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Development of a semi-automated covalent binding methodology

Master of Science Thesis in Chemistry and Bioscience

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Abstract

Idiosyncratic adverse drug reactions (IADRs) are the number one cause for pharmaceuticals being withdrawn from the market today. Since IADRs occurs in only 1-10 out of 100 000 people and with a delayed onset of up to a year the drug is likely to pass preclinical safety studies before being discovered as harmful. The formation of reactive metabolites during biotransformation of a drug can result in covalent modification of cellular macromolecules such as enzymes or other proteins, thereby causing cell damage. Covalent binding to proteins is proposed to be involved in pathways leading to IADRs and has therefore been implemented as part of the screening criteria for drug candidates. In this work human hepatocyte and human liver microsome incubations were investigated as *in vitro* methods for studying the existing drugs acetaminophen (paracetamol), amodiaquine, carbamazepine, clozapine, diclofenac, troglitazone and zomepirac from a biotransformation and covalent binding point of view. Applicability of a zone classification system proposed by Nakayama, et al., 2009 was evaluated, taking into consideration daily dose of each drug together with the amount of covalent binding. The results from the assay revealed good reproducibility and an acceptable coherence with previously reported experiments. LC-MS analysis was used to determine metabolic profiles of the compounds. Issues regarding hepatic enzyme activities have been addressed and proposed as a possible factor for variation of covalent binding data. It is concluded that covalent binding screening criteria may be constructed upon expansion of the incubated compound data set.

Keywords: covalent binding, idiosyncratic adverse drug reaction, biotransformation, reactive metabolite, glucuronidation, incubation, human hepatocytes, human liver microsomes, radiolabeled compounds.

Reference:

Nakayama,S., Atsumi, R., Takahusa, H., Kobayashi, Y., Kurihara, A., Nagai, Y., et al. (2009). A Zone Classification System for Risk Assessment of Idiosyncratic Drug Toxicity Using Daily Dose and Covalent Binding. *Drug Metabolism and Disposition*, 37: 1970-1977.

Abbreviations in alphabetical order

ACN – Acetonitrile

BCA – Bicinconinic acid

BEH – Bridged ethyl hybrid

C – Molar concentration

CPM – Counts per minute

CYP – Cytochrome P450

DILI – Drug induced liver injury

DPM – Disintegration per minute

DMSO – Dimethyl sulphoxide

FA – Formic acid

GSH – Glutathione, tripeptide antioxidant

HBSS – Hanks balanced salt solution

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLM – Human liver microsomes

HSM – Hepatocyte suspension media

IADR – Idiosyncratic adverse drug reaction

NADPH – Reduced β-nicotine amide adenine dinucleotide phosphate

NSAID – Nonsteroidal anti-inflammatory drug

RC – Radioactive concentration

RP – Radioactive purity

SA – Specific activity

SD – Standard deviation

SDS – Sodium dodecyl sulphate

UDPGA – Uridine 5'-diphospho-α-D-glucuronic acid

UGT – Uridine 5’-diphospho-α-D-glucuronosyltranferase

UPLC – Ultra performance liquid chromatography

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1 Introduction

There are a number of different fates for a drug molecule that enters our body. From a drug developers point of view it is essential to be able to understand and minimize the pathways that are potentially harmful. A common potentially hazardous event in drug metabolism is the formation of reactive metabolites (Pohl & Pumford, 1996). Our cells are equipped to tolerate and adapt to a certain amount of reactive metabolites as they are naturally formed via our diet, however therapeutic use of pharmaceuticals may greatly increase the amount of reactive metabolites (Park, Kitteringham, Maggs, Pirmohamed, & Williams, 2005). The reactions between reactive metabolites and macromolecules such as proteins can result in covalent modification of the target. This in turn may cause severe detrimental effects in the function of the protein, trigger a downstream response or lead to cell damage or cell death. Ultimately this may result in drug induced liver injury (DILI) or even death (Uetrecht, 2006). Idiosyncratic adverse drug reactions (IADRs) are rare and often serious drug related adverse events observed in 1-10 out of 100 000 people. Covalent binding of reactive metabolites to macromolecules is thought to increase the risk of IADRs (Liebler & Guengerich, 2005). Due to the rareness of IADRs and/or human specificity it is unlikely that they will be picked up in any preclinical safety studies and the candidate drug is likely to be put to clinical use before being discovered as harmful. This is of major concern for the pharmaceutical industry as a significant amount of research and effort has been put in the making of the drug at this stage. There are several examples of drugs being withdrawn from the market or black box labeled as a result of IADRs including carbamazepine, clozapine, troglitazone and acetaminophen (Kalgutkar, Fate, Didiuk, & Bauman, 2008). To avoid this, effective ways of assessing the potential for covalent binding of candidate drugs at an early stage of development is needed.

1.1 Aim

The aim of this project is to set up a zone classification system based on covalent binding to proteins and daily dose using the system developed by Daiichi (Nakayama, et al., 2009) as a starting point. A semi-automated *in vitro* covalent binding assay with the marketed compounds acetaminophen, amodiaquine, carbamazepine, clozapine, diclofenac, troglitazone, and zomepirac has been used to create the framework of the classification system. These compounds have either a record of having a warning, a black box warning or have been withdrawn from the market. By determining the amount of covalent binding to proteins in an *in vitro* assay with these existing drugs in relation to the recommended daily dose it may be

possible to categorize future drug candidates based on the results from the same assay. This would provide a powerful tool for assessing the risk of covalent binding for drug candidates, facilitating the screening of potentially harmful compounds in drug discovery.

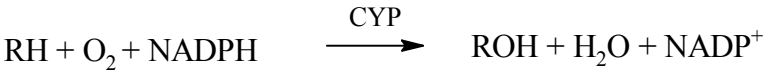
1.2 Scientific background

1.2.1 General drug metabolism

A majority of the xenobiotics entering our body are metabolized in the liver. The entering molecule is modified by an intricate network of enzymatic and non-enzymatic reactions to increase its hydrophilicity hence facilitating renal and biliary excretion (Testa & Krämer, 2008). This chain of events is known as biotransformation and has traditionally been subdivided into Phase I and Phase II reactions. Phase I reactions involve oxidations, reductions, hydrolyses or a combination of the three while Phase II reactions consists of conjugations with glucuronic acids, glutathione, sulphates etc. (Williams T. R., 1959). Phase I and Phase II reactions often, but not always, occur sequentially depending on the structure of the xenobiotic. This terminology has been criticized due to lack of stringence but remain widely applied today (Josephy, Guengerich, & Miners, 2007).

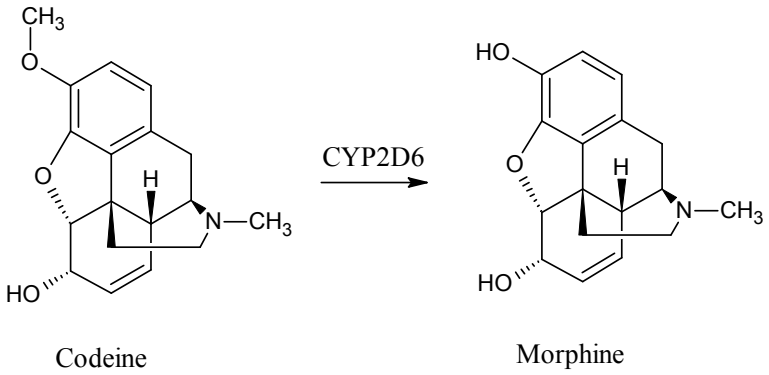
1.2.1.1 Cytochrome P450 (CYP)

Most of the oxidizing enzymes acting in the Phase I reactions belong to the CYP family. CYPs constitute a group of over 10 000 different enzymes present in many species (Nelson, 2009). CYPs can catalyze hydroxylations, formation of reactive species such as an epoxide or dealkylations as well as heteroatom oxidations. CYPs are hemecontaining enzymes meaning that they have a coordinated Fe^{3+}/Fe^{2+} in the active site and they are involved in a catalytic cyclic redox reaction where an organic substrate (the xenobiotic) is oxidized while oxygene is reduced to water. The electrons needed for the reaction are liberated when NADPH is simultaneously reduced to $NADP^{+}$, making NADPH a cofactor to CYPs. The net reaction is shown in **Reaction 1**. Metabolic reactions catalyzed by CYPs are termed CYP mediated metabolic pathways (Isin & Guengerich, 2008).



Reaction 1. Net reaction of CYP mediated hydroxylation.

Because each new drug candidate will have a unique structure its biotransformation pathway and metabolic profile may also be unique. It is not uncommon for a drug to be inactive in its parent form, only to be activated through biotransformation. In these cases the compounds are called prodrugs. A descriptive example of a prodrug is the bioactivation of codeine to morphine that proceeds via a demethylation as shown in **Reaction 2** (Dayer, Desmeules, Leemann, & Striberni, 1988).

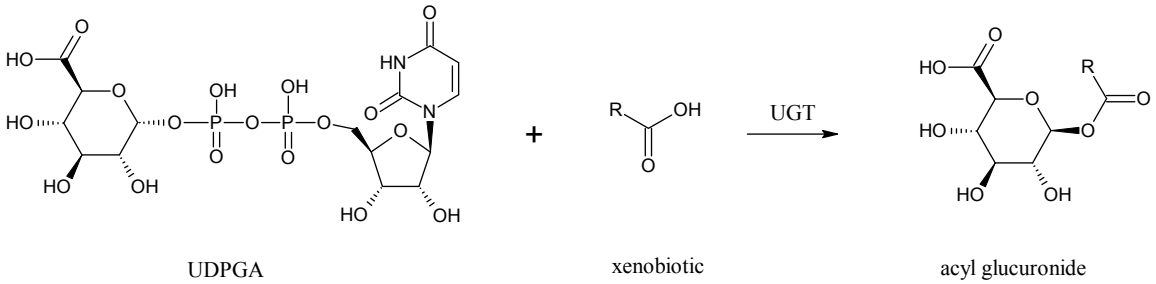


Codeine Morphine

Reaction 2. Biotransformation of the prodrug codeine to its active form morphine by oxidative dealkylation. The reaction is catalyzed by the enzyme CYP2D6.

1.2.1.2 Glucuronidation

Glucuronidation is a Phase II conjugation reaction catalyzed by the enzyme family uridine 5'-diphospho-α-D-glucuronosyltranferases (UGTs). In a glucuronidation reaction uridine 5'-diphospho-α-D-glucuronic acid (UDPGA) reacts with the xenobiotic in such a way that glucoronic acid from UDPGA binds to the xenobiotic, yielding the corresponding glucuronide (Stachulski, Harding, Lindon, Maggs, Park, & Wilson, 2006). This is shown in **Reaction 3**.



Reaction 3. The formation of acyl glucuronides.

1.2.2 *In vitro* studies

To study xenobiotic metabolism in vitro, systems acting similar to those found in the body are desired. There are different approaches for achieving this, resulting in different information. Some common tools are studies with expressed enzyme systems, subcellular preparations, cell cultures, liver slices and perfused organs (Testa & Krämer, 2008).

1.2.2.1 Hepatocytes

The human liver consists of 70-80% hepatocytes, which are considered the most important cells for biotransformation of xenobiotics. The use of hepatocytes in *in vitro* metabolism studies is a powerful tool providing information that correlates well with corresponding *in vivo* experiments (Asha & Vidyavathi, 2010). Since the entire liver cell is used, both Phase I and Phase II reactions as well as membrane transport factors have to be considered (Brown, Griffin, & Houston, 2007). A disadvantage using hepatocytes is the interindividual variation amoung donors.

1.2.2.2 Human liver microsomes (HLMs)

Human liver microsomes are subcellular preparations. They are extracted by homogenization and subsequent differential centrifugation of human liver, primary hepatocytes or liver cell cultures. Its major constituent is the endoplasmatic reticulum of the hepatic cells (Li, 2005). HLMs have a high content of the drug-metabolizing enzymes CYP, flavin monooxygenases, carboxyl esterases, and epoxide hydrolase (Asha & Vidyavathi, 2010). HLMs are used to study mainly Phase I reactions.

1.2.3 Reactive metabolites

Reactive metabolites are formed continously in our body for instance when dietary compounds are metabolized in our cells. Reactive metabolites may take the molecular form of an epoxide, free radical or other reactive moiety. The potential danger a reactive metabolite poses is by modifying functional intracellular macromolecules such as DNA, RNA, enzymes or other proteins. To cope with this we are equipped with a relatively high cellular concentration of glutathione (GSH), a naturally occuring tripeptide antioxidant. The structure of GSH is shown in **Figure 1**.

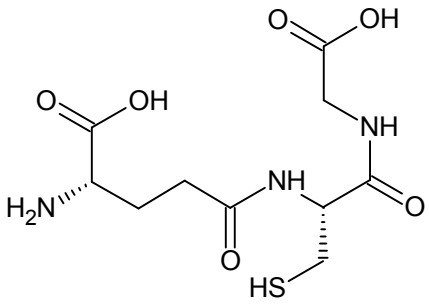


Figure 1. Structure of GSH.

