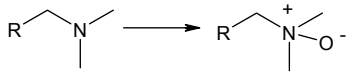
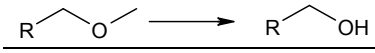
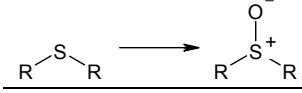
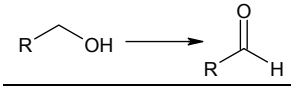
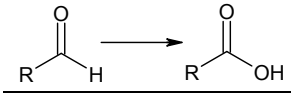
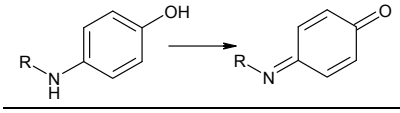


Figure 4.2. Structures of test compounds.

Table 4.1. Enzymatic oxidations in HLM/RLM and means of mimicry.

Enzymatic oxidation	Reaction mimicked?	Oxidation method
a) Aliphatic hydroxylation e.g. testosterone 	Yes	EC-Fenton Porphyrin
b) Benzylic hydroxylation e.g. metoprolol 	Yes	EC EC-Fenton Porphyrin
c) Aromatic hydroxylation Ex.1) with e^--donating groups e.g. N0437, metoprolol 	Yes	EC EC-Fenton Porphyrin
Ex. 2) without e^--donating groups e.g. mephenytoin 	Yes	EC-Fenton Porphyrin

Enzymatic oxidation	Reaction mimicked?	Oxidation method
d) *N*-dealkylation Ex. 1) tertiary amine e.g. lidocaine 	Yes	EC
Ex. 2) secondary amine e.g. metoprolol 	Yes	EC EC-Fenton Porphyrin
e) *N*-oxidation e.g. lidocaine 	Yes	EC EC-Fenton Porphyrin
f) *O*-dealkylation e.g. metoprolol, 7-ethoxycoumarin 	Yes	EC EC-Fenton Porphyrin
g) *S*-oxidation e.g. *S*-methylthiopurine 	Yes	EC EC-Fenton Porphyrin
h) Alcohol oxidation e.g. metoprolol 	Yes	Porphyrin
i) Aldehyde oxidation e.g. metoprolol 	Yes	Porphyrin
j) Dehydrogenation e.g. amodiaquine 	Yes	EC EC-Fenton Porphyrin

4.1.3 Recommendation of oxidation system

For alcohol oxidation, aldehyde oxidation, *O*-dealkylation, *N*-oxidation and hydroxylation of aromatic rings without electron donating groups, the porphyrin system would generally be the preferred system. The porphyrin system and the EC-Fenton system are recommended for mimicking aliphatic and benzylic hydroxylations. The EC-Fenton system may also be recommended as the most suitable system for mimicking hydroxylation of aromatic compounds substituted with electron donating groups. Electrochemical oxidation is preferred to achieve dehydrogenation, *S*-oxidation and *N*-dealkylation of both secondary and tertiary amines.

In order to synthesize metabolites, e.g. for identification by NMR spectroscopy, the oxidation systems need to be used on a larger scale. This may be difficult using the EC-Fenton system as this system requires a low flow through the electrochemical cell. From this perspective, the porphyrin system and the electrochemical system would be more appropriate. The porphyrin system and the EC-Fenton system often yield a mixture of many different oxidation products. Formation of a mixture of oxidation products may lead to a low yield of a certain metabolite of interest and also separating this metabolite from other components may be difficult. With the electrochemical system it is often easier to achieve higher yields of a specific product with fewer by-products compared to the other systems. This may in part be due to the possibility to monitor the oxidation products in the on-line setup (EC/MS) where the electrochemical conditions can rapidly be changed to optimize the yield of a certain oxidation product.

4.1.4 Concluding discussion

This study presents an evaluation of the utility of electrochemical oxidation, electrochemically assisted Fenton chemistry and synthetic metalloporphyrins to mimic liver microsomal phase I oxidations. The combination of these three chemical oxidation systems was sufficient to mimic the most relevant metabolic oxidations. The systems may complement classic synthetic chemistry and isolation of metabolites from *in vivo* samples, especially when the synthetic work is time-consuming or when the metabolites are difficult to isolate from complicated biological matrices.

5 Electrochemical Applications on Reactive Drug Metabolites

This section highlights applications of mimicking CYP-catalyzed oxidations by electrochemical oxidation, focused on synthesis, trapping reactions and comparison of oxidative pathways. Based on the evaluation of the three systems previously described, the electrochemical system was chosen as the most suitable method to use for these applications. There is a great potential in using electrochemistry to oxidize drugs on a preparative scale, e.g. due to the possibility to optimize for a certain reaction or product. Another advantage is the ability to use an on-line electrochemical setup to characterize short-lived, reactive intermediates.

5.1 STUDIES ON AMODIAQUINE METABOLISM (PAPER II)

The effective antimalarial drug amodiaquine (AQ, Figure 5.1) was withdrawn from clinical use in the 1970s due to its toxicity[34], including severe side effects such as agranulocytosis and hepatitis. Earlier studies have identified a quinoneimine metabolite (AQQI) as the likely cause of amodiaquine toxicity, proposed to originate from the reactivity of this electrophilic metabolite.[35-41] In a previous study, screening a panel of 13 human rCYPs showed that CYP2C8 was the main hepatic isoform responsible for the elimination of AQ and the formation of desethylamodiaquine (M1). It was also found that CYP1A1 and CYP1B1 mediated the formation of AQ to an unidentified metabolite, M2. This metabolite was not observed in human liver microsomal incubations.[42,43] An electrochemical oxidation product with the same molecular mass as M2 (MH^+ at m/z 299) has been generated in two different studies and an aldehyde structure has been proposed.[28,29]

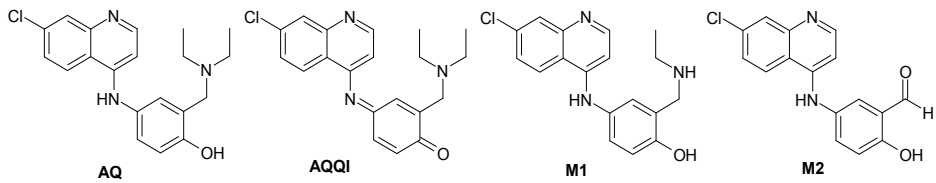


Figure 5.1. Structures of amodiaquine and metabolites relevant for this study.

The aim of this study was to identify and characterize M2. Incubations with rCYPs in the presence of different trapping reagents were carried out to investigate the potential reactivity of the metabolite and possible intermediates. The electrochemical system was further optimized for synthesizing metabolites in higher quantities to allow for structural determination by NMR spectroscopy. Furthermore, incubations with noninduced vs. induced liver microsomes, were performed to study the hepatic significance and the effect of induction.

5.1.1 Electrochemical mimicry of rCYP oxidations

Electrochemical oxidation of amodiaquine resulted in a product, eluting at 1.75 min., detected as an MH^+ at m/z 299. For comparison of the electrochemically generated oxidation product with the unidentified metabolite M2, incubations with rCYP1A1 and rCYP1B1 were performed. From the corresponding retention times, accurate mass and product ion spectra, the oxidation product formed electrochemically was shown to be identical with the major metabolite formed by these rCYPs (see Figure 5.2). This metabolite/oxidation product was identical with metabolite M2 from the work by Li et al.[42] since it was detected as an MH^+ at m/z 299, with a long retention time compared to amodiaquine and was the major metabolite formed via rCYP1A1 and rCYP1B1.

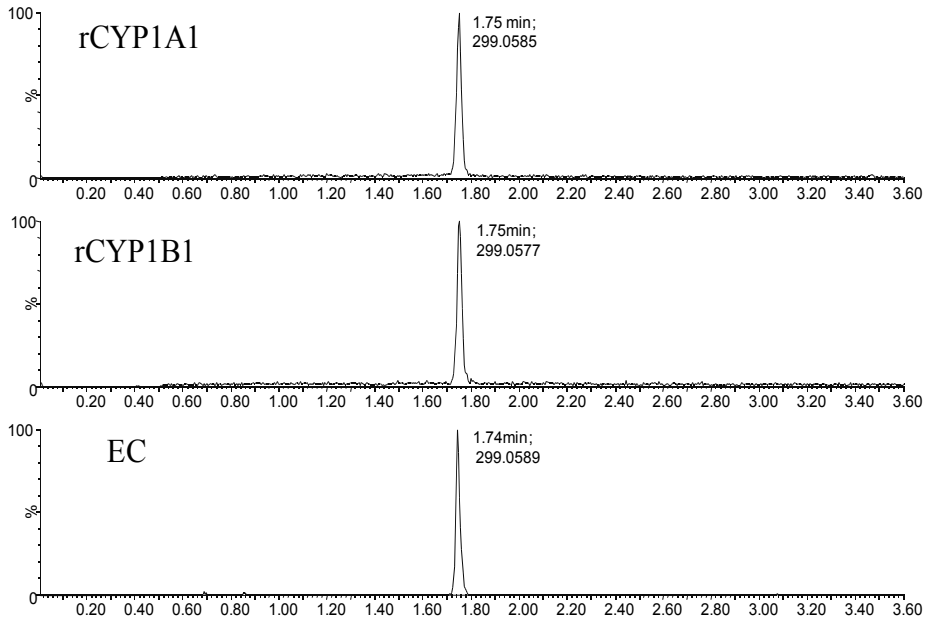


Figure 5.2. Metabolite M2 formed via rCYP1A1, rCYP1B1 and by electrochemical oxidation. Extracted ion chromatograms of m/z 299.

5.1.2 Trapping experiments with methoxylamine

The accurate mass and product ion spectra (data not shown) of M2 indicated that the structure corresponded to that of an aldehyde. In order to test this hypothesis, trapping experiments were performed with NAL and MA. These trapping reagents have previously been utilized for trapping of aldehydes [44,45]. Trapping with NAL did not give any adducts whereas trapping with MA resulted in an adduct, M3, both in the electrochemical oxidation experiments and rCYP incubations. In the experiments when MA was added, the MH^+ at m/z 299 completely disappeared and a new MH^+ at m/z 328 appeared in the chromatograms. The shift in mass was 29 Da, implying that addition of MA to the putative aldehyde had taken place.