Biotransformation of a xenobiotic may or may not yield a reactive metabolite depending on the structure of the xenobiotic and the enzymes present in the cell, which in turn reflects the genetic material of the induvidual. Individual dosage requirements for many frequently used drugs can differ more than 20-fold depending on the genotype or the enzyme expression in the cell (Ingelman-Sundberg, 2001). GSH acts by covalently binding to the reactive metabolite, making it less reactive and more easily excreetable. Excessive intake of pharmaceuticals or other xenobiotics may therefore greatly decrease GSH levels, leading to an increased risk of covalent modification of macromolecules and potential cell damage (Park, Kitteringham, Maggs, Pirmohamed, & Williams, 2005).

1.2.4 Covalent binding studies

Covalent binding in a drug metabolism context is the result of a reactive metabolite reacting with an intracellular target. Covalent binding is believed to contribute to the drug induced toxicity occasionally exhibited in our cells (Masubuchi, Makino, & Murayama, 2007). This has led to the use of covalent binding studies as an attempt to discriminate potential harmful drug candidates. Allthough there are exceptions to the thesis that a high amount of covalent

binding leads to increased toxicity several articles has shown some correlations between toxicity and covalent binding for a variety of compounds (Nakayama, et al., 2009). Some recent studies consider the amount of covalent binding together with the expected daily dose. This has proven even more succesful in trying to separate known harmful compounds from safe ones based on the amount of covalent binding (Nakayama, et al., 2009), (Usui, Mise, Hashizume, Yabuki, & Komuro, 2009). The idea has been to place compounds known to cause (or not cause) toxicity in a 2-dimensional zone system with covalent binding on the Y-axis and therapeutic daily dose on the X-axis, thereafter set up cut off values to distinguish safe from non safe zones. Once the zones are in place the assessment of future compounds depends on how the compound places in the 2-dimensional system. The concept is visualized in **Figure 2**.

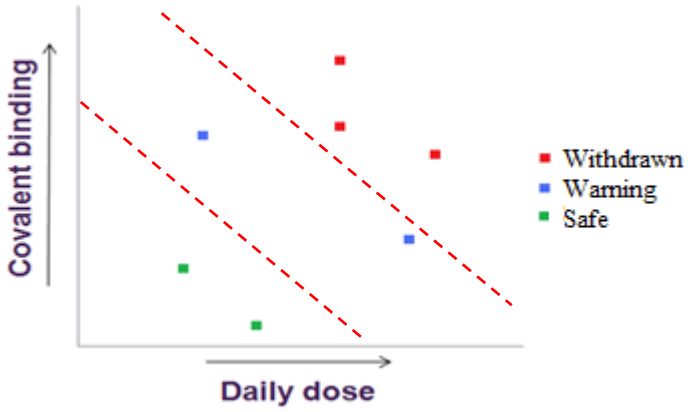


Figure 2. Conceptual basis for the covalent binding and daily dose zone system. The color of the compounds in the graph illustrates how drugs associated with different danger levels could group. The dashed lines are hypothetical cut off values to distinguish the zones.

Expected daily dose of the candidate drug is based on the expected potency of the drug as well as bioavailability and clearence, this value often becomes more accurate further down the development chain. Covalent binding is frequently reported as picomol equivalent per milligram protein (pmoleq/mg). One equivalent (eq) refers to one covalently bound molecule originating from the parent, i.e. the parent itself or a metabolite of the parent. To get an accurate quantification of the picomol equivalent, radiolabeled compounds are used. The amount of protein can be determined using chelating techniques such as the Lowry or Bradford method which includes absorbance spectroscopy measurements.

1.2.5 Liquid scintillation counting

Liquid scintillation counting is a common technique to quantify the bound radiolabeled metabolites and/or parent in covalent binding studies. The radioactive beta decay taking place in a ^{3}H or ^{14}C nucleus is as shown in **Reaction 4** (Harrison, Rundt, & Oikari).

$$\text{neutron} \longrightarrow \text{proton} + \beta^{-} + \gamma$$

Reaction 4. Beta decay producing a β^{-} particle (electron) and a γ particle (anti-neutrino).

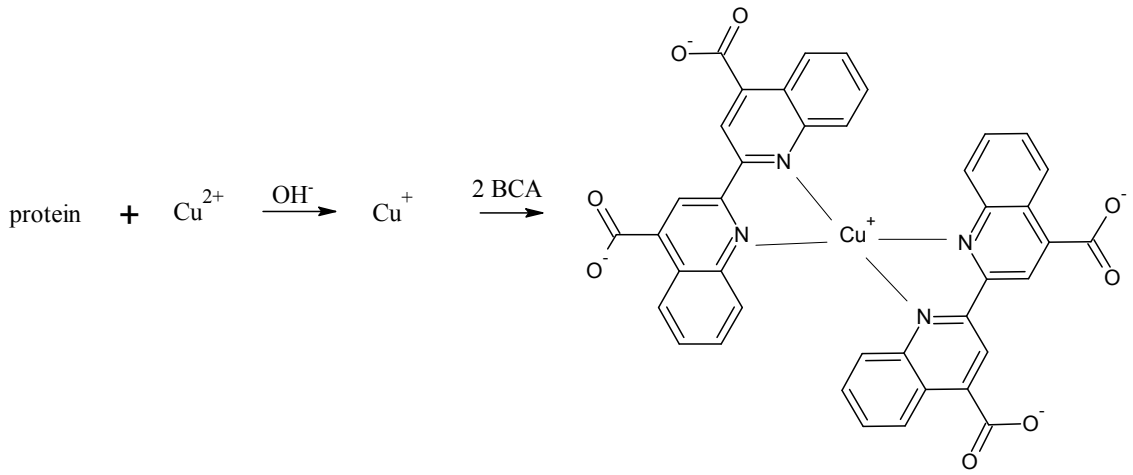
Scintillation cocktail contains phosphors, molecules capable of absorbing energy and emitting it as light, in organic solvent. The solvent in the cocktail is designed to absorb the energy emitted by the radioisotopes and transfer it to the phosphors. The phosphors then re-emit the energy as light. A photomultiplier tube amplifies the light signal which is in turn recorded by a detector as counts per minute (CPM). CPM is converted to disintegration per minute (DPM), by taking into account the counting efficiency of the scintillation fluid from its losses during energy transfer according to **Equation 1**. Counting efficiencies varies with the amount of quenching in the system. Quenching is affected by isotopes, sample compositions and the scintillation apparatus. It can be compensated for using fine tuning curves.

$$\text{CPM} = \text{DPM} \times \text{counting efficiency} \qquad \textbf{(1)}$$

By adding scintillation cocktail to a sample and analyzing it on a liquid scintillation counter it is possible to accurately quantify the amount of isotopes present in the sample (National Diagnostics Laboratory Staff, 2004).

1.2.6 Protein determination

The protein determination assay used in this project is a copper based method utilizing the copper complexation reaction with bicinconinic acid (BCA). First protein reduces Cu^{2+} to Cu^{+} in an alkaline medium. Two molecules of bicinconinic acid (BCA) then chelate to the Cu^{+} and the resulting complex exhibits a strong purple color absorbing light at 562 nm (Smith, et al., 1985). The working range of the Pierce BCA protein determination assay is 20 – 2000 µg protein/mL (Thermo Scientific, 2009). The two step reaction is illustrated in **Reaction 5**.



Reaction 5. Reaction schematic for the BCA protein determination adopted from Smith, et.al., 1985.

1.2.7 Compounds

The following section intends to provide a brief description of the compounds used in the project

1.2.7.1 Acetaminophen

Acetaminophen (also known as paracetamol) is a non-opiate analgesic and antipyretic. It was first introduced in the US as a safe alternative to aspirin for children. It has been used extensively since its introduction in 1955 and is today one of the most consumed drugs on the market. Another common use of acetaminophen is as a partner drug to opiate analgesics (Scottish intercollegiate guidelines network, 2008). Acetaminophen is the number one cause of drug induced acute liver failure in the US and UK today (Lee, 2008).

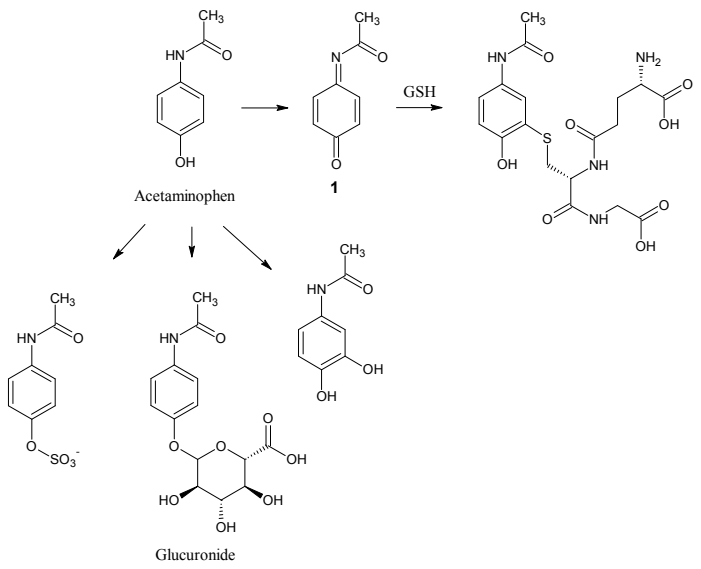


Figure 3. Bioactivation and detoxication of acetaminophen, adopted from Laine, Auriola, Pasanen, & Juvonen, 2009.

The benzoquinone imine (**1, Figure 3)**, which is capable of binding to cellular macromolecules and will accumulate upon GSH depletion, is thought to be responsible for the toxic effects caused by acetaminophen (Cocordan, Mitchell, Vaishnav, & Horning, 1984). Acetaminophen carries a warning.

1.2.7.2 Amodiaquine

Amodiaquine is an antimalarial compound for treatment of acute malaria and for prophylactic use. It is a member of the 4-aminoquinoline class of anti-malarial drugs. The main adverse effects include agranulocytosis, hepatitis and peripheral neuropathy (Hatton, et al., 1986).

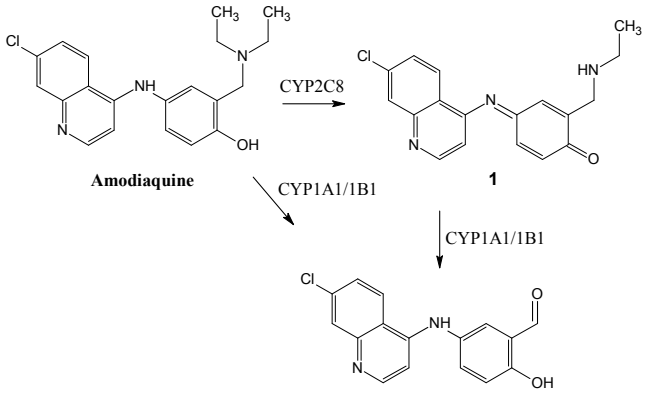


Figure 4. Proposed metabolic pathways of amodiaquine in HLMs adopted from Johansson, Jurva, Grönberg, Weidolf, & Masimirembwa, 2009.

The major metabolite of amodiaquine is desethylamodiaquine (**1, Figure 4)** which is responsible for the larger part of the antimalarial activity observed, making amodiaquine a prodrug (Winstanley, Edwards, Orme, & Breckenridge, 1987). Amodiaquine was withdrawn from prophylactic use because of its adverse effects but is being reconsidered as a potentially good partner drug to artesunate (Taylor & White, 2004).

1.2.7.3 Carbamazepine

Carbamazepine was first synthesized in the 1960s and has been approved for commercial use in the UK since 1965. It is used primarily as an anticonvulsant for treating epilepsy but also as an antidepressant for treatment of bipolar disorder. Carbamazepine is associated with a number of adverse effects including skin rash, fever, hepatitis and hematologic abnormalities (Shear & Spielberg, 1988).

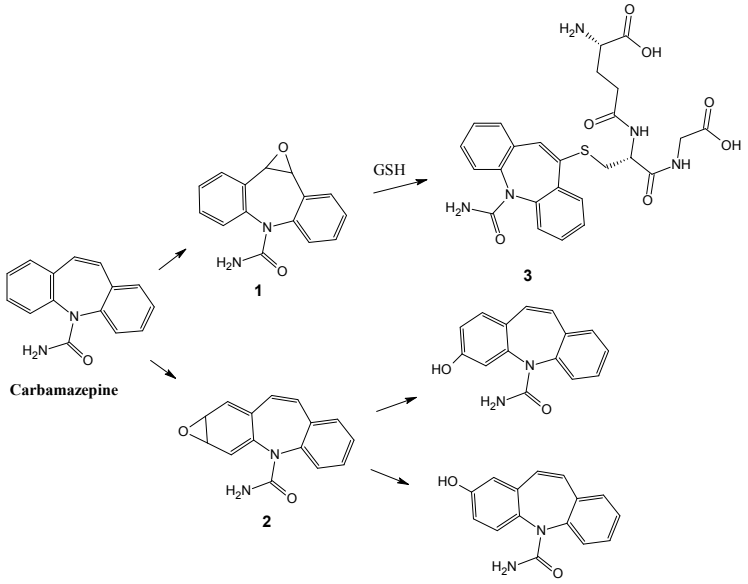


Figure 5. Proposed metabolic pathways of carbamazepine in HLMs adopted from Pearce, Lu, Wang, Uetrecht, Correia, & Leeder, 2008.

There are several reactive metabolites of carbamazepine thought to be responsible for the observed adverse effects. The areneoxides carbamazepine 10,11-epoxide (**1, Figure 5)** and carbamazepine 2,3-epoxide (**2, Figure 5)** has been observed as both GSH adducts, one of which is shown as **3, Figure 5** and covalently bound to proteins in HLMs (Bu, Kang, Deese, Zhao, & Pool, 2005). Carbamazepine is still being sold today but with a black box warning.

1.2.7.4 Clozapine

Clozapine is an antipsychotic drug for treatment of schizophrenia. It was the first of the so called atypical antipsychotics. After its introduction in 1971 several cases of severe agranulocytosis led to its withdrawal four years later (Opgen-Rhein & Dettling, 2008). Failure to produce an equally potent substance led to the reinstatement of clozapine as a last resort treatment for refractory schizophrenia (Meltzer, 1997).

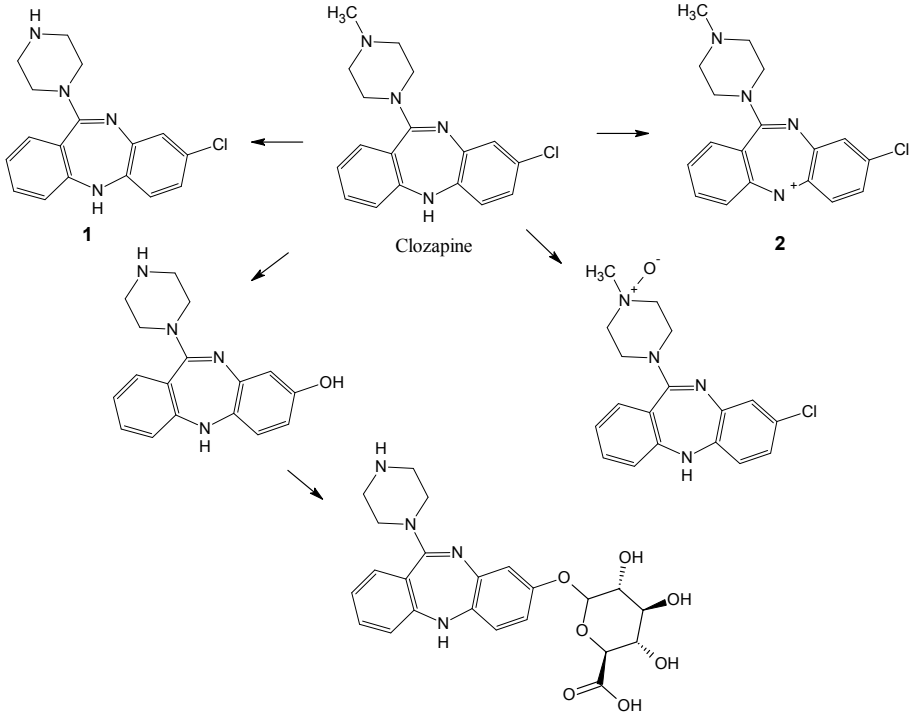


Figure 6. Clozapine metabolites observed in HLM and in vivo in humans, adopted from Dain, Nicoletti, & Ballard, 1997 and Williams, Pirmohamed, Naisbitt, Uetrecht, & Park, 2000.

The reactive nitrenium ion **(2, Figure 6)** and the *N*-desmethylclozapine (**1, Figure 6)** are suspected to be involved in the severe agranulocytosis cases observed. (Williams, Pirmohamed, Naisbitt, Uetrecht, & Park, 2000)

1.2.7.5 Diclofenac

Diclofenac is a widely used nonsteroidal anti-inflammatory drug (NSAID) with antipyretic properties. It was introduced in the UK in 1973. Diclofenac has been reported to cause mild to severe hepatotoxicity in a small but significant number of patients (Helfgott, Sandberg-Cook, Zakimand, & Nestler, 1990).

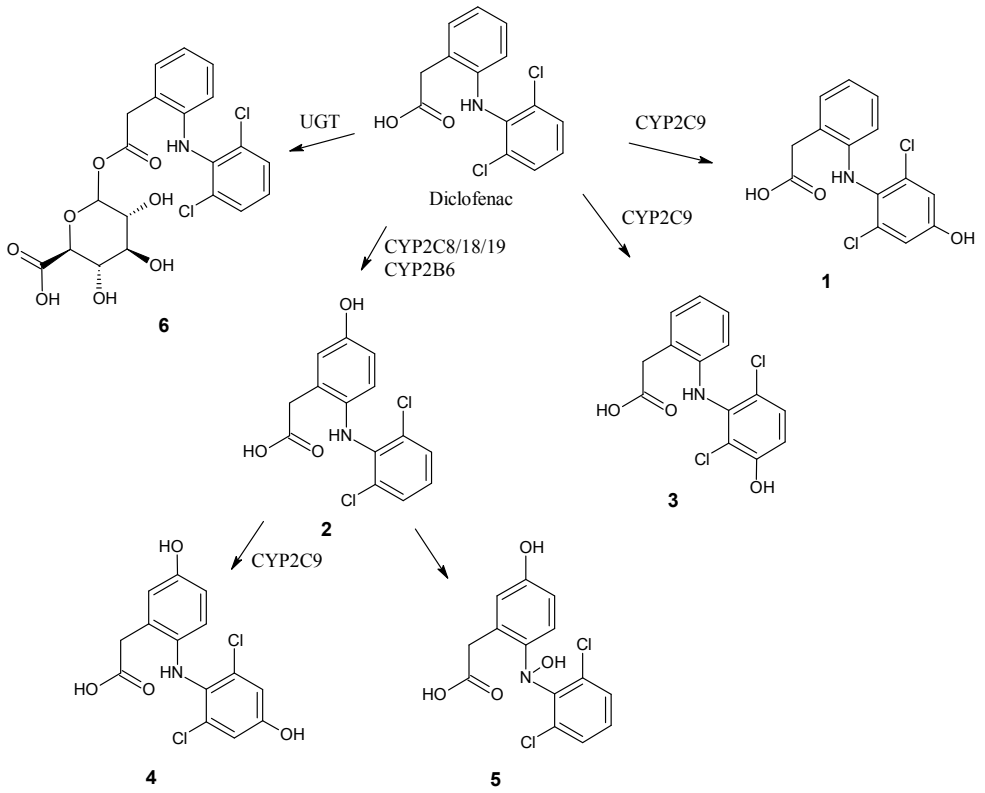


Figure 7. Biotransformation of diclofenac in human hepatocytes, adopted from Bort, Macé, Boobis, Gómez-Lechón, Pfeifer, & Castell, 1999.

It is suspected that a CYP mediated metabolite **(1-5, Figure 7)** to diclofenac is involved in covalent binding to proteins in HLMs (Pohl, 1996). Acyl glucuronides **(6, Figure 7)** have also been observed.

1.2.7.6 Troglitazone

Troglitazone is an anti-inflammatory and anti-diabetic drug. It was introduced into the market in the 1990s but withdrawn shortly after as it seemed to cause IADRs manifested as drug induced hepatitis (Masubuchi Y. , 2006). The GSH conjugates from CYP mediated reactive metabolite formation are shown as **1-5, Figure 8**.

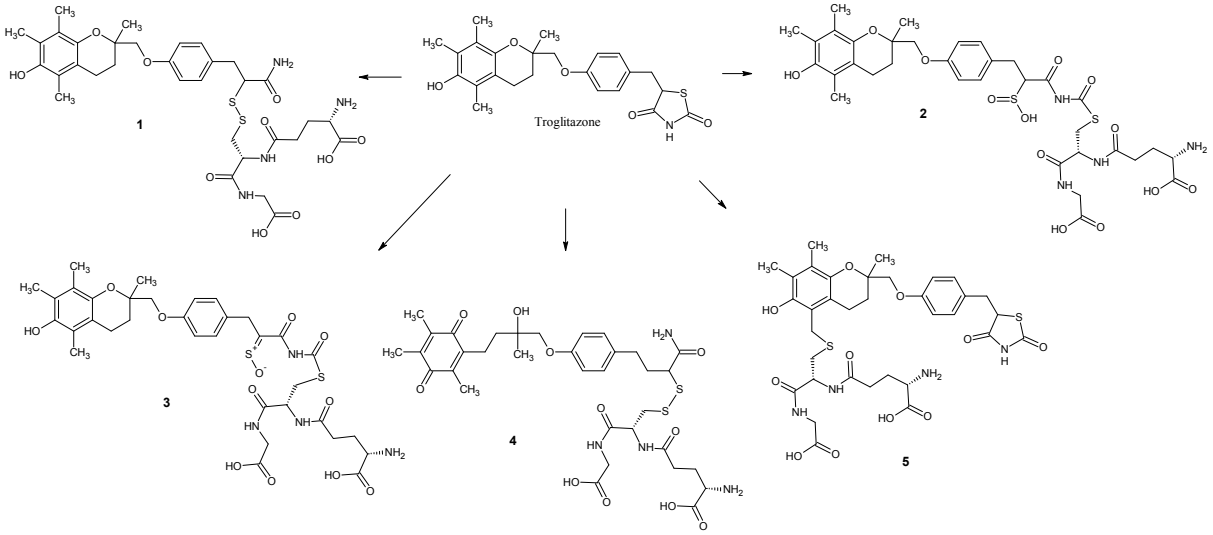


Figure 8. Proposed reactive metabolites trapped as GSH conjugates produced in HLMs. Adopted from Kassahun, et al., 2001.

1.2.7.7 Zomepirac

Zomepirac is an analgesic for treating mild to severe pain and it belongs to the NSAIDs. When zomepirac was first approved by the American Food and Drug Administration in 1980 it served as a popular substitute for narcotic analgesics such as morphine (Lewis, 1981).

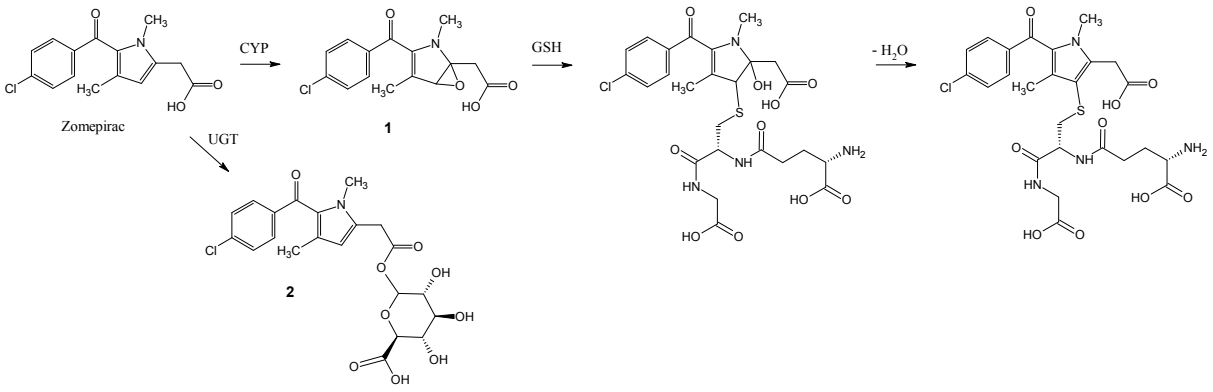


Figure 9. Proposed oxidative biotransformation of zomepirac and formation of acyl glucuronides. Adopted from Chen, et al., 2006.

Biotransformation of zomepirac in activated HLMs yields a reactive epoxide **(1, Figure 9)** (Chen, et al., 2006). Biotransformation of zomepirac also leads to the formation of acyl glucuronides (**2, Figure 9)** (Smith, McDonagh, & Henet, 1986). Zomepirac was withdrawn from the market in 1983 after causing rare but severe anaphylactic shocks in patients (Darwish, et al., 1984).

1.2.8 Summary of compounds

Figure 10 summarizes the compounds used in this project and the danger level they are associated with. Three classes have been distinguished as; “Warning”, “Black box warning” and “Withdrawn” according to the Physicians' desk reference, 2010.