5.1.3 Electrochemical synthesis and NMR spectroscopy

The electrochemical oxidation of amodiaquine to the putative aldehyde product was optimized to achieve high yields with minimal amounts of by-products. Structure identification of the electrochemically synthesized product was enabled after lyophilization, followed by LC-MS-solid phase extraction (SPE)-NMR. The aldehyde structure was confirmed by the NMR data. The ^1H NMR spectrum of the aldehyde metabolite of amodiaquine is shown in Figure 5.3.

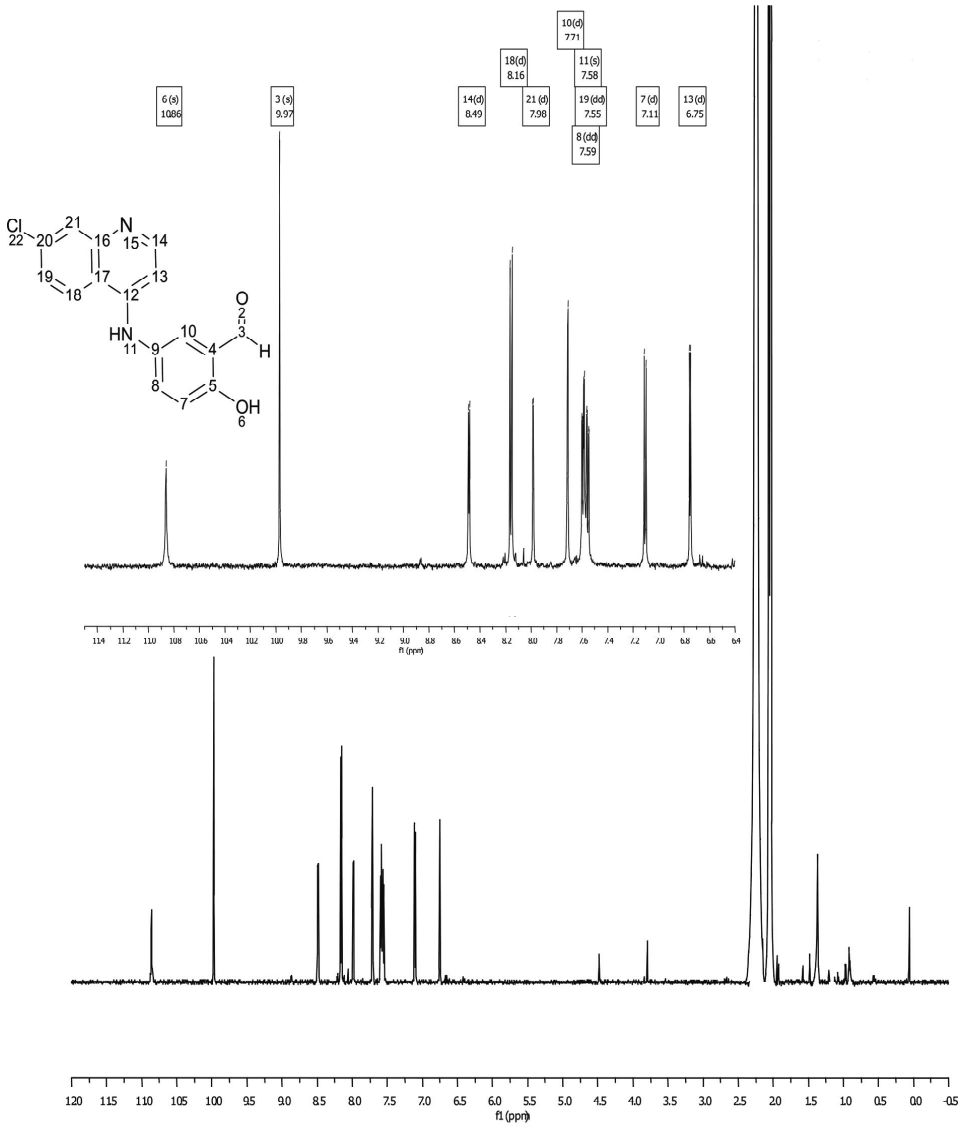


Figure 5.3. ^{1}H NMR spectrum of the aldehyde metabolite of amodiaquine. Detailed view in insert.

5.1.4 Trapping experiments with *N*-acetylcysteine

Electrochemical experiments and the rCYP incubations were repeated in the presence of the trapping agent NAC to elucidate whether quinoneimines were formed in the same systems as the aldehyde metabolite. Trapping with NAC resulted in two adducts, formed in the rCYP incubations as well as in the electrochemical experiments. The

adducts, M4 and M5, were detected as MH^+ at m/z 460, corresponding to an addition of NAC to a quinoneimine species of the aldehyde (M6). The product ion spectra were almost identical and displayed a neutral loss of 129 Da, resulting from cleavage of the thioether of *N*-acetylcysteine (see Figure 5.4). In addition, the quinoneimines of amodiaquine and desethylamodiaquine were trapped by NAC in the electrochemical experiments and the rCYP incubations.

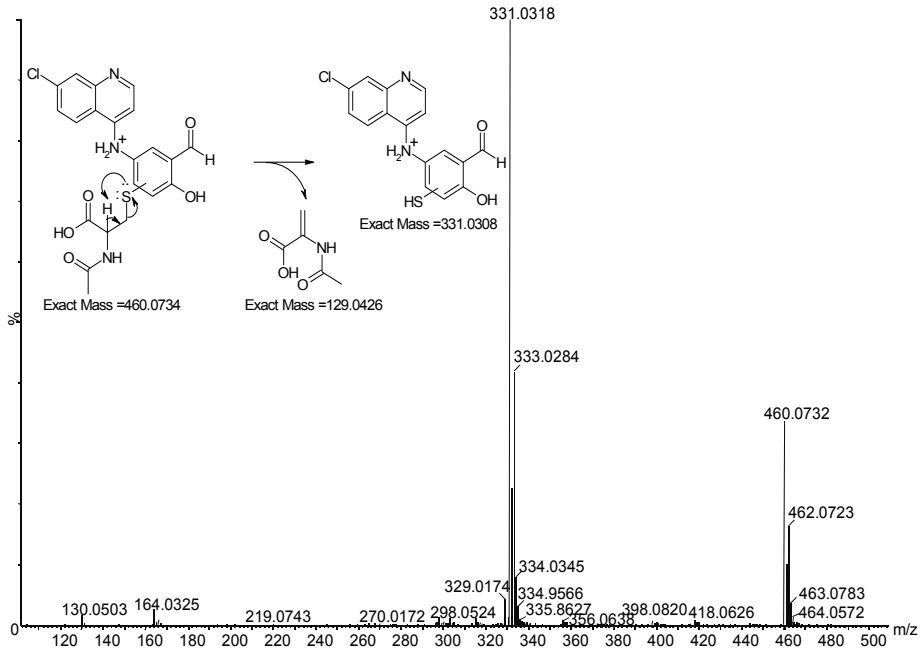


Figure 5.4. Product ion spectrum of M4/M5 and proposed mechanism for the fragment formation.

All experiments, the incubations as well as the electrochemical experiments, were repeated with desethylamodiaquine (M1). AQ is rapidly converted to M1 whereas the elimination of the metabolite is slow with a long terminal half-life.[34,46] This, in addition to the fact that both parent and metabolite have antimalarial activity, motivated the inclusion of M1 in the study. From M1, the same metabolites and adducts were formed as in the experiments with AQ. The observed metabolism involving the aldehyde, mediated by rCYPs and mimicked by electrochemical oxidation, is summarized in Figure 5.5.

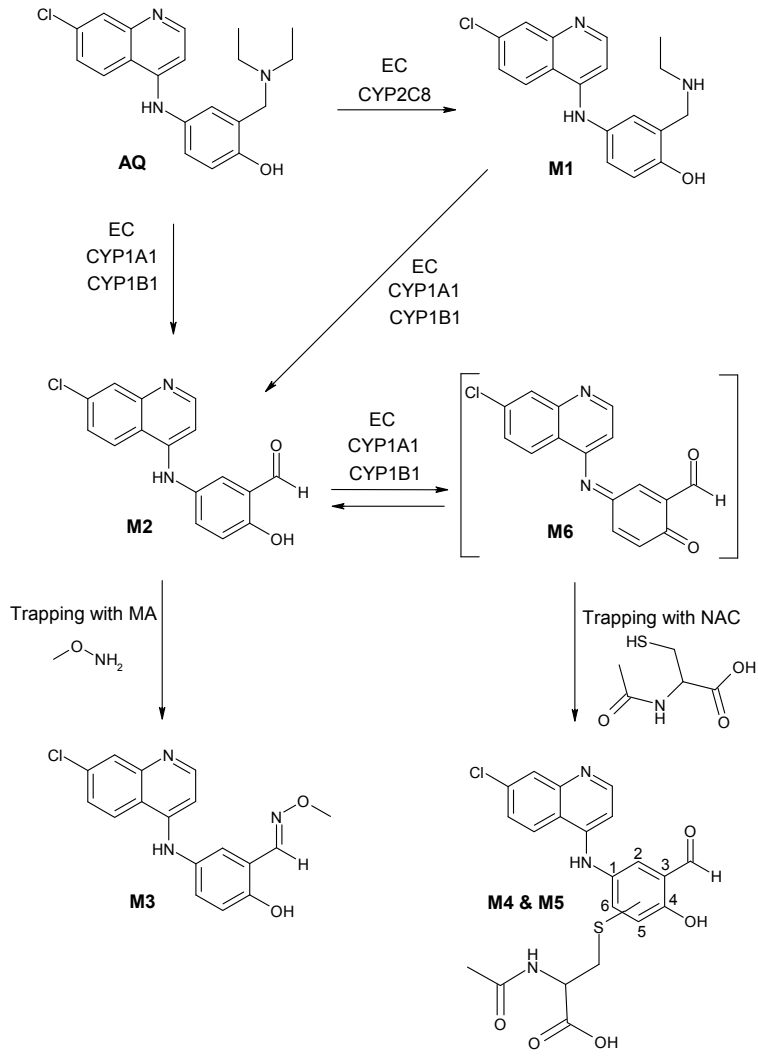


Figure 5.5. Observed amodiaquine (AQ) metabolism via rCYP1A1 and rCYP1B1 including trapping reactions. Formation of all metabolites and adducts were mimicked by the electrochemical system.