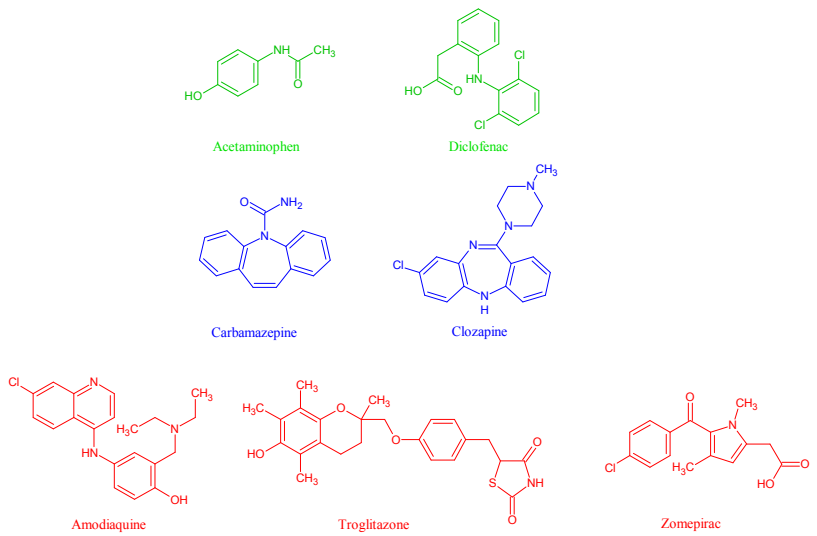


Figure 10. The compounds used in the project and their associated danger level. Green compounds (acetaminophen and diclofenac) carry a warning, blue compounds (carbamazepine and clozapine) carry a black box warning and red compounds (amodiaquine, troglitazone and zomepirac) have been withdrawn from the market.

1.2.9 Reported daily dose

The reported therapeutic daily doses for the compounds in the project are shown as intervals. The intervals denote the lowest and highest doses recommended for therapeutic treatment. For the analysis of the covalent binding data to daily dose the highest of these values has been chosen. **Table 1** summarizes the daily doses reported in the Physicians' desk reference, 2010.

Table 1. Therapeutic daily dose of the compounds. *Only maximum daily dose shown.

Compound	Daily dose [mg]
Acetaminophen	900–4000
Amodiaquine	1750–2450
Carbamazepine	600–1200
Clozapine	100–900
Diclofenac	75–200
Troglitazone	600*
Zomepirac	200–600

2 Experimental

2.1 Materials

2.1.1 Radiolabeled compounds

^{3}H-acetaminophen (RP: 99%; SA: 679 kBq/nmol; RC: 9.5 MBq/mL), ^{3}H-amodiaquine (RP: 99%; SA: 421 kBq/nmol; RC: 41.43 MBq/mL), ^{14}C-carbamazepine (RP: 99%; SA: 4.7 kBq/nmol; RC: 24.7 MBq/mL), ^{14}C-troglitazone (RP: 98%; SA: 1.5 kBq/nmol; RC: 8.04 MBq/mL) and ^{3}H-zomepirac (RP: 99%; SA: 2226 kBq/nmol; RC: 26.46 MBq/mL) were all synthesized in Isotope Chemistry, in-house, AstraZeneca R&D (Mölndal, Sweden). ^{14}C-diclofenac (RP: 97%; SA: 4.7 kBq/nmol; RC: 4.2 MBq/mL) and ^{14}C-clozapine (RP: 99%; SA: 2.0 kBq/nmol; RC: 5.5 MBq/mL) were synthesized in-house, AstraZeneca R&D (Wilmington, Sweden). **Figure 11** shows the labeling position for the compounds used.

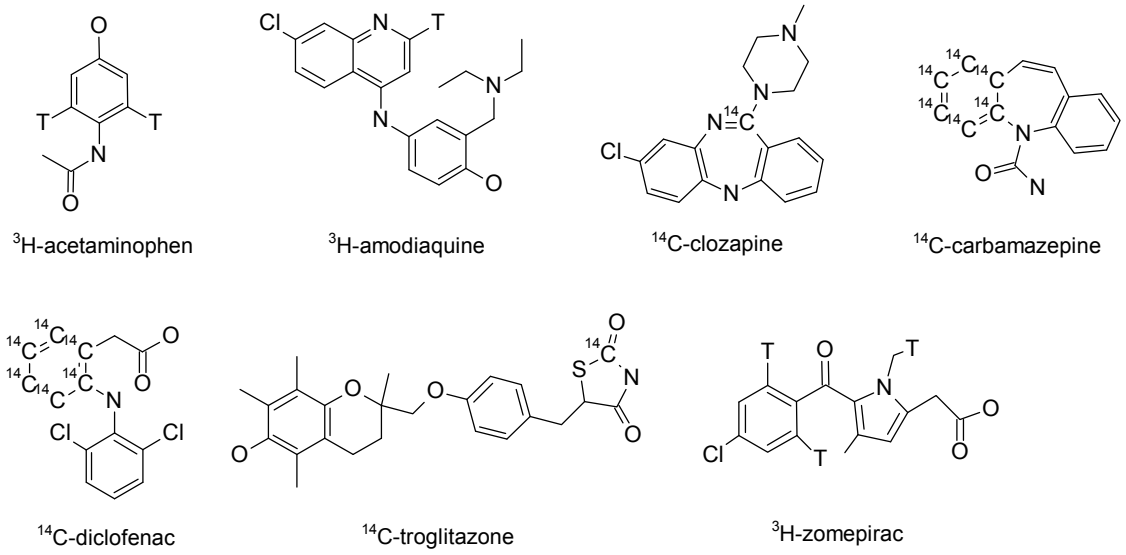


Figure 11. Structures of the radiolabeled compounds used in the incubations, T = tritium.

2.1.2 Non-labeled compounds

Amodiaquine dihydrochloride dihydrate ($C_{20}H_{22}ClN_3O \cdot 2HCl \cdot 2H_2O$) zomepirac sodium ($C_{15}H_{13}ClNO_3Na$) and acetaminophen ($C_8H_9NO_2$) were all purchased from Sigma Aldrich Chemie, (Steinheim, Germany).

2.1.3 Incubation chemicals

The hepatocytes were purchased from Celsis/IVT (Chicago, USA). Hepatocyte incubation medium (hepatocyte suspension medium, HSM) was prepared from Williams Medium E purchased from Sigma Aldrich Chemie, (Steinheim, Germany), 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) purchased from In VitroGen, (Paisley, UK) and *L*-Glutamine (in-house, AstraZeneca R&D Mölndal, Sweden). Easycoll Separating Solution was purchased from Biochrom AG (Berlin Germany). 10 × Hanks Balanced Salt Solution (10 × HBSS) was purchased from In VitroGen, (Paisley, UK). The HLMs were purchased from BD Biosciences (Erembodegen, Belgium). β-nicotine amide adenine dinucleotide phosphate, reduced (NADPH) was purchased from Sigma Aldrich Chemie, (Steinheim, Germany). $K_xH_yPO_4$ buffer 0.1 M, pH 7.36 (Kphos buffer) was prepared in-house, AstraZeneca R&D (Mölndal, Sweden) from K_2HPO_4 and KH_2PO_4, both purchased from Merck (Darmstadt, Germany).

2.1.4 Additional chemicals

Other chemicals used were acetone purchased from Rathburn Chemicals (Walkerburn, Scotland), formic acid 98-100% (FA) purchased from Merck (Darmstadt, Germany), and methanol HPLC grade (MeOH) purchased from Fisher Scientific (Loughborough, UK). acetonitrile LC-MS Chromasolv® (ACN), dimethylsulphoxide Hybri-max® (DMSO) and sodium dodecyl sulphate (SDS) were all purchased from Sigma Aldrich Chemie, (Steinheim, Germany). Purified water (H_2O) was produced using ELGA Purelab Ultra system.

2.2 Instrumentation

2.2.1 LC-MS

The LC-MS analysis of the hepatocyte incubations was performed with a Waters Acquity UPLC™ in series with a Waters Synapt HDMS. The LC-MS analysis of the HLM incubations was performed with a Waters Acquity UPLC™ in series with a Waters LCT Premier™ XE. The data analysis was done using Waters MassLynx and Metabolynx software.

2.2.2 Liquid scintillation counting

The liquid scintillation counting was carried out on a Wallac 1409 Liquid Scintillation Counter (Turku, Finland). The scintillation liquid used was Optiphase ´Hi safe´ 2 purchased from PerkinElmer Life and Analytical Sciences (Shelton, USA)

2.2.3 Cell Harvester

Harvesting was carried out using a Brandel Cell Harvester model ML-48TI and Whatman GF/B (fired) filter paper, both purchased from Biomedical Research and Development Laboratories (Gaithersburg, USA).

2.2.4 Absorbance spectrophotometer and protein determination kit

For the absorption measurements a Spectra MAX 190 plate reader from Molecular Devices (Workingham, UK) was used. The implemented software was Softmax® Pro 4.8. For the protein determination procedure a BCA™ Protein Assay kit containing albumin standard

ampoules (bovine serum albumin at 2 mg/mL in 0.9% saline and 0.05% sodium azide), "reagent A" (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide) and "reagent B" (4% cupric sulphate) was purchased from Pierce Biotechnology (Rockford, USA).

2.3 Experimental procedures

2.3.1 Hepatocyte incubation procedure

HSM was prepared by mixing 5 mL (200 mM) *L*-glutamine in water with 12.5 mL (1 M) HEPES and 482.5 mL Williams Medium E, the pH was set to 7.4. Cryopreserved hepatocytes were thawed and prepared to a 1 000 000 cells/mL solution according to **Appendix 1**. The radiolabeled compounds ^{3}H-acetaminophen, ^{3}H-amodiaquine, ^{3}H-zomepirac (all 100 µM) were prepared according to **Appendix 2**. The radiolabeled compounds ^{14}C-clozapine, ^{14}C-diclofenac, ^{14}C-troglitazone and ^{14}C-carbamazepine (all 100 µM) were prepared according to **Appendix 3**. To a 24 well Corning® Costar® flat bottomed cell culture plate 360 µL (1 000 000 cells/mL) hepatocyte solution was added and preincubated for 6 minutes at 37°C, under a 5% CO_2 atmosphere. To initiate the incubation 40 µL (100 µM) preheated (37°C) radiolabeled compound was added to give a final compound concentration of 10 µM. ^{3}H-amodiaquine,^{14}C-carbamazepine, ^{14}C-clozapine, ^{14}C-diclofenac and ^{14}C-troglitazone were incubated for 2 h in 37°C, under a 5% CO_2 atmosphere. ^{3}H-acetaminophen and ^{3}H-zomepirac were incubated for 4 h in 37°C, under a 5% CO_2 atmosphere. All incubations were carried out in triplicates. The incubation conditions are summarized in **Table 2**.

Table 2. Summarized incubation conditions for hepatocyte incubations.

Final compound concentration	10 µM
Final cell concentration	900 000 cells/mL
Incubation volume	400 µL
Final DMSO concentration	0.1 vol-%
Final ACN concentration	1 vol-%

2.3.1.1 Preparation of aliquots for LC-MS analysis

2.3.1.1.1 Zero minute (t_0) sample

To produce a t_0 sample for the LC-MS analysis 45 µL (1 000 000 cells/mL) preincubated hepatocytes was quenched with 150 µL ice-cold ACN containing 0.2 vol-% FA followed by an addition of 5 µL (100 µM) compound. The quenched solution was put in the freezer (-20°C) for 20 minutes and then centrifuged for 20 minutes at 3220 *g*, 4°C. From the centrifuged solution 80 µL supernatant was withdrawn and added to 80 µL H_2O.

2.3.1.1.2 Other time points

To quench the incubation for LC analysis at other time points 50 µL aliquot was taken from the incubation and quenched with 150 µL ice-cold ACN containing 0.2 vol-% FA. The quenched solution was put in the freezer (-20°C) for 20 minutes and then centrifuged for 20 minutes at 3220 *g*, 4°C. From the centrifuged solution 80 µL supernatant was withdrawn and added to 80 µL H_2O.

2.3.1.2 Sample preparation for covalent binding determination

To quench the incubation for covalent binding quantification, 200 µL of the incubation mix was added to 300 µL acetone, this was then vortexed for 30 seconds, followed by an addition of 500 µL acetone and a 60 second vortex. The quenched incubation was then placed in the refrigerator (8°C) for 1 h. To prepare for the harvesting step the refrigerated solution was centrifuged for 5 minutes at 50 *g* followed by a brief vortex (3 seconds).

2.3.1.3 Negative controls for covalent binding

Each compound was incubated in triplicates and for each triplicate a parallel back added incubation was performed as follows; Hepatocyte solution (360 µL, 1 000 000 cells/mL) were incubated in the absence of test compound. After the designated incubation time passed 180 µL aliquots were removed and added onto 300 µL acetone. The resulting mixture was then vortexed for 30 seconds, followed by an addition of 500 µL acetone and a 60 second vortex. At this point 20 µL (100 µM) compound was added. The quenched back added incubation was then placed in the refrigerator (8°C) for 1 h. To prepare for the harvesting step the refrigerated solution was centrifuged for 5 minutes at 50 *g* followed by a brief vortex (3 seconds).