5.1.5 Liver microsomal incubations

Amodiaquine was also incubated with NADPH-supplemented human and rat liver microsomes. The aldehyde metabolite was observed in both the human and the rat liver microsomal incubations, although at low levels. Identification of the metabolite in liver microsomes was made by comparing the retention times and the product ion spectra with those of the electrochemical standard (see Figure 5.6). The observation of the aldehyde metabolite in the human liver microsomes, contrary to the previous study

by Li et al.[42], may be explained by the use of much more sensitive detection methods. CYP1A1 and CYP1B1 mainly are found in extrahepatic tissues but they are in fact expressed in low levels in human liver[47-49] and at least CYP1A1 is expressed in low levels in rat liver.[50,51] CYP1A1 and CYP1B1 are inducible enzymes and amodiaquine was incubated with induced rat liver microsomes to investigate if the small amounts of the aldehyde metabolite would increase using induced liver microsomes compared to ordinary microsomes. Commercially available 3-methylcholanthrene-induced rat liver microsomes were used as 3-methylcholanthrene is an inducer of CYP1A1 and CYP1B1 in rat liver[52] and CYP1A1 in human hepatocytes.[53] A 6-fold increase of formed aldehyde metabolite was observed in the liver microsomes from 3-methylcholanthrene-induced rats compared to the non-induced rat liver microsomes. This number is an estimation of the difference in contribution of the aldehyde to the total metabolism, based on integrated peak areas of extracted ion chromatograms and averaged from duplicate incubations.

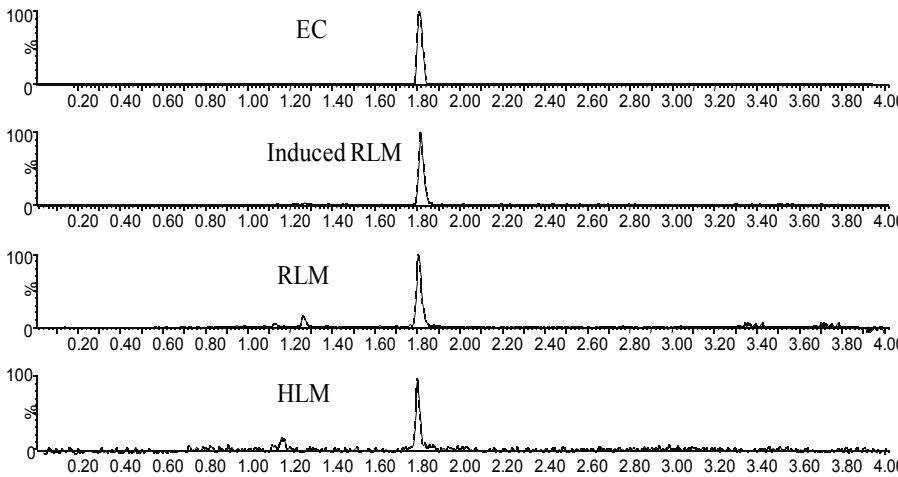


Figure 5.6. Metabolite M2 formed in liver microsomal incubations and by electrochemical oxidation. Extracted ion chromatograms of m/z 299.

5.1.6 Concluding discussion

This investigation highlights the possibility to use electrochemistry for generating the same oxidation products as those formed by different biological systems and, after optimizing the reaction on a preparative scale, synthesize enough material for identification by NMR spectroscopy. An alternative approach for metabolite generation could be to perform large liver microsomal incubations. However, the disadvantages of this method compared to the electrochemical system are the greater expense, a more complex matrix generated and that optimizing for a certain metabolite would be difficult. Another example is to use other enzyme technologies, such as bacterial CYP enzymes, which may be exemplified by the commercially available

Codexis MicroCyps.[54] Clearly, this method is much more expensive than the electrochemical system.

This study has shown that the aldehyde metabolite of amodiaquine was formed in rCYP1A1, rCYP1B1, human liver microsomes, both non-induced and 3-methylcholanthrene-induced rat liver microsomes and generated electrochemically. In the liver microsomal incubations the aldehyde was a minor metabolite whereas in rCYP1A1 and rCYP1B1 it was the major metabolite. These enzymes, CYP1A1 and CYP1B1, are expressed in extrahepatic tissues such as human blood cells rather than in the liver. The expression of different CYPs may vary between individuals and CYP1A1 and CYP1B1 are induced by a series of polyaromatic hydrocarbons.[55] Consequently, this study has demonstrated new potentially toxic metabolites of amodiaquine, other than the previously identified amodiaquine quinoneimine. Metabolites of particular concern are proposed to be the aldehyde metabolite (M2) and the aldehyde quinoneimine metabolite (M6). These metabolites may be involved in the adverse drug reactions in addition to or instead of the quinoneimine of amodiaquine, although further studies are needed to establish their contribution to the *in vivo* toxicity.

In summary, this study on the well-known antimalarial agent amodiaquine presents previously not identified and potentially toxic metabolites, formed in different *in vitro* systems. The investigation also shows how the electrochemical technique may be used for generating metabolites, enabling further characterization and identification.

5.2 STUDIES ON HALOPERIDOL METABOLISM (PAPER III)

Haloperidol (**1**) is a dopamine (D_2) receptor antagonist of butyrophenone-type, introduced into clinical practice in the late 1950s. At present, haloperidol is used in the therapy of patients with schizophrenia and also in cases of acute psychosis, even though it may cause extrapyramidal side effects including tardive dyskinesia, akathisia, dystonia and parkinsonism.[56,57] The pyridinium metabolite of haloperidol (**2**$^+$) has been proposed to contribute to these severe side effects due to its structural similarity with MPP^+(**3**$^+$), the neurotoxic metabolite of the parkinsonian-inducing agent MPTP (**4**) (see Figure 5.7).[58,59] The nigrostriatal toxicity of **3**$^+$ is caused by inhibition of Complex I in the mitochondrial respiratory chain, and it has also been shown that **2**$^+$ inhibits mitochondrial respiration.[60]

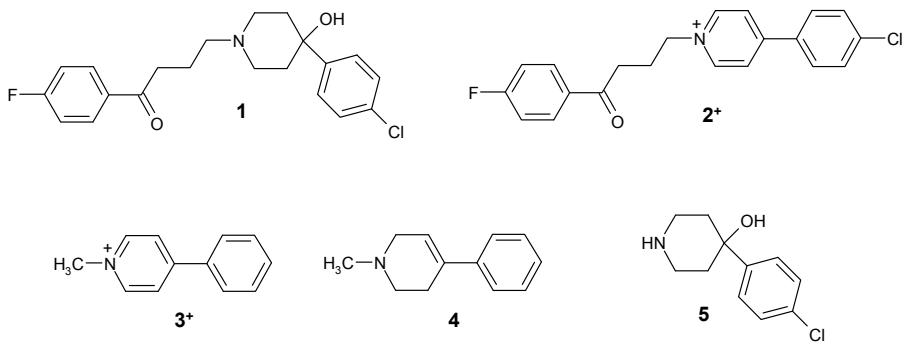


Figure 5.7. Structures of haloperidol (**1**), MPTP (**3**$^+$) and metabolites relevant for this study.

The conversion of haloperidol to its pyridinium metabolite has been extensively studied[59,61-70] and for this biotransformation, two different pathways have been proposed (see Figure 5.8).[59,68]

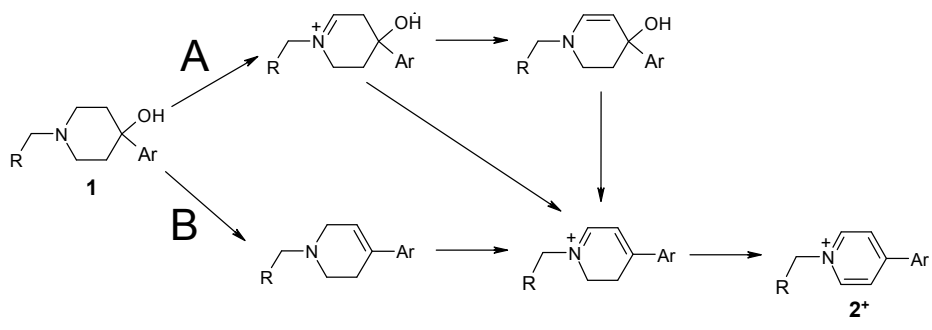


Figure 5.8. Proposed metabolic pathways of haloperidol to the pyridinium metabolite from the literature. R = $(CH_2)_2COC_6H_4$-*p*-F; Ar = C_6H_4-*p*-Cl

In the first pathway (**A**) haloperidol undergoes α-carbon hydroxylation followed by elimination of water to yield an iminium ion that directly or, via its enamine conjugate base, is oxidized to a dihydropyridinium intermediate. Alternatively (**B**), dehydration of haloperidol yields a tetrahydropyridinyl intermediate that undergoes *a*-carbon hydroxylation and subsequent elimination of water to yield the common dihydropyridinium intermediate. In the final step this dihydropyridinium species is converted to $\mathbf{2}^{+}$, a reaction that has been proposed to proceed via autoxidation.[64]

In addition to the formation of the pyridinium species, *N*-dealkylation of haloperidol is another significant biotransformation route, being a major metabolic pathway both *in vitro* and *in vivo*.[71-73] The oxidative *N*-dealkylation to the secondary amine (**5**, Figure 5.7) and the ring oxidation to pyridinium are both catalyzed by CYPs.[74,75]

The aim of this investigation was to study CYP-catalyzed oxidation of haloperidol to its pyridinium and *N*-dealkylation products. The oxidation of haloperidol was studied electrochemically and in liver microsomes, also in the presence of trapping agents to catch short-lived intermediates not possible to isolate.

5.2.1 Electrochemical mimicry of CYP oxidations

The major metabolites of haloperidol, $\mathbf{2}^{+}$ and **5**, formed in RLM and HLM, were also generated by electrochemical oxidation at pH 7. In the electrochemical experiment, four additional oxidation products were observed. The two products eluting shortly after **5** were tentatively assigned as 4(4-chlorophenyl)-2,3-dihydropyridine (**6**) and 4(4-chlorophenyl)pyridine (**7**), proposed to originate from *N*-dealkylation and oxidation of the piperidine ring. The oxidation product eluting at 2.60 min was tentatively assigned as a lactam (**8**). Proposed structures of the electrochemical oxidation products **6**-**7** and **8** are included in Figures 5.14 and 5.13, respectively. The latest eluting product (**9**) has not yet been identified. Extracted ion chromatograms of haloperidol and metabolites or oxidation products formed in HLM, RLM and by electrochemistry (EC) are shown in Figure 5.9.