2.3.2 HLM incubations

The microsomal incubations in the presence and absence of NADPH were carried out with ^{3}H-amodiaquine (10 µM final concentration) prepared according to **Appendix 4**. HLMs (20 mg/mL) were thawed and diluted to 5 mg/mL with Kphos buffer. Kphos buffer (340 µL, 100 mM, pH 7.36), 100 µL HLM (5 mg/mL), 10 µL (500 µM) ^{3}H-amodiaquine was added to a 96 well Nunc DeepWell™ plate. The plate was preincubated for 6 minutes at 37°C, with shaking at 0.5 *g*. To initiate the incubation 50 µL (10 mM) preincubated NADPH was added. For the non-NADPH incubations, 50 µL preincubated H_2O was added instead of NADPH. The solutions were incubated for 60 minutes. The incubations were performed in sets of 12, i.e. 12 NADPH and 12 non-NADPH incubations. The incubation conditions are summarized in **Table 3.**

Table 3. Summary of incubation conditions for HLM incubations.

Final compound concentration	10 µM
Final HLM concentration	1 mg/mL
Incubation volume	500 µL
Final NADPH concentration	1 mM
Final DMSO concentration	0.1 vol-%
Final ACN concentration	1 vol-%

2.3.2.1 Preparation of aliquots for LC-MS analysis

2.3.2.1.1 Zero minute (t_0) sample

To produce t_0 samples for the LC-MS analysis a 45 µL aliquot from the preincubated incubation mix was quenched with 50 µL ice-cold ACN containing 0.2 vol-% FA. An addition of 5 µL NADPH (10 mM) to one set and 5 µL H_2O to the other produced a t_0 for both the NADPH and non-NADPH incubations. The quenched solutions were centrifuged for 20 minutes at 3220 *g*, 4°C. From the centrifuged solutions 50 µL supernatants were withdrawn and added to 50 µL H_2O.

2.3.2.1.2 Sixty minute (t_{60}) sample

To quench the incubation for LC analysis at 60 minutes a 50 µL aliquot was taken and quenched with 50 µL ACN containing ice-cold 0.2 vol-% FA. The quenched solutions were centrifuged for 20 minutes at 3220 *g*, 4°C. From the centrifuged solutions 50 µL supernatant was withdrawn and added to 50 µL H_2O.

2.3.2.2 Sample preparation for covalent binding determination

To quench the incubation for covalent binding quantization 200 µL aliquots of the incubation mixtures was added onto 800 µL acetone, this was then vortexed for 30 seconds. The quenched incubations were then placed in the refrigerator (8°C) for 1 h.

2.3.2.3 Negative control for covalent binding

The ^{3}H-amodiaquine was incubated in two sets of 12 (12 NADPH and 12 non-NADPH). For each of these 12 incubations a parallel back added incubation was performed (i.e. 12 back added) by first preincubating a mixture of 340 µL Kphos buffer (pH 7.36) and 100 µL HLM (5 mg/mL), then adding 50 µL NADPH (10 mM) and incubating for 60 minutes. To quench the back added incubations for covalent binding quantification a 200 µL aliquot of the back added incubation mixture was added onto 800 µL acetone, this was then vortexed for 30 seconds. Finally 4 µL ^{3}H-amodiaquine (500 µM) was added to the quenched incubations. The quenched back added incubations were then placed in the refrigerator (8°C) for 1 h.

2.3.3 Harvesting and solubilization

The quenched incubation mixtures were harvested using the Brandel harvester onto a filter paper and washed with 20 mL MeOH (80 vol-%) in H_2O. The filter paper was transferred to a 20 mL glass scintillation vial and 1 mL SDS (5 weight-%) in H_2O was added. The scintillation vial was then put on a shaking water bath set at 55°C for 20 h.

2.3.4 Liquid Scintillation Counting

To a 10 mL scintillation vial 250 µL of the SDS solution was added. Scintillation liquid (5 mL) was added to the scintillation vial which was then shaken manually to achieve mixing. The vial was left standing until no bubbles were seen in the liquid and then run in the liquid scintillation counter.

2.3.5 Protein determination

A standard concentration curve was constructed for the protein determination starting from a 2 mg/mL albumin capsule (Stock). The solution used as diluent was pooled SDS solutions from solubilized filter paper from incubations done with incubation media (HSM) only. The dilutions are demonstrated in **Table 4**.

Table 4. Dilution scheme for standard curve for protein determination. Stock is a 2 mg/mL albumin solution.

Solution No	End conc [µg/mL]	Volume [µL]	Solution	Solution conc [µg/mL]	Diluent [µL]
7	250	100	Stock	2000	700
6	200	600	No 7	250	150
5	150	550	No 6	200	183
4	100	500	No 5	150	250
3	75	500	No 4	100	167
2	50	425	No 3	75	212
1	25	300	No 2	50	300

The analysis samples were drawn from the corresponding SDS solutions; a blank sample drawn from the pooled diluent was also used. For each blank / standard concentration point / sample 25 µL was added in triplicates to a 96 well Corning® Costar® flat bottomed cell culture plate. Working reagent (200 µL), prepared by mixing reagent A and reagent B in a 50:1 volume ratio, was added to each well. The plate was incubated for 30 minutes, at 37°C, with shaking at 0.5 *g*. The absorbance measurement was performed at λ = 540 nm after the plate was left standing to cool to 22°C.

2.3.6 LC-MS analysis of hepatocyte incubations

The hepatocyte LC-MS analysis was done using a Waters Acquity UPLC™ in series with a Waters Synapt HDMS. Ionization was carried out in positive mode using electro spray technique (ES^+); V-mode was used for the mass spectral analysis. The cone and capillary voltage was 35 V and 3000 V respectively. The LockSpray™ reference system for compensation of mass and environmental variations used a leucine-enkephalin solution (^{12}C peak m/z = 556.2771) with a flow of 40 µL /min. The employed column was a 2.1 × 50 mm, 1.7 µm diameter Waters Acquity UPLC™ BEH C18 column. The sample injection volume was 5 µL. The mobile phase consisted of 0.1 vol-% FA in H_2O (A) and ACN (B).

Amodiaquine samples were analyzed with the gradient shown in **Table 5**, all other samples from incubated compounds were analyzed with the gradient shown in **Table 6.** Run time was 7 minutes.

Table 5. LC mobile phase used for amodiaquine in the hepatocyte and HLM incubation analysis.

Time	A [%]	B [%]
0	98	2
6	65	35
6.01	10	90
6.71	98	2

Table 6. LC mobile phase used in the hepatocyte incubation analyses with the exception of amodiaquine.

Time	A [%]	B [%]
0	95	5
6	65	35
6.01	10	90
6.71	95	5

2.3.7 LC-MS analysis of amodiaquine HLM incubations

LC-MS analysis of samples from incubation of amodiaquine with HLM was performed on a Waters Acquity UPLC™ in series with a Waters LCT Premier™ XE. The ionization was carried out in positive mode using electro spray technique (ES^+); W-mode was used for the mass spectral analysis. The sample cone and capillary voltage was 35 V and 3000 V respectively. The LockSpray™ reference system for compensation of mass and environmental variations used a leucine-enkephalin solution (^{13}C peak m/z = 557.2802) with a flow of 40 µL /min. The employed column was a 2.1 × 50 mm, 1.7 µm diameter Waters Acquity UPLC™ BEH C18 column. The sample injection volume was 15 µL. The mobile phase consisted of 0.1 vol-% FA in H_2O (A) and ACN (B), the gradient is shown above in **Table 5.** Run time was 7 minutes.

2.4 Calculations

The covalent binding calculations are based on the results from the liquid scintillation counting (described in **Section 1.2.5** and **Section 2.3.4**) and protein determination (described in **Section 1.2.6** and **Section 2.3.5**).

2.4.1 Liquid scintillation counting

The output obtained from the liquid scintillation counting was expressed as DPM. The concentration of covalently bound compound (C_{CB}) is expressed in mol equivalent/L SDS.

The calculations were performed as follows; First the DPM read-out from the scintillation counting (DPM_{scint}) was back calculated to DPM in the SDS solution (DPM_{SDS}) by relating the volumes used. This is shown in **Equation 2**.

$$DPM_{SDS} = DPM_{scint} \times \frac{V_{SDS}}{V_{scint}} \quad \textbf{(2)}$$

where

V_{SDS} = volume SDS used in the solubilization [L]

V_{scint} = volume SDS solution used in the liquid scintillation counting [L]

After that DPM_{SDS} is converted to Becquerel in the SDS solution (Bq_{SDS}) using **Equation 3**.

$$Bq_{SDS} = DPM_{SDS} \times k_c \quad \textbf{(3)}$$

where

k_c = 0.0166834 = conversion factor DPM → Bq

In the next step the Bq_{SDS} was related to the specific activity of the compound (SA) to quantify the concentration labeled compound in the SDS solution (C_{CBiso}) according to **Equation 4**.

$$C_{CBiso} = \frac{Bq_{SDS}}{SA} \quad \textbf{(4)}$$

where

SA = the specific activity of the compound [Bq/mol]

Finally C_{CB} was calculated by taking into account the percentage labeled compound from the preparations, using **Equation 5**.

$$C_{CB} = \frac{C_{CBiso}}{k_{iso}} \quad \textbf{(5)}$$

where

k_{iso} = fraction of labeled compound in the incubation

2.4.2 Protein determination

By comparing the absorbance from the samples with the standard curve a protein concentration ($C_{protein}$) is attained as g protein/L SDS.

2.4.3 Covalent binding

Covalent binding of the incubated samples (CB_{sample}) is calculated by relating the C_{CB} of the incubated samples ($C_{CBsample}$) to $C_{protein}$ according to **Equation 6**.

$$CB_{sample} = \frac{C_{CBsample}}{C_{protein}} \quad \textbf{(6)}$$

Covalent binding of the back added incubations ($CB_{back\ added}$) is calculated by relating the C_{CB} of the back added incubations ($C_{CBback\ added}$) to $C_{protein}$ according to **Equation 7**.

$$CB_{back\ added} = \frac{C_{CBback\ added}}{C_{protein}} \quad \textbf{(7)}$$

To take into account the non-specific binding generated in the incubation when calculating the covalent binding (Net CB), the $CB_{back\ added}$ is subtracted from the CB_{sample} as shown in **Equation 8**.

$$\text{Net CB} = CB_{sample} - CB_{back\ added} \quad \textbf{(8)}$$

3 Results

3.1 Covalent binding from hepatocyte incubations

The covalent binding is presented as a mean of the triplicates ± standard deviation (SD). The covalent binding from the hepatocyte incubations is summarized in **Table 7** and **Figure 12**.

Table 7. Covalent binding from hepatocyte incubations (mean ± SD where available). * (Nakayama, et al., 2009), ** (Usui, Mise, Hashizume, Yabuki, & Komuro, 2009).

Compound	Net CB [pmoleq/mg protein]	$CB_{back\ added}$ [pmoleq/mg protein]	Net CB, literature value [pmoleq/mg protein]	Incubation time [h]
^{3}H-acetaminophen	2.2 ± 0.7	4.4 ± 0.5	8.4 ± 1.5*	4
^{3}H-amodiaquine	72.6 ± 21.0	19.5 ± 0.2	91 ± 6.1*	2
^{14}C-carbamazepine	2.4 ± 0.9	15.6 ± 2.0	18**	2
^{3}H-clozapine	86.0 ± 3.9	6.2 ± 0.7	82.7 ± 7.7*	2
^{14}C-diclofenac	111.7 ± 8.9	9.5 ± 0.9	53 ± 2.6*	2
^{14}C-troglitazone	100.1 ± 8.8	8.6 ± 0.5	118**	2
^{3}H-zomepirac	22.1 ± 1.2	1.1 ± 0.1	7.2 ± 0.4*	4