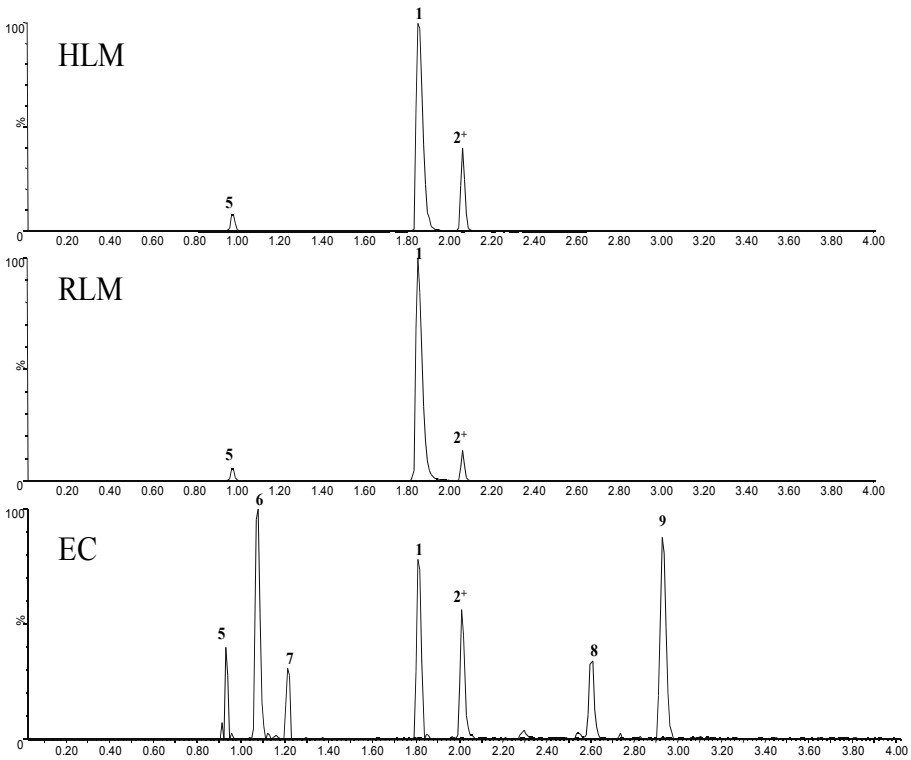


Figure 5.9. Overlaid extracted ion chromatograms of haloperidol and metabolites or oxidation products formed in HLM, RLM and electrochemically, respectively.

5.2.2 On-line electrochemistry mass spectrometry of short-lived species

In the electrochemical on-line setup, the outlet of the electrochemical cell is coupled directly to a mass spectrometer without any separation taking place. Oxidation of haloperidol led to the detection of an MH^+ at m/z 374, corresponding to a decrease of 2 Da compared to haloperidol. The product ion spectrum of m/z 374 acquired on-line is shown in Figure 5.10. This oxidation product was not stable enough to allow for detection by LC-MS. The MH^+ at m/z 374 is proposed to correspond to one or more short-lived iminium species and trapping experiments are necessary for further elucidation.

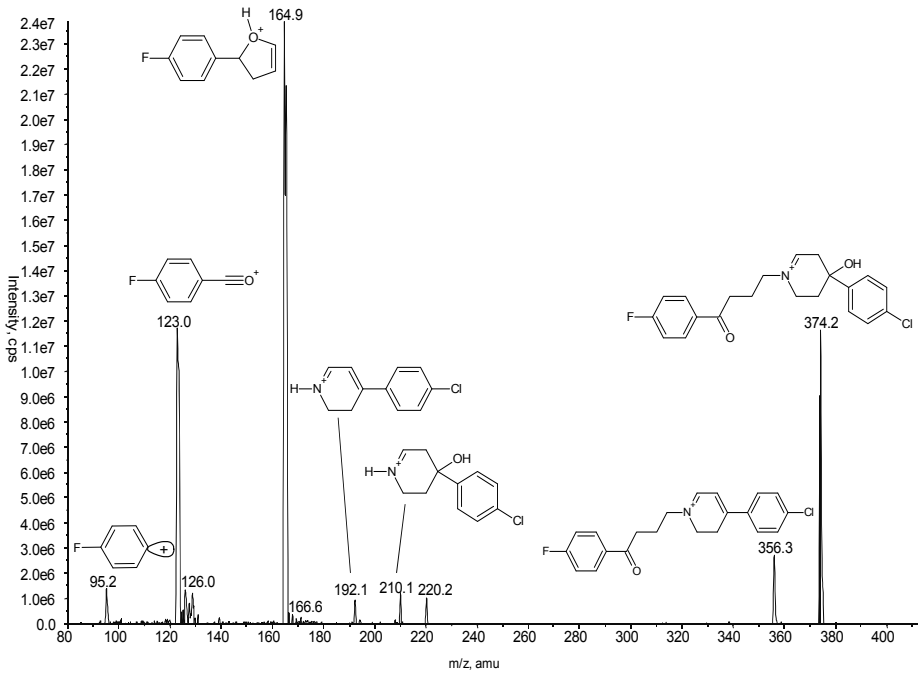


Figure 5.10. Product ion spectrum and proposed fragment ions of m/z 374, achieved from on-line electrochemical experiments. Note that this spectrum was obtained at acidic conditions, and hence an iminium species is proposed over the corresponding enamine.

5.2.3 Trapping studies with cyanide

In order to trap intermediates in the CYP-mediated metabolic pathways of haloperidol, liver microsomal incubations in the presence of different trapping agents were performed. Trapping with NAC, NAT, GSH or MA did not result in any detected adducts. Incubations performed in the presence of KCN, resulted in two products, which were not observed in the absence of NADPH or KCN. These cyano adducts eluted at 3.10 and 3.20 min, respectively, and were detected as MH^+ at m/z 401 (see Figure 5.11). The two well-separated adducts displayed the same product ion spectrum and were tentatively assigned as the diastereomeric 2-cyano adducts of haloperidol (**10A**/**10B**, Figure 5.11). Based on the product ion spectra, these cyano adducts were proposed to be generated from trapping of the endocyclic iminium species of haloperidol.

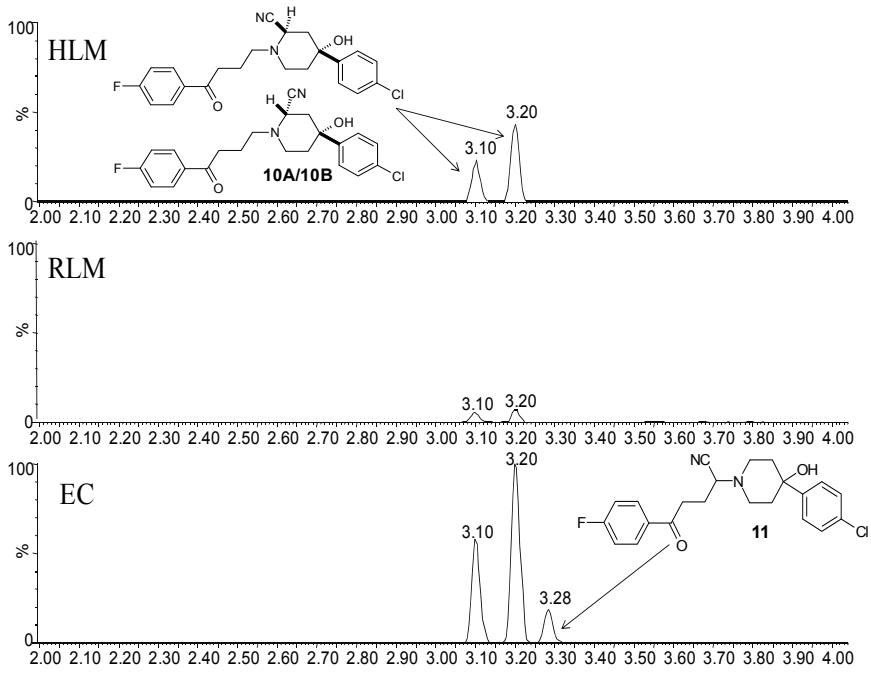


Figure 5.11. Extracted ion chromatograms of the cyano adducts (m/z 401) of haloperidol formed in HLM, RLM and electrochemically. Structures are shown with relative configurational assignments only.

Electrochemical oxidation of haloperidol at pH 7 was also performed in the presence of trapping reagents. In accordance with results from the liver microsomal incubations, the trapping attempts with NAC, NAT, GSH or MA did not give any detected adducts. Electrochemical oxidation of haloperidol in the presence of KCN resulted in three well-separated products (MH^+ at m/z 401). Two of them were identified as the same adducts as formed in the liver microsomal trapping experiments, based on the retention times and product ion spectra. The third adduct eluted at 3.28 min and the product ion spectrum differed significantly from those of the diastereomeric cyano adducts. Based on interpretation of the product ion spectrum, this cyano adduct (**11**) is proposed to be the α-cyanoamine, generated from trapping of an exocyclic iminium species of haloperidol.