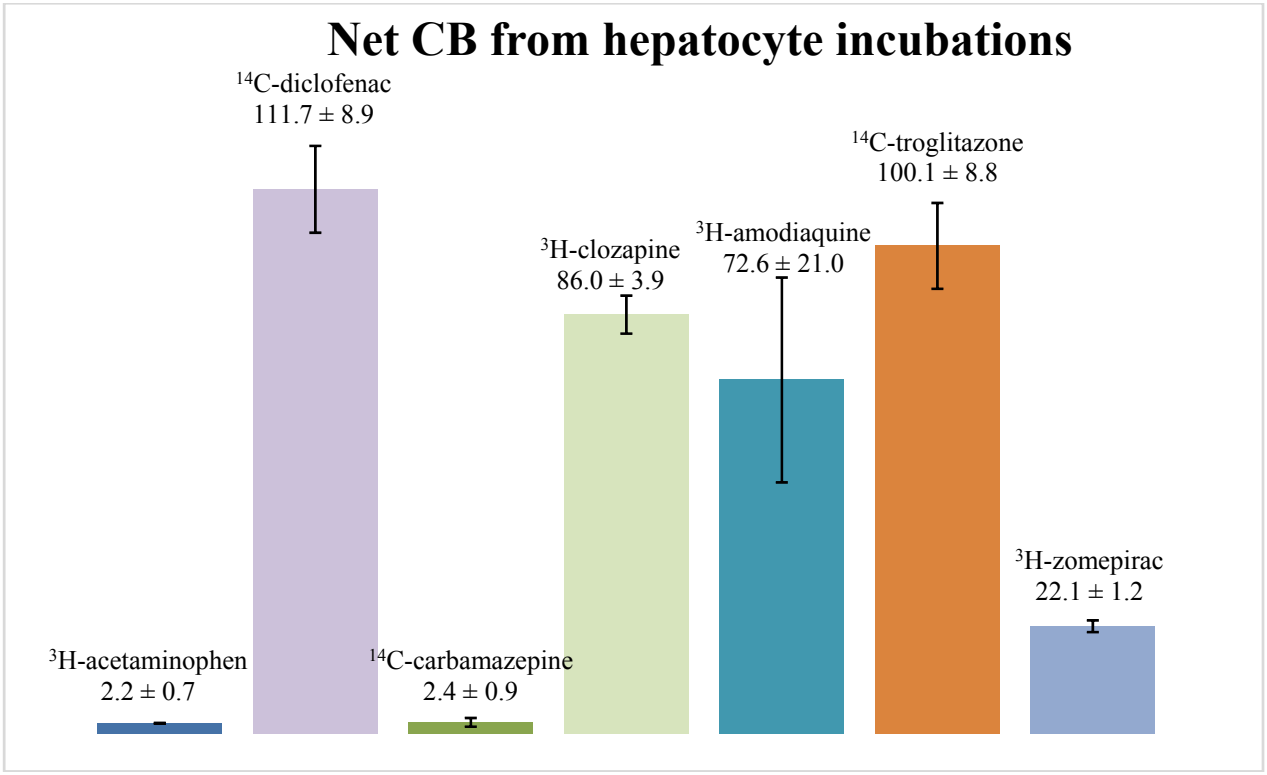


Figure 12. Covalent binding from hepatocyte incubations (mean ± SD).

When covalent binding is put in context with therapeutic daily dose (from **Table 1**) another dimension is added. The resulting logarithmic plot is presented in **Figure 13**.

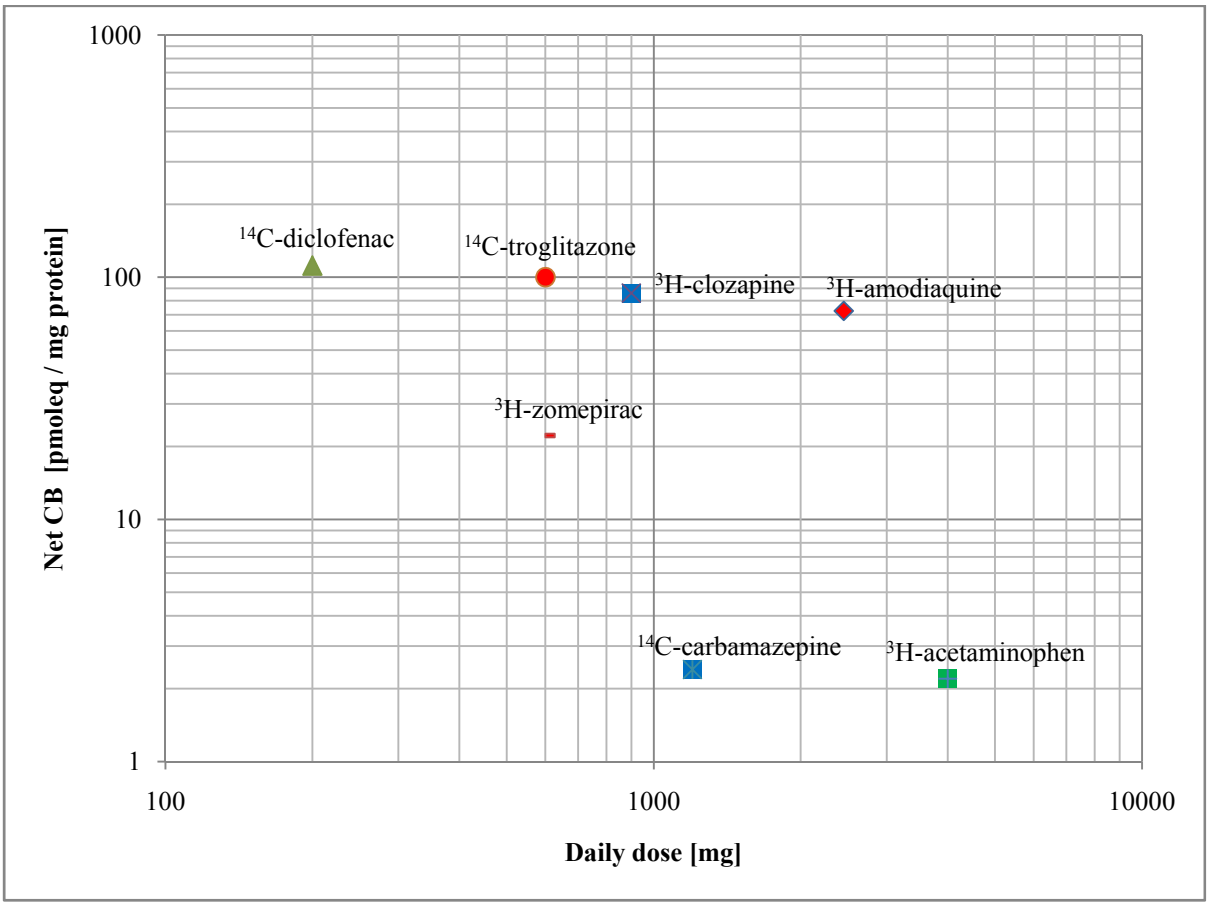


Figure 13. Covalent binding from the hepatocyte incubations vs. daily dose. The highest therapeutic daily dose of the intervals presented in Table 1 is applied. The compounds are colored according to danger level, see Section 1.2.8.

3.2 Covalent binding from HLM incubations

Amodiaquine was the only compound tested in the HLM incubations. Generated covalent binding data are shown in **Table 8.**

Table 8. Covalent binding from HLM incubation of amodiaquine (mean ± SD). * (Nakayama, et al., 2009), the presented literature value is covalent binding from incubations with NADPH present.

Compound	Net CB +NADPH [pmoleq/mg protein]	Net CB –NADPH [pmoleq/mg protein]	$CB_{back\ added}$ [pmoleq/mg protein]	Net CB, literature value [pmoleq/mg protein]
^{3}H-amodiaquine	162.7 ± 23.2	209.6 ± 31.3	65.1 ± 6.8	208.1 ± 13.4*

3.3 Metabolic profiles from incubations

The LC-MS data generated from the hepatocyte and HLM incubation analyses have been processed in Metabolynx and are presented below in the form of extracted ion combined metabolic trace chromatograms. m/z values for the parent ions ($[M+H]^+$) observed for the metabolites are used for proposing structures for the metabolites formed. Numbering of metabolites is based on the integrated area of the chromatograms, largest to smallest.

3.3.1 Extracted ion chromatograms for hepatocyte incubations

3.3.1.1 Acetaminophen

Acetaminophen was not detected in the applied LC-MS system. Due to time constraints optimization of the chromatography and/or mass spectrometry has not been attempted.

3.3.1.2 Amodiaquine

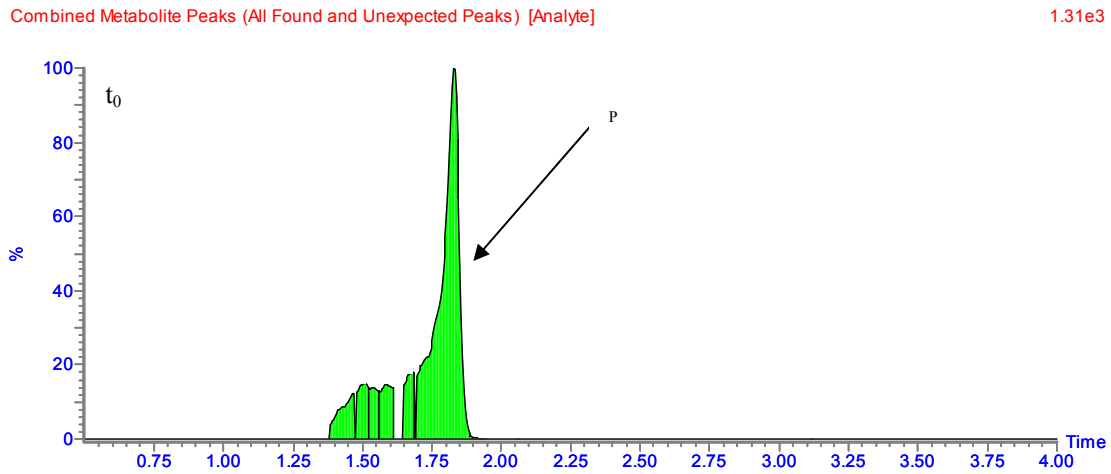


Figure 14. Extracted ion chromatogram of t_0 sample of amodiaquine from the hepatocyte incubation.

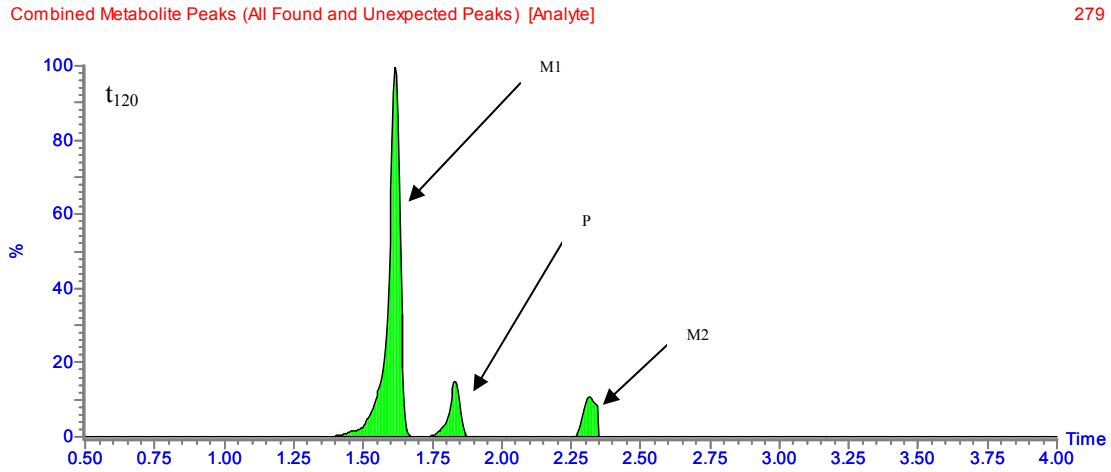


Figure 15. Extracted ion chromatogram of t_{120} sample of amodiaquine from the hepatocyte incubation.

Table 9. Summary of the metabolites of amodiaquine from the hepatocyte incubation.

Metabolite	Retention time [min]	$[M+H]^+$	Biotransformation
P	1.83	356.1537	Parent
M1	1.61	327.1138	Deethylation
M2	2.32	285.0656	Parent – C_4H_9N

3.3.1.3 Carbamazepine

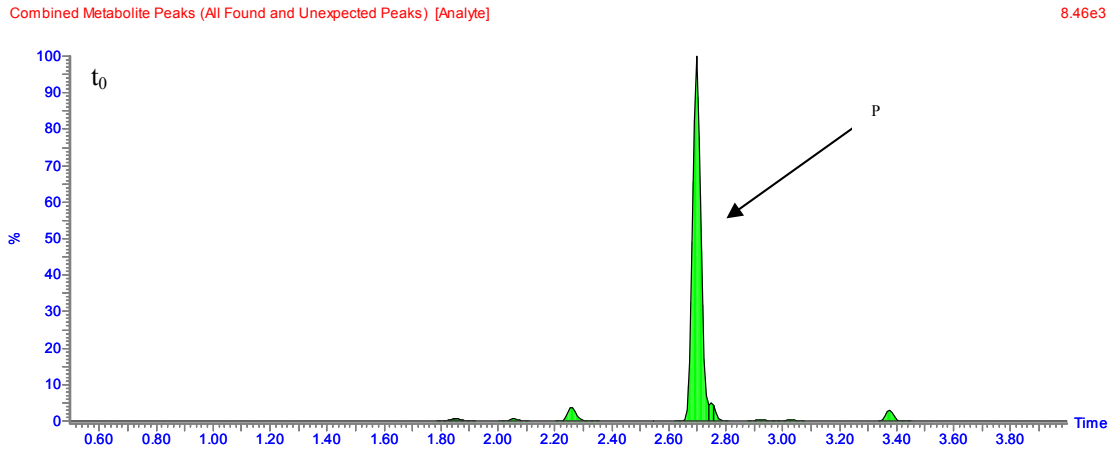


Figure 16. Extracted ion chromatogram of t_0 sample of carbamazepine from the hepatocyte incubation.

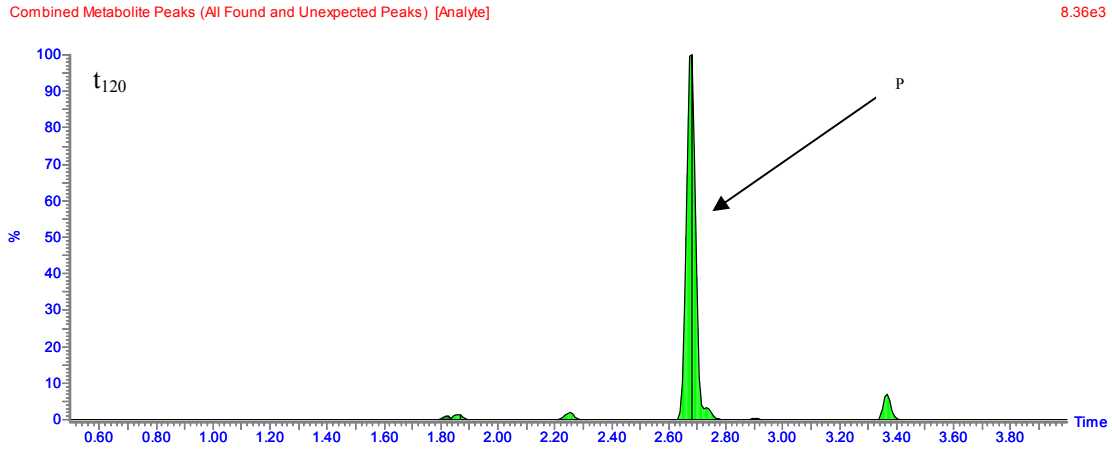


Figure 17. Extracted ion chromatogram of t_{120} sample of carbamazepine from the hepatocyte incubation.

Based on the LC-MS data obtained it is apparent that carbamazepine has not been turned over in the 2 h incubation and therefore did not yield any metabolites.

Table 10. Summary of the metabolites of carbamazepine from the hepatocyte incubation.

Metabolite	Retention time [min]	$[M+H]^+$	Biotransformation
P	2.68	237.0997	Parent

3.3.1.4 Clozapine

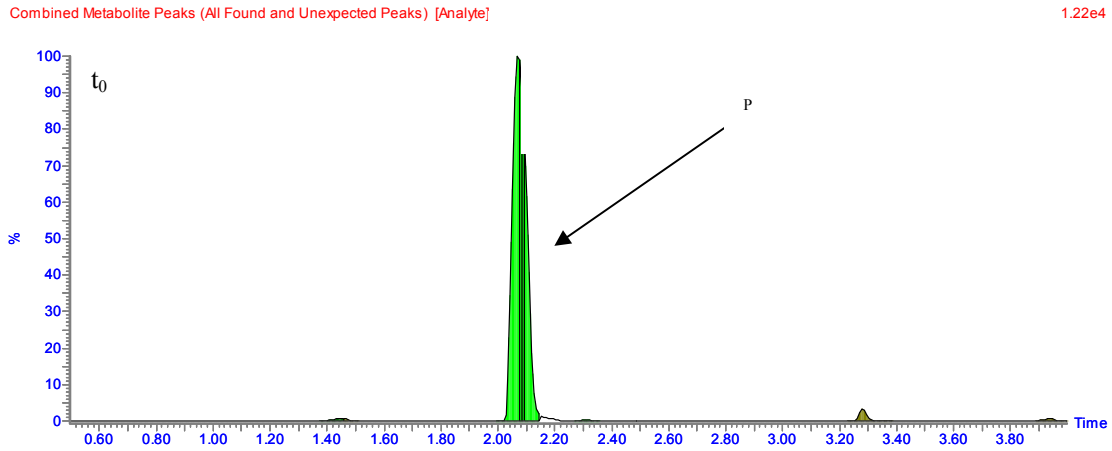


Figure 18. Extracted ion chromatogram of t_0 sample of clozapine from the hepatocyte incubation.

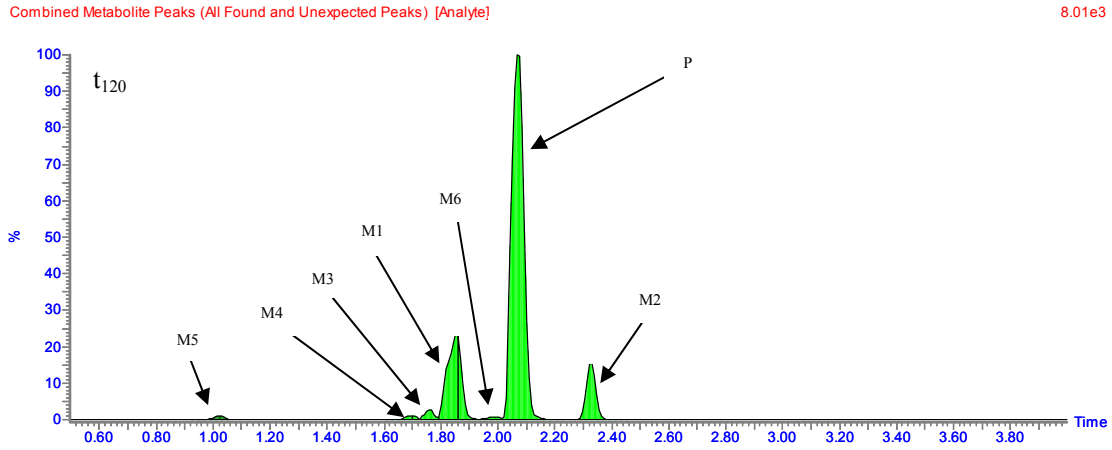


Figure 19. Extracted ion chromatogram of t_{120} sample of clozapine from the hepatocyte incubation.

Table 11. Summary of the metabolites of clozapine from the hepatocyte incubation.

Metabolite	Retention time [min]	$[M+H]^+$	Biotransformation
P	2.08	329.1425	Parent
M1	1.85	315.1240	Demethylation
M2	2.33	345.1345	*N*-Oxide formation
M3	1.85	505.1739	*N*-Glucuronidation
M4	1.69	505.1721	*N*-Glucuronidation
M5	1.02	345.1346	Hydroxylation
M6	1.99	331.1238	Demethylation + hydroxylation

3.3.1.5 Diclofenac

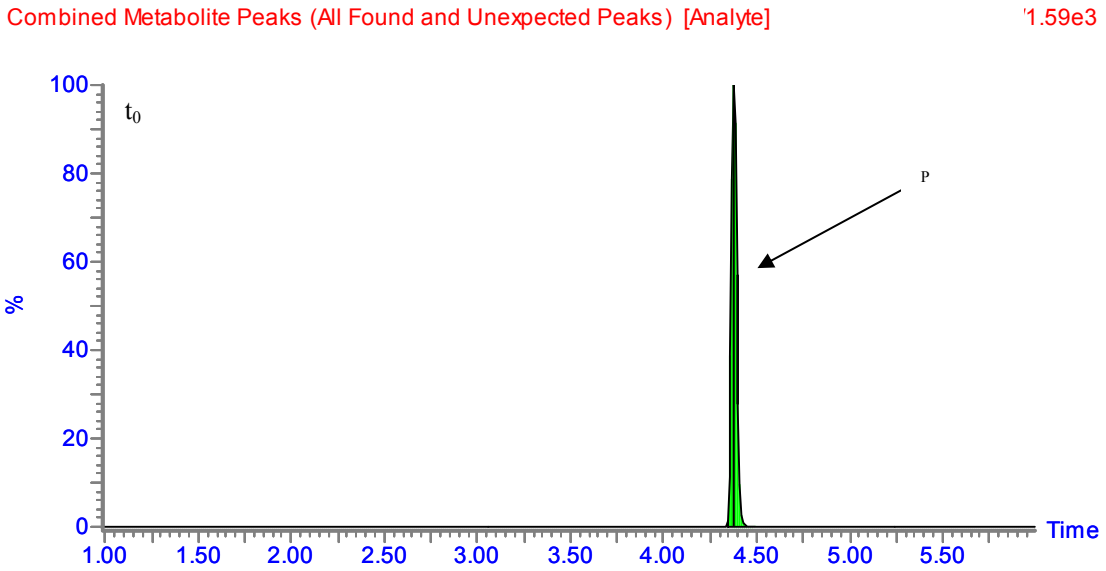


Figure 20. Extracted ion chromatogram of t_0 sample of diclofenac from the hepatocyte incubation.

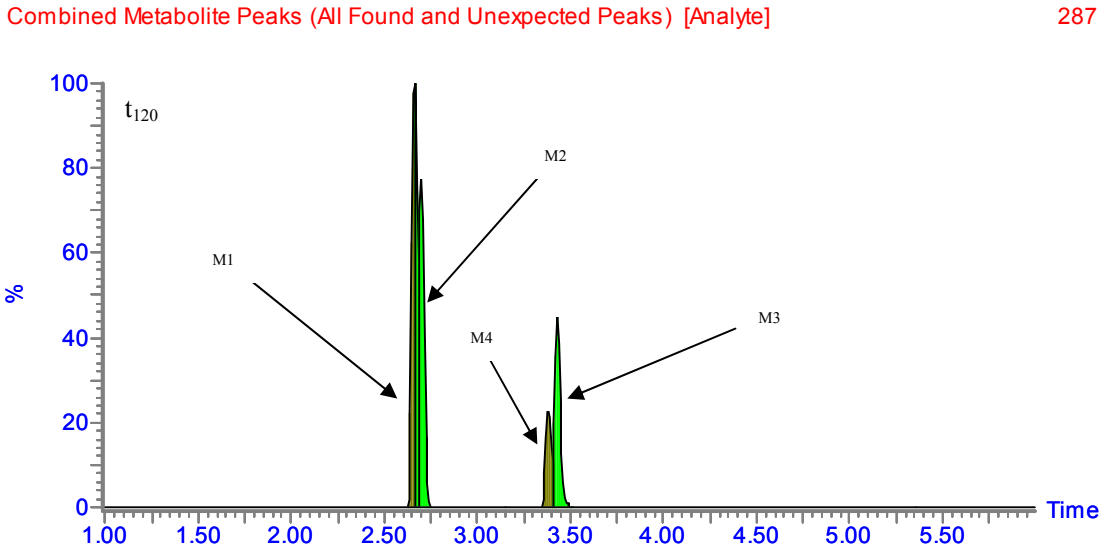


Figure 21. Extracted ion chromatogram of t_{120} sample of diclofenac from the hepatocyte incubation.

Table 12. Summary of the metabolites of diclofenac from the hepatocyte incubation.

Metabolite	Retention time [min]	$[M+H]^+$	Biotransformation
P	4.38	296.0236	Parent
M1	2.67	488.0512	Hydroxylation + glucuronidation
M2	2.70	312.0197	Hydroxylation
M3	3.43	471.0488	Glucuronidation
M4	3.38	335.0155	unknown

3.3.1.6 Troglitazone

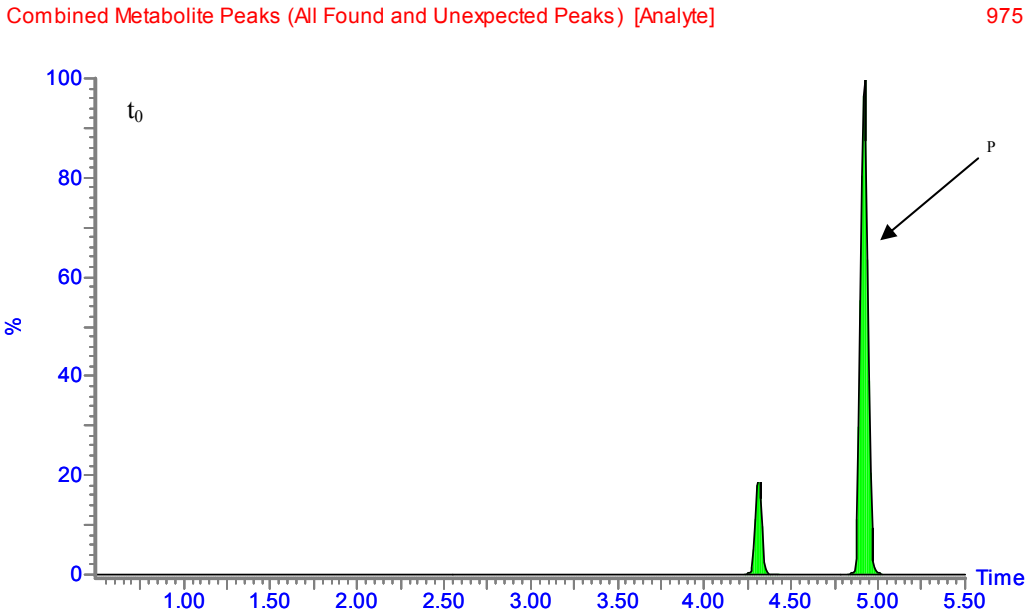


Figure 22. Extracted ion chromatogram of t_0 sample of troglitazone from the hepatocyte incubation.