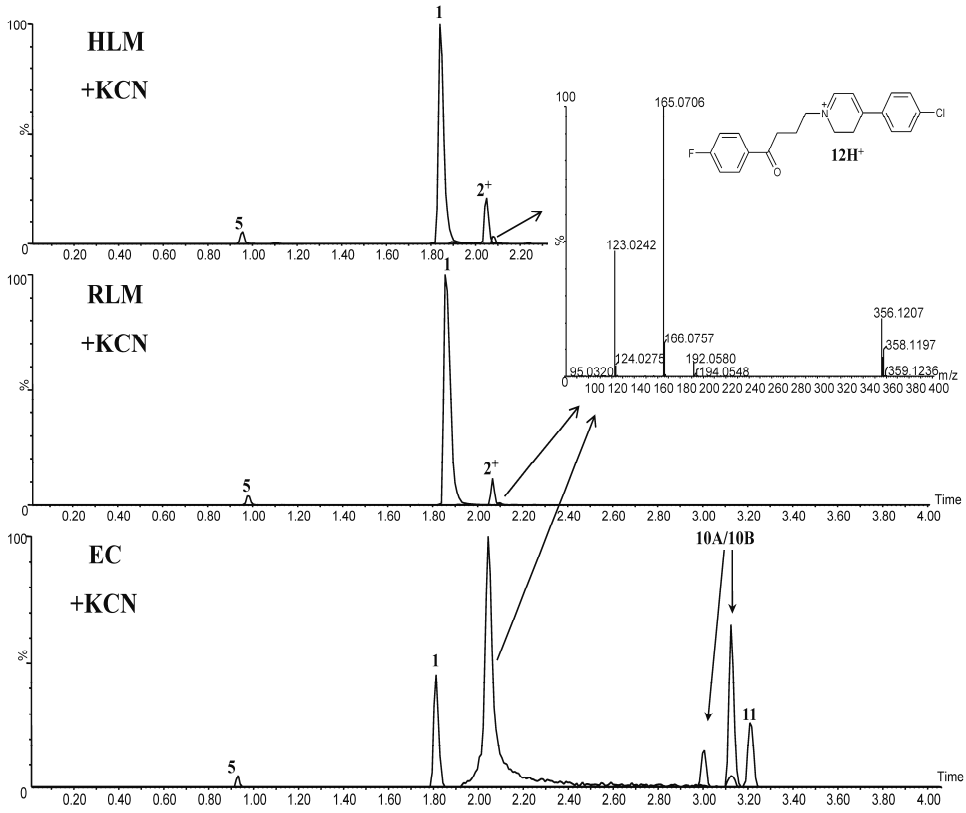


Figure 5.12. Overlaid extracted ion chromatograms of remaining parent (haloperidol), metabolites or oxidation products and cyano adducts formed in HLM, RLM and by electrochemical oxidation, respectively, in the presence of KCN. Note that the levels of cyano adducts formed in HLM and RLM were too low to be visible in these chromatograms.

In addition to the three cyano adducts, the presence of KCN during electrochemical oxidation resulted in another previously not observed species. This product was identified as the dihydropyridinium species of haloperidol **12H**$^+$ (see Figure 5.12), by comparison of its product ion spectrum and retention time with those of the authentic synthetic standard. Also, the formation of the other electrochemical oxidation products was affected by the addition of KCN. An almost complete disappearance of the pyridinium species **2**$^+$, the *N*-dealkylation products (**5**, **6** and **7**) and the putative lactam **8** was observed, compared to being the major products of oxidation formed in the absence of KCN. In addition to the two cyano adducts, the dihydropyridinium species of haloperidol **12H**$^+$ was observed in the microsomal incubations in the presence of KCN. The major shift in product formation observed in the electrochemical system, a result of the KCN addition, was not observed in the liver microsomal incubations. In both rat and human liver microsomal incubations, the metabolites **5** and **2**$^+$ were the major metabolites formed in the absence as well as in the presence of KCN.

5.2.4 Proposed mechanisms of ring oxidation and *N*-dealkylation

Due to a low yield of the cyano adducts in the KCN-supplemented liver microsomal incubations comments on the CYP-catalyzed ring oxidations of haloperidol are not conclusive. Thus, haloperidol may be oxidized via the iminium species to the dihydropyridinium species and further to the pyridinium species (pathway **A**), but pathway **B** initiated by dehydration cannot be excluded.

Proposed pathways for the electrochemically mediated ring oxidations of haloperidol are shown in Figure 5.13. The obligatory one-electron abstraction, deprotonation to **13$^{\bullet}$** and subsequent loss of a second electron, forms the iminium product **14H^{+}**. The cyano adducts are formed by addition of KCN to **14H^{+}**, whereas addition of water gives the carbinolamine **15**, which is further oxidized to the putative lactam. In addition, the iminium species is oxidized further to the pyridinium product **2^{+}**, a reaction likely to proceed through the dienamine free base **12**, which is formed by loss of H_2O from the aminoenol **14**.

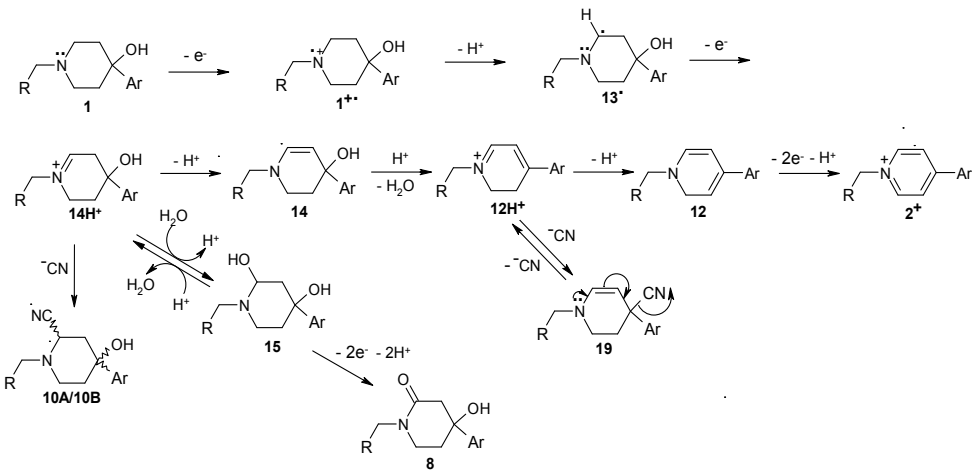


Figure 5.13. Proposed pathways for the electrochemically mediated ring oxidation of haloperidol. R=$(CH_2)_2COC_6H_4$(*p*)F and Ar=C_6H_4(*p*)Cl

The CYP-catalyzed *N*-dealkylation of haloperidol may proceed via the single electron transfer (**1** → **1$^{\bullet+}$** → **16$^{\bullet}$**) or α-carbon hydrogen atom transfer (**1** → **16$^{\bullet}$**) pathways (see Figure 5.14). The carbinolamine **17**, formed by oxygen rebound to **16$^{\bullet}$**, will spontaneously cleave to an aldehyde (RCHO) and the secondary amine. The electrochemically mediated *N*-dealkylation is obligated, however, to proceed via **1$^{\bullet+}$** and **16$^{\bullet}$**. Loss of the second electron from **16$^{\bullet}$** generates the exocyclic iminium species **18H^{+}**, which is trapped in the presence of KCN. The carbinolamine **17**, formed by hydration of **18H^{+}**, gives the aldehyde and the secondary amine as cleavage products, i.e. the same products as formed in the CYP-catalyzed dealkylation reaction. The formation of the exocyclic cyano adduct **11** at pH 7 in the electrochemical oxidation

experiments and not in the liver microsomal incubations suggests that the iminium intermediate **18H**$^+$ may not be generated in CYP-catalyzed reaction.

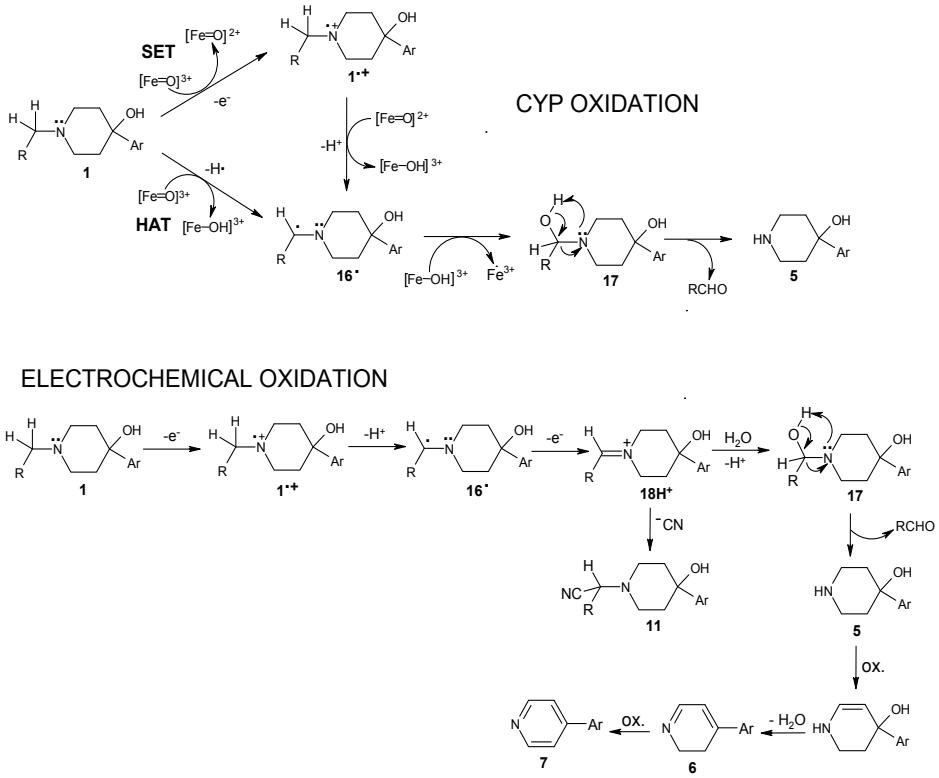


Figure 5.14. Proposed mechanisms for the CYP-catalyzed and electrochemically mediated oxidative *N*-dealkylation of haloperidol. SET=single electron transfer, HAT=hydrogen atom transfer, R=$(CH_2)_2COC_6H_4(p)F$ and Ar=$C_6H_4(p)Cl$

In the electrochemical experiments, addition of KCN resulted in the formation of three cyano adducts and the dihydropyridinium species **12H**$^+$, whereas a significant decrease in formation of the other oxidation products was observed. The cyanide anions are likely to accumulate at the positively charged electrode surface. Hence, at the electrode surface where the oxidation takes place, the iminium species **14H**$^+$ and **18H**$^+$ are proposed to be efficiently trapped by cyanide leaving only a small fraction to react with water, giving the lactam and the *N*-dealkylated products, respectively. Obviously, the detected cyano adducts were stable enough to be swept away from the electrode surface into the solution and allow for subsequent detection by LC-MS. However, the product of cyanide addition to the dihydropyridinium species **12H**$^+$, the putative cyano adduct **19** (see Figure 5.13), was not detected. Because **12H**$^+$ was a major product in the presence of KCN and not observed in the absence of KCN, it was assumed that **19** was formed as an unstable cyano adduct. The formation of **19**

would hinder further oxidation to **2**$^+$ at the electrode surface. When **19** is swept away from the electrode into the solution, the relative concentration of cyanide drops and the equilibrium between **19** and **12H**$^+$ is shifted towards **12H**$^+$, regaining the favorable conjugated system. Also in the liver microsomal incubations, the dihydropyridinium species **12H**$^+$ was observed only in the presence of KCN. The attempts to detect **12H**$^+$ as the corresponding cyano adduct were unsuccessful, both in these incubations and also with the available authentic standard of **12H**$^+$. This is consistent with previous studies reporting the failure to trap the haloperidol-2,3-dihydropyridinium species (**12H**$^+$) and the structurally related loperamide-2,3-dihydropyridinium species.[59,64,76]

5.2.5 Concluding discussion

The major metabolites formed by liver microsomes, the *N*-dealkylation and the pyridinium species, and the two diastereomeric cyano adducts formed by KCN trapping, were also generated by electrochemical oxidation. The absence of the third cyano adduct in liver microsomal incubations, indicates that the exocyclic iminium species, an obligatory intermediate in the electrochemically mediated *N*-dealkylation reaction, may not be formed in the CYP-catalyzed reaction. The dihydropyridinium species of haloperidol was detected exclusively in the presence of KCN, both in the electrochemical and the liver microsomal experiments. The detection of the dihydropyridinium species may be the result of unstable cyano adducts, where cyanide has been eliminated to regain the favorable conjugated system. In the electrochemical trapping experiments, the yields of cyano adducts, were significantly higher than those in the corresponding microsomal incubations. Consistent with this, the addition of KCN in the electrochemical experiments resulted in an almost complete lack of formation of other oxidation products, whereas a similar shift in product formation was not observed in the liver microsomal incubations.

Efforts to trap the electrochemically generated iminium species with KCN at pH 3 were unsuccessful. At this pH, the iminium species will be favored over the corresponding enamines but since the pK_a of HCN is 9.4, the concentration of cyanide ions may be too low for the trapping reactions to succeed. The corresponding trapping experiments performed at pH 10 resulted in slightly higher yields of cyano adducts compared to pH 7. The cyanide ion concentration is higher in a more basic environment and these ions may also accumulate at the positive electrode surface, reacting immediately when the iminium species are formed.

In summary, this is a study on the well-known drug haloperidol that presents new intermediates formed *in vitro* and via electrochemical oxidation. The investigation exemplifies the usefulness of electrochemistry for generating metabolites and/or intermediates, making trapping and further characterization possible.

6
Avoiding Reactive or Toxic Metabolites

So far, the focus of this thesis has been on mimicking, synthesizing and characterizing drug metabolites. In order to avoid undesired metabolic activation, it is important to use knowledge gained on reactive metabolites from previous failures, terminated projects and with-drawn drugs. As stated earlier, there are several different approaches to avoid or minimize the formation of reactive or toxic metabolites during drug discovery. The following section will exemplify one of these strategies, namely carbon/silicon exchange.

6.1 CHANGING METABOLIC PATHWAYS BY CARBON/SILICON SWITCHING (PAPER IV)

The neurotoxic, extrapyramidal side effects observed for the neuroleptic drug haloperidol have been associated with its pyridinium metabolite.[59] A silicon analogue of haloperidol, sila-haloperidol, containing a silicon atom instead of the carbon atom in the 4-position of the piperidine ring, has been synthesized.[77] Like haloperidol, trifluperidol is a potent antipsychotic agent with significant extrapyramidal side effects. Sila-trifluperidol, resulting from replacing a carbon atom with a silicon atom (see Figure 6.1), has recently been synthesized.[78] The term carbon/silicon switch, also called sila-substitution, is used when a silicon analogue of a known drug is synthesized where one carbon atom is replaced by a silicon atom, leaving the rest of the structure unchanged. Previously, silicon switches of marketed drugs have been reviewed, and examples of silicon switches are (besides sila-haloperidol and sila-trifluperidol) sila-venlafaxine, sila-fexofenadine and disila-bexarotene, studied in different *in vitro* systems.[79]

The aim of this study was to perform a thorough investigation of the *in vitro* metabolic pathways of two sila-substituted compounds compared to their carbon analogues (see Figure 6.1), including identification of differences and/or similarities in formation of reactive and potentially toxic metabolites, e.g. pyridinium metabolites.

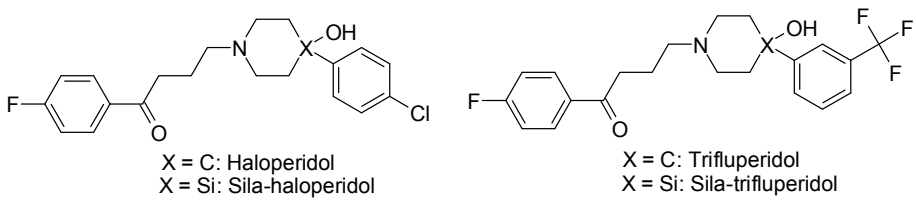


Figure 6.1. Structures of the test compounds.

6.1.1 The metabolism of C/Si-haloperidol in liver microsomes

The phase I metabolism of haloperidol and sila-haloperidol was studied in rat and human liver microsomes. Relative metabolite amounts of the fraction metabolized of haloperidol and sila-haloperidol are shown in Table 6.1. Cross-species metabolite schemes obtained from microsomal and hepatocyte incubations of haloperidol and sila-haloperidol are shown in Figure 6.2 and Figure 6.3, respectively.

For haloperidol, the major metabolite formed in the microsomal incubations was the pyridinium metabolite. Other metabolites formed were tentatively assigned as two hydroxylations (OH1 and OH2), two diastereomeric *N*-oxides (*N*-oxide1 and *N*-oxide2), reduced haloperidol (Red) and an *N*-dealkylated metabolite (*N*-dealk).

The metabolism of the silicon analogue, sila-haloperidol, in RLM and HLM was significantly different compared to that of haloperidol. No silapyridinium metabolite was formed in the liver microsomes, instead metabolites detected as MH^+ at m/z 364 and m/z 380, respectively, were formed. These two metabolites, Ring-opened1 and Ring-opened2, were the major metabolites in HLM and were proposed to originate from opening of the piperidine ring. Other metabolites formed in HLM were tentatively assigned to be an *N*-dealkylated metabolite (*N*-dealk), hydroxylation product of Ring-opened1 (Ring-opened1+OH), reduced sila-haloperidol (Red) and a hydroxylated metabolite (OH). The metabolite pattern was different in the RLM, where the hydroxylation and the *N*-dealkylated metabolite were the major metabolites. Minor metabolites were tentatively assigned as reduced sila-haloperidol, the two ring-opened metabolites and the hydroxylation product of Ring-opened1.

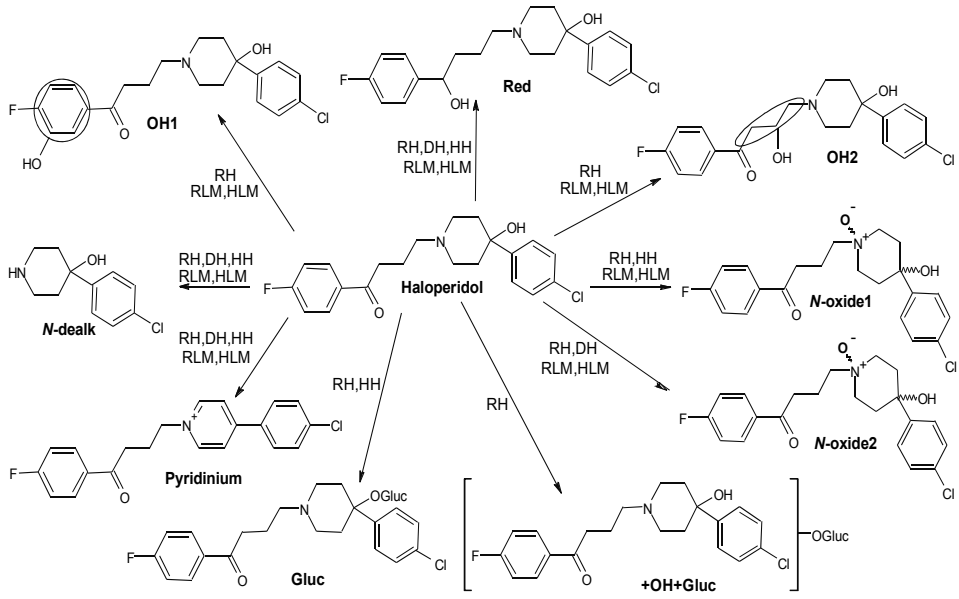


Figure 6.2. Proposed overall metabolism of haloperidol in HLM, RLM, and hepatocytes (HH, human; DH, dog; RH, rat).

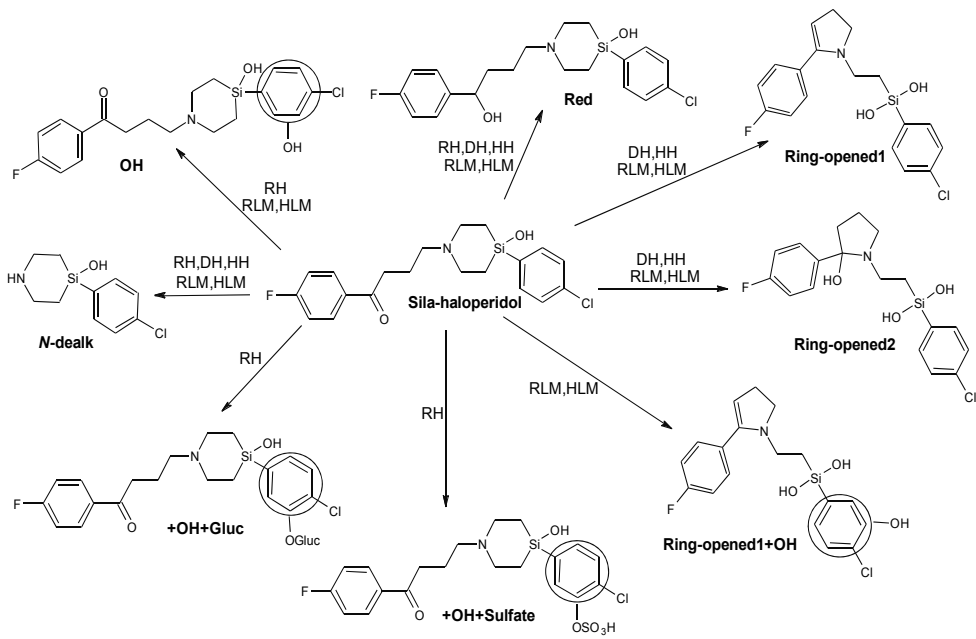


Figure 6.3. Proposed overall metabolism of sila-haloperidol in HLM, RLM, and hepatocytes (HH, human; DH, dog; RH, rat).

TABLE 6.1. Relative metabolite amounts of the fraction metabolized of haloperidol and sila-haloperidol, respectively. Data estimated from integration of extracted ion chromatograms.

				Haloperidol			
Metabolite	*m/z*	t_R	RLM	HLM	rat heps	dog heps	human heps
Relative proportions of metabolites after incubation of haloperidol for 60/120 min (%)							
N-dealk	212	1.06	7	9	32	37	37
+OH+Gluc	568	1.43	N.D.	N.D.	2	0	0
Gluc	552	1.62	N.D.	N.D.	10	0	0
OH1	392	1.71	3	2	1	0	0
OH2	392	1.78	3	1	1	0	0
Red	378	1.84	9	2	12	40	40
N-oxide1	392	2.08	1	1	1	0	0
Pyridinium	354	2.18	73	83	39	14	14
N-oxide2	392	2.23	4	1	4	9	9
Fraction of parent drug remaining after incubation (%)							
Haloperidol	376	1.97	55	59	77	81	86
				Sila-haloperidol			
Metabolite	*m/z*	t_R	RLM	HLM	rat heps	dog heps	human heps
Relative proportions of metabolites after incubation of haloperidol for 60/120 min (%)							
N-dealk	228	1.22	21	11	44	5	12
Ring-opened1+OH	380	1.28	2	2	0	0	0
+OH+Gluc	584	1.52	N.D.	N.D.	39	0	0
Ring-opened1	364	1.74	4	63	0	10	25
+OH+Sulfate	488	1.82	N.D.	N.D.	3	0	0
OH	408	1.90	71	3	5	0	0
Ring-opened2	382	1.94	1	20	0	2	7
Red	394	2.01	1	1	9	84	56
Fraction of parent drug remaining after incubation (%)							
Sila-haloperidol	392	2.18	83	65	80	61	94

N.D., not determined (no addition of UDPGA in this experiment)

6.1.2 The metabolism of C/Si-trifluperidol in liver microsomes

For trifluperidol, the major metabolites formed in RLM and HLM were the pyridinium metabolite and an *N*-dealkylated metabolite (*N*-dealk). Other metabolites formed were tentatively assigned as two hydroxylations (OH1 and OH2), two diastereomeric *N*-oxides (*N*-oxide1 and *N*-oxide2) and reduced trifluperidol (Red). In other words, the results were almost identical as those of haloperidol. For the silicon analogue, sila-trifluperidol, the metabolism in the liver microsomal incubations differed from that of trifluperidol. In the HLM, the major metabolites were one metabolite resulting from ring opening (Ring-opened1) and an *N*-dealkylated metabolite (*N*-dealk). Other metabolites formed in HLM were a second metabolite resulting from ring opening (Ring-opened2) and a hydroxylated metabolite (OH).

The major metabolite of sila-trifluperidol formed in RLM was the *N*-dealkylated metabolite, whereas other metabolites were the two ring-opened metabolites, a hydroxylated metabolite (OH) and the hydroxylation product of Ring-opened1 (Ring-opened1+OH). No pyridinium metabolite of sila-trifluperidol was formed in the liver microsomes.

6.1.3 The metabolism of C/Si-haloperidol in hepatocytes

Haloperidol and sila-haloperidol were also incubated with rat, dog and human hepatocytes, in order to study the phase II metabolism. For haloperidol, the major metabolites in hepatocytes were tentatively assigned to be reduced haloperidol (Red), the *N*-dealkylated metabolite (*N*-dealk), the pyridinium metabolite (Pyridinium) and the direct glucuronidation metabolite (Gluc). The direct glucuronidation metabolite, resulting from glucuronidation of the hydroxy group bound to the piperidine ring, was formed by rat and human hepatocytes but not by dog hepatocytes. Minor metabolites in the hepatocytes were tentatively assigned as hydroxylations, *N*-oxides and a second glucuronidation metabolite (+OH+Gluc).

For sila-haloperidol, reduced sila-haloperidol (Red) was the major metabolite in the dog and human hepatocytes, while the *N*-dealkylated metabolite (*N*-dealk) and a metabolite originating from hydroxylation and glucuronidation (+OH+Gluc) were the major metabolites in rat hepatocytes. Two metabolites, detected as MH^+ at m/z 488 and m/z 584 respectively, appeared in the rat hepatocyte incubations but not in dog and human hepatocyte incubations. The metabolite detected as an MH^+ at m/z 488, an addition of 96 Da to sila-haloperidol, corresponds to a hydroxylation and sulfate conjugation (+OH+Sulfate). The metabolite detected as an MH^+ at m/z 584, an addition of 192 Da to sila-haloperidol, corresponds to a hydroxylation and glucuronide conjugation (+OH+Gluc). In addition, minor metabolites formed were metabolites resulting from hydroxylation and opening of the piperidine ring, respectively. No metabolite resulting from a direct glucuronidation of the SiOH group and no pyridinium metabolite were observed for sila-haloperidol.

6.1.4 The metabolism of C/Si-trifluperidol in hepatocytes

The phase II metabolism of trifluperidol and sila-trifluperidol was studied in rat and human hepatocytes. For trifluperidol, the major metabolites in hepatocytes were tentatively assigned to be the pyridinium metabolite (Pyridinium), the *N*-dealkylated metabolite (*N*-dealk) and reduced haloperidol (Red). Minor metabolites were the direct glucuronidation metabolite (Gluc), a hydroxylated metabolite, *N*-oxides and a second glucuronidation metabolite (+OH+Gluc).

For sila-trifluperidol, one of the major metabolites in the rat hepatocytes was a metabolite originating from hydroxylation and glucuronidation (+OH+Gluc), whereas in the human hepatocytes the ring-opened metabolite (Ring-opened1) was a major metabolite. In addition, the *N*-dealkylated metabolite was a major metabolite in the hepatocytes from both species. Minor metabolites were resulting from hydroxylation, opening of the piperidine ring and reduction, respectively. No pyridinium metabolite of sila-trifluperidol was formed in the hepatocytes.

6.1.5 UDPGA-supplemented liver microsomes

Rat and human liver microsomal incubations supplemented with UDPGA were performed. UDPGA is a cofactor necessary for glucuronidations to occur. These

experiments were performed to exclude the possibility that the difference in direct glucuronidation between the carbon and the silicon analogue of haloperidol was due to switching the metabolism to another part of the molecule. Because no NADPH was added to the incubations, only the direct glucuronidation reaction was studied.

The direct glucuronidation metabolite of haloperidol was observed in the rat and human hepatocytes. This metabolite was also formed by the UDPGA-supplemented rat and human liver microsomes. For sila-haloperidol, no direct glucuronidation of the SiOH group was observed in the hepatocyte incubations. In the liver microsomes activated with UDPGA, no direct glucuronidation metabolite was present.

For trifluperidol, small amounts of the direct glucuronidation metabolite were observed in the human hepatocyte incubation. An explanation to the difference in direct glucuronidation between haloperidol and trifluperidol may be that the trifluoromethyl group in the *meta* position of trifluperidol constitutes a larger steric hindrance compared to the chlorine atom in the *para* position of haloperidol. The results from the UDPGA-supplemented liver microsomal incubations showed that the direct glucuronidation metabolite was formed by the human liver microsomes. For sila-trifluperidol, no direct glucuronidation of the SiOH group was observed in the hepatocyte incubations or in the liver microsomes activated with UDPGA.

6.1.6 Concluding discussion

Replacement of the carbon in the 4-position of the piperidine ring of haloperidol and trifluperidol by a silicon atom significantly altered the metabolic fate, especially regarding the formation of the pyridinium metabolite. No sila-pyridinium metabolites were formed since silicon does not form stable double bonds to carbon in an aqueous environment. Thus, this exemplifies how a deliberate change to a position important for the biotransformation of a drug candidate, may provide a means of avoiding a potential toxicity issue. In addition, a considerable difference in formation of a direct glucuronidation metabolite was observed between haloperidol and its silicon analogue (see Figure 6.4). This difference was not equally obvious for C/Si-trifluperidol. The decrease in direct glucuronidation for trifluperidol compared to haloperidol is proposed to be due to an enhanced steric hindrance from the trifluoromethyl group. In accordance with the results for sila-haloperidol, no direct glucuronidation metabolite was observed for sila-trifluperidol in the hepatocyte incubations or the UDPGA-supplemented microsomes. If the silanol (SiOH) groups in the investigated sila-compounds were glucuronidated, the resulting conjugates with their hydrolytically sensitive Si-OC bonds, would then undergo hydrolysis to the silanol and free glucuronic acid. If silanol groups do not form stable glucuronide conjugates, this would provide a possibility to introduce hydrophilicity in drug molecules, e.g. by replacing a C-H group by a Si-OH group, without increasing phase II metabolism.

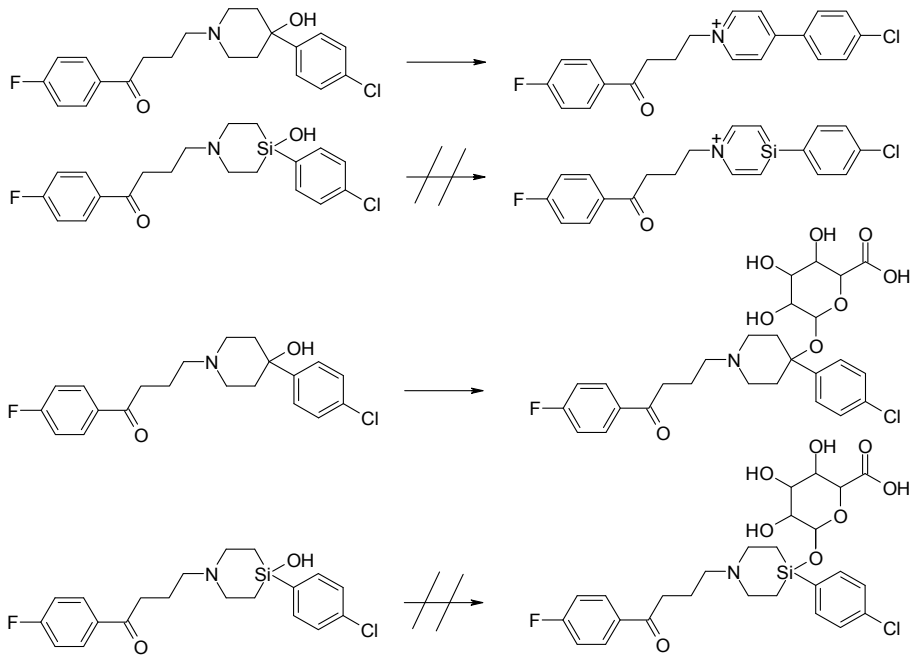


Figure 6.4. Observed differences in pyridinium and glucuronide formation between C/Si-haloperidol.

The use of sila-substitution in medicinal chemistry does not present a universal solution to avoid formation of reactive or toxic metabolites but it may complement more traditional approaches during the lead optimization process. Sila-substitution may also provide opportunities regarding intellectual property. Because the original carbon-based drug has been studied extensively, the corresponding silicon analogue may be able to shortcut the discovery part of drug development. Some silicon-containing drugs have progressed to clinical studies[80,81] and following the outcome of those trials will be intriguing, especially regarding pharmocodynamic properties and safety profiles.

In conclusion, by replacing one single carbon atom in haloperidol by a silicon atom the metabolic pathways, including the pyridinium metabolite formation but also the glucuronide formation, changed significantly. Studies on the structurally related C/Si-trifluperidol displayed the same difference in metabolic pathways, although regarding the direct glucuronidation formation the difference was not as significant as for C/Si-haloperidol.

7
Concluding Discussion and Future Perspectives

The work presented in this thesis emphasizes the importance of considering reactive metabolite formation early in the development of new drug candidates. The focus of the studies was put on cytochrome P450 enzymes since these enzymes are considered to be the major catalysts in drug metabolism, including metabolic activation.

The three different chemical oxidation systems together successfully mimicked all relevant phase I drug reactions. Due to the ability to scale up the porphyrin and the electrochemical system, these systems have a greater potential to be used for metabolite synthesis compared to the electrochemically assisted Fenton system. For the reactive metabolite studies the electrochemical oxidation system was assessed as the most useful system, due to the possibilities for rapid optimizations and on-line setups.

Direct applications of the electrochemical system in reactive metabolite studies were shown in the investigations of the well-known drugs amodiaquine and haloperidol. In the amodiaquine study, electrochemical synthesis enabled structural determination of a previously not identified metabolite by NMR spectroscopy. This new metabolite may be involved in the adverse drug reactions of amodiaquine, including agranulocytosis. In the studies of the oxidative metabolic pathways of haloperidol, the proposed iminium species was first observed in the electrochemical on-line setup and were later trapped as the corresponding cyano adducts. This study underpinned the possibility to use the electrochemical system for mechanistic studies.

Although it is of great importance to detect reactive or toxic metabolites and to elucidate their mechanism of formation, it may be even more crucial to have strategies to avoid them. Sila-substitution was shown to successfully avoid a toxicity related biotransformation for the drugs haloperidol and trifluperidol. The strategy of sila-substitution is interesting and might be useful, not only as a means to avoid reactive or toxic metabolite formation and/or to introduce hydrophilicity in drug molecules without increasing the phase II metabolism from glucuronidation, but also from an intellectual property point of view.

From the perspective of present investigations, the most effective application of electrochemistry in pharmaceutical industries may be synthesis of metabolites during drug development. Electrochemistry may complement classic synthetic chemistry by providing a rapid tool to oxidize or reduce drug compounds to biologically relevant metabolites. Furthermore, due to the recently published Guidance for Industry on Safety Testing of Drug Metabolites, issued by the US Food and Drug Administration, the accessibility of synthetic metabolite standards and subsequent safety testing are likely to increase.

The studies in this thesis present qualitative metabolite data, mainly on reactive metabolites. From a safety perspective, quantitative studies of reactive metabolites are needed, e.g. covalent binding studies, where the binding to macromolecules is assessed. In addition, studies of reactive metabolites or intermediates and their potential targets are needed, in order to understand how they may covalently modify these macromolecules. Another possibility may be to use electrochemistry in other reactive metabolite studies of biological significance than nucleophile trapping, e.g. to use electrochemically generated metabolite standards in cell and gene toxicity assays or in mechanism based inhibition studies.

8
Populärvetenskaplig Sammanfattning

När ett läkemedel har tagits upp i kroppen och utövat sin effekt, bryts det ned för att slutligen lämna kroppen genom utsöndring. Nedbrytning av kroppsfrämmande ämnen såsom läkemedel kallas för metabolism. Läkemedelsmetabolism betraktas ofta som en process som förändrar läkemedel till ofarliga, vattenlösliga nedbrytningsprodukter som lätt kan lämna kroppen, t.ex. via urinen. Läkemedel kan emellertid även omvandlas till reaktiva och potentiellt toxiska nedbrytningsprodukter (metaboliter) genom metabolism. Sådana reaktiva metaboliter kan binda till olika proteiner eller till DNA i kroppen, vilket kan ge biverkningar och skador hos patienter. Reaktiva metaboliter försvårar på detta sätt användandet och utvecklingen av nya läkemedel. Det är därför viktigt att utveckla metoder för att tidigt i utvecklingen av läkemedel identifiera samt undvika reaktiva metaboliter.

Denna avhandling utreder hur elektrokemiska metoder kan användas för att studera reaktiva metaboliter. I **delarbete I** vidareutvecklades och förfinades metoder för att efterlikna metabolismen av läkemedel i kroppen med hjälp av tre olika kemiska system. Systemen kompletterade varandra och alla relevanta typer av metaboliska reaktioner efterliknades med minst ett av de tre systemen. I **delarbete II** och **III** studerades metabolismen av två välkända läkemedel, amodiakin och haloperidol. Amodiakin var ett malarialäkemedel som blev indraget från marknaden på grund av dess allvarliga biverkningar. I detta arbete identifierades hittills okända metaboliter som skulle kunna orsaka eller bidra till amodiakins biverkningar. Elektrokemisk framställning av en av metaboliterna i tillräckliga mängder, möjliggjorde att metabolitens struktur kunde bestämmas. Haloperidol är ett läkemedel som används i behandling av schizofreni och svåra psykoser. Idag är användandet av detta läkemedel begränsat på grund av dess oönskade biverkningar, som tros bero på en av haloperidols metaboliter. I **delarbete III** studerades bildningen av denna metabolit och nya, kortlivade produkter observerades som visade på mellansteg i denna bildning.

Om man i utvecklingen av ett läkemedel upptäcker att en eller flera reaktiva eller toxiska metaboliter bildas vid nedbrytningen i kroppen, finns det olika sätt att hantera problemet. Ett exempel är att på olika sätt ändra på läkemedlets struktur. I **delarbete IV** ändrades haloperidols struktur för att undvika att den toxiska metaboliten bildas och därmed minska dess allvarliga biverkningar.

Acknowledgements

I would like to express my sincere gratitude to the following people, you have all in different ways contributed to the preparation of this thesis:

My supervisors Dr Lars Weidolf and Dr Ulrik Jurva for giving me the great opportunity to perform my master thesis work in your group and later for taking me on as a Ph.D. student.

Dr Lars Weidolf for always being able to see the overall picture and for contributing with your large knowledge. Thank you for sharing interesting project problems, thorough proofreading of manuscripts and for taking on the responsibility as main supervisor. You have been acting as my mentor as well as my supervisor.

Dr Ulrik Jurva for your endless enthusiasm and encouragement! Thank you for sharing your knowledge of electrochemistry and mass spectrometry, and for always taking time to discuss results or experimental problems. You are one of the most helpful, positive and kindest persons I know!

Dr Kristina Luthman for support and encouragement, always with a warm and positive attitude.

To the Biotransformation group, the old and the new one, for showing interest in my work and for helping me with equipment and questions. Especially thanks to Bo Lindmark, Carina Leandersson and Anna Abrahamsson for answering many questions about the QTOF instruments. Thanks also to Emre Isin for clarifying reactivity issues.

Dr Jennie Georgsson for your patient guidance during the synthetic work. Although it did not lead to any publication, I am now able to claim that I have synthesized 200 compounds. Thanks also to the group at KK2 during that time.

Dr Gunnar Grönberg for always being very helpful and open to new, exciting ideas. We did get some really nice NMR spectra!

Dr Collen Masimirembwa for a nice collaboration on the amodiaquine paper. Nothing is impossible when you work with Collen!

Professor Reinhold Tacke for the fruitful collaboration on the sila-compounds.

Professor Neal Castagnoli, Jr. for contributing with your knowledge of haloperidol and MS/MS fragmentation, and for generously providing synthetic reference compounds.

Dr Martin Kjerrulf for help with setting up a method for isolation of human leukocytes.

Ingvar Nilsson for helping me with the lyophilization with a happy smile!

Dr Anders Tunek, Dr Richard Thompson and Dr Hugues Dolgos for supporting this project.

To all the people that I have worked with during these years, thank you for your support and guidance but also for making the time at AstraZeneca interesting and joyful.

My family and friends:

Jag skulle vilja tacka alla mina släktingar och vänner för ert stöd och för att ni får mig att tänka på andra saker än arbetet.

Uppsalaflickorna, våra årliga träffar har gett mig välbehövlig energi och det är alltid lika trevligt att umgås med er.

Tack till min familj, för stöd i tuffa perioder och för firande av glada stunder. Pappa och Elisabet för att ni alltid lyssnar, stöttar och tror på mig. Min mamma Grethe, du finns alltid i mina tankar och jag önskar att du fick uppleva det här.

Mormor, du betyder så mycket för mig.

Mailis och Niilo, för er omtanke, stöd och intresse i mitt arbete.

Samuel, utan dig hade detta inte varit möjligt. Tack för att du alltid tror på mig, stöttar när det är svårt och gläds när det går bra. Du uppmuntrade mig att söka doktorandtjänsten och du har peppat mig hela vägen. You and me, me and you.

The Swedish Academy of Pharmaceutical Sciences, Stiftelsen Kjellbergska Flickskolans Donationer, Adlerbertska Foundation Travel Grant for travel and conference support.

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