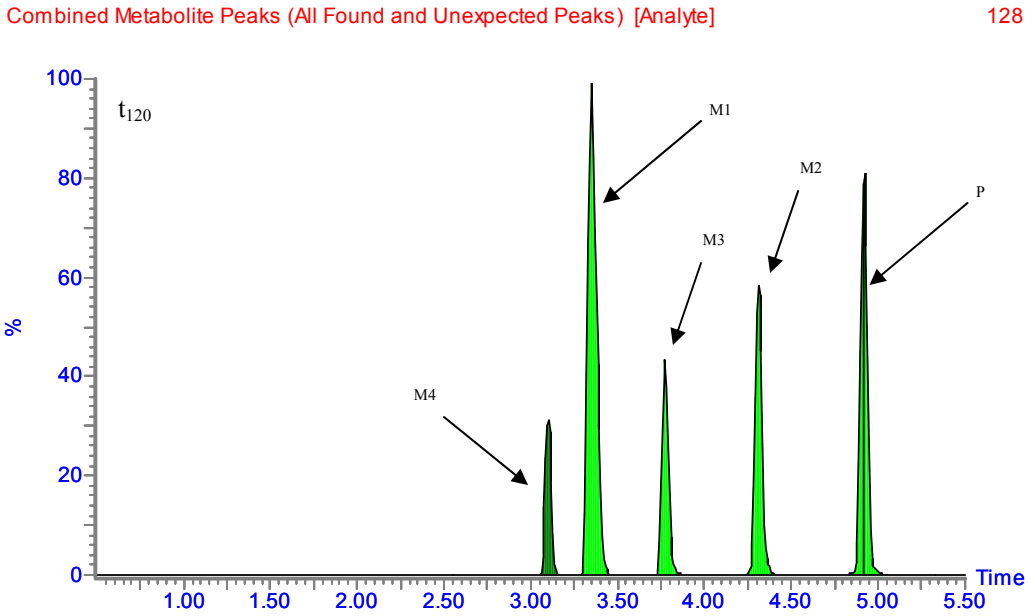


Figure 23. Extracted ion chromatogram of t_{120} sample of troglitazone from the hepatocyte incubation.

Table 13. Summary of the metabolites of troglitazone from the hepatocyte incubation.

Metabolite	Retention time [min]	$[M+H]^+$	Biotransformation
P	4.92	444.1718	Parent
M1	3.35	749.2418	*S*-Glutathione conjugation
M2	4.31	460.1608	Hydroxylation
M3	3.77	460.1742	Hydroxylation
M4	3.10	476.1615	2 × hydroxylation

3.3.1.7 Zomepirac

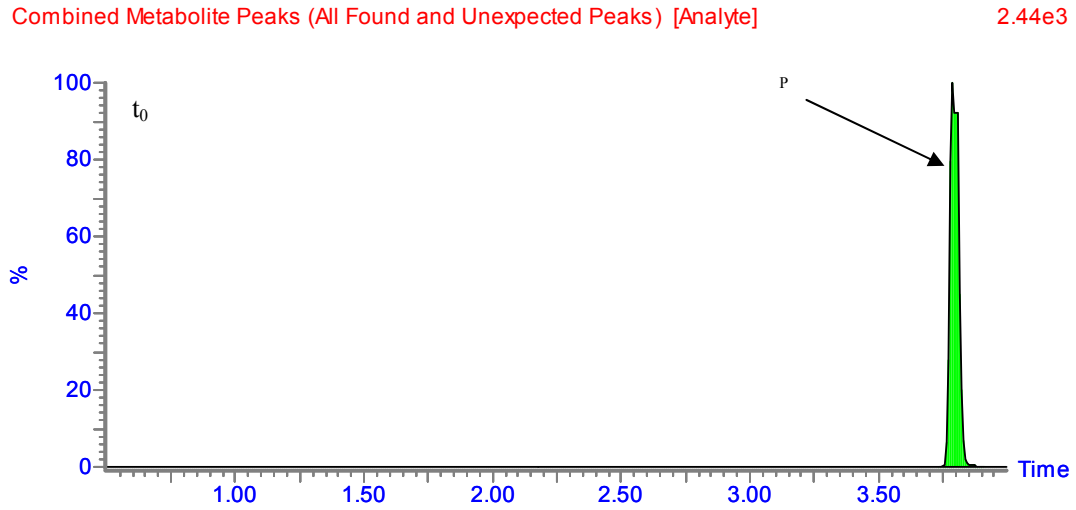


Figure 24. Extracted ion chromatogram of t_0 sample of zomepirac from the hepatocyte incubation.

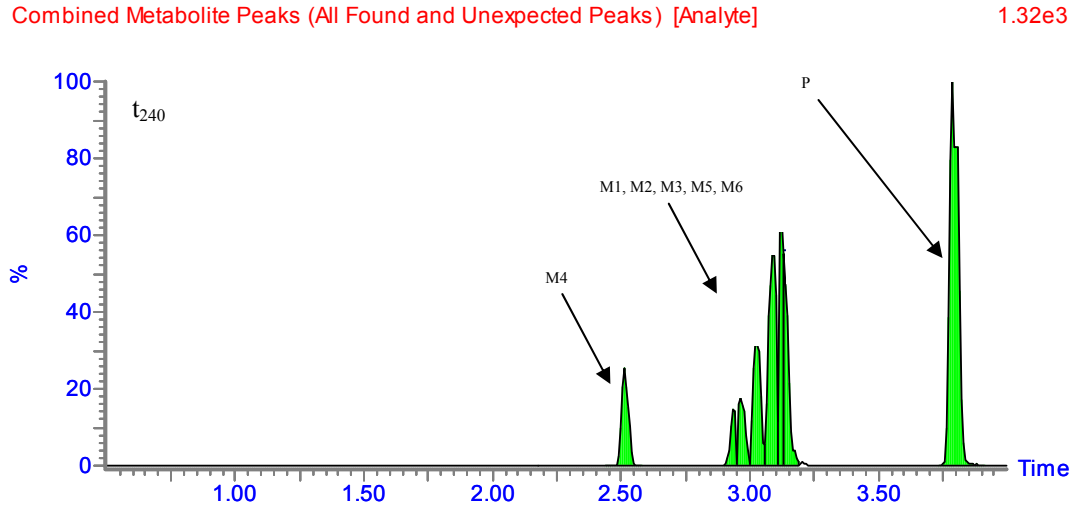


Figure 25. Extracted ion chromatogram of t_{240} sample of zomepirac from the hepatocyte incubation.

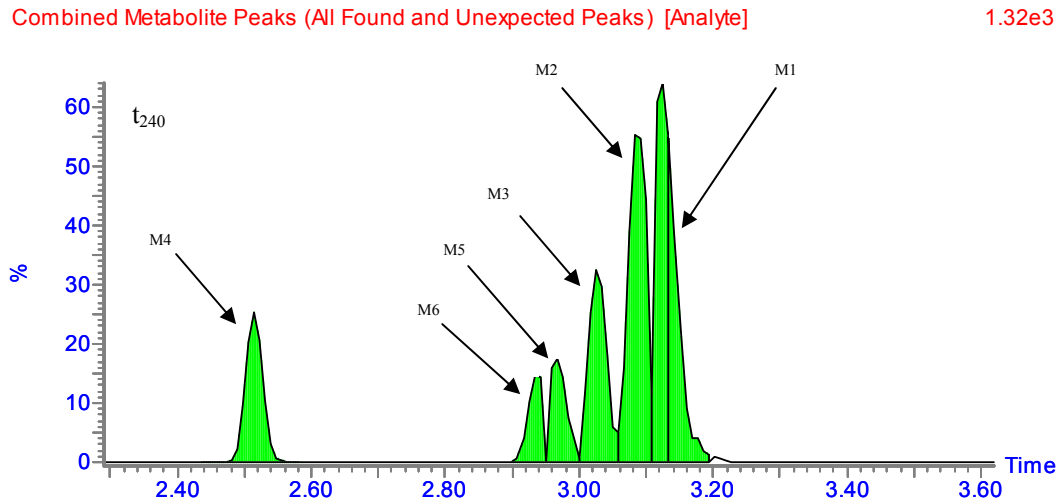


Figure 26. Extracted ion chromatogram of the t_{240} sample of zomepirac from the hepatocyte incubation (retention time 2.30-3.60 minutes is shown).

Table 14. Summary of the metabolites of zomepirac from the hepatocyte incubation.

Metabolite	Retention time [min]	$[M+H]^+$	Biotransformation
P	3.79	292.0724	Parent
M1	3.12	468.1053	Glucuronidation
M2	3.09	468.1059	Glucuronidation
M3	3.03	468.1065	Glucuronidation
M4	2.51	290.0589	Desaturation
M5	2.97	468.1053	Glucuronidation
M6	2.93	468.1067	Glucuronidation

3.3.2 Extracted ion chromatograms for HLM incubations

3.3.2.1 Amodiaquine without NADPH present

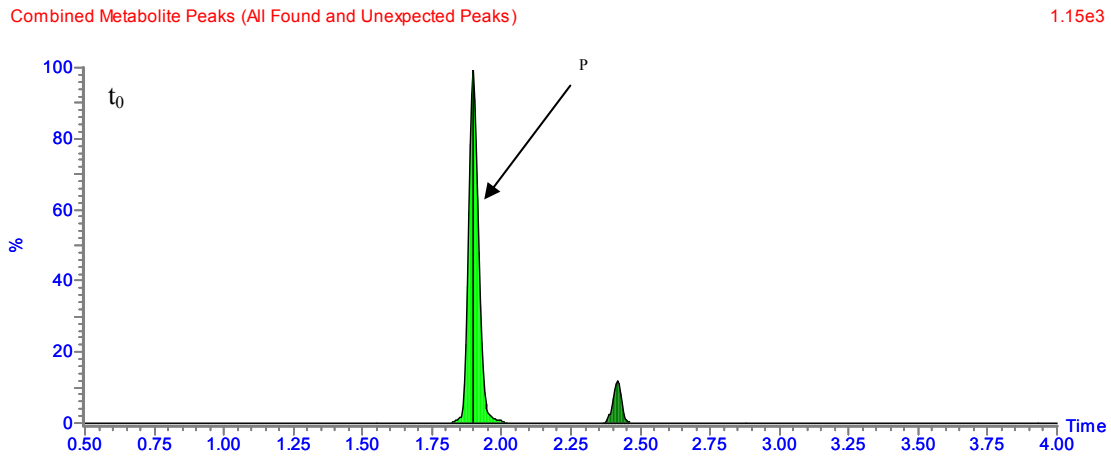


Figure 27. Extracted ion chromatogram of t_0 sample of amodiaquine from the HLM incubation without NADPH present.

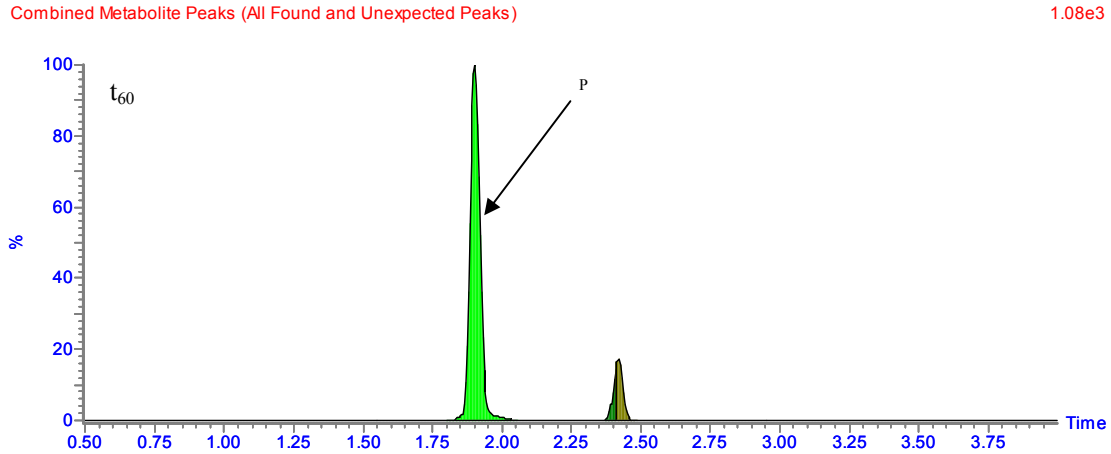


Figure 28. Extracted ion chromatogram of t_{60} sample of amodiaquine from the HLM incubation without NADPH present.

Some disappearance of parent is noted. No detectable metabolites were formed in the incubation without NADPH.

Table 15. Summary of the metabolites of amodiaquine from the HLM incubation without NADPH present.

Metabolite	Retention time [min]	$[M+H]^+$	Biotransformation
P	1.90	356.1519	Parent

3.3.2.2 Amodiaquine with NADPH present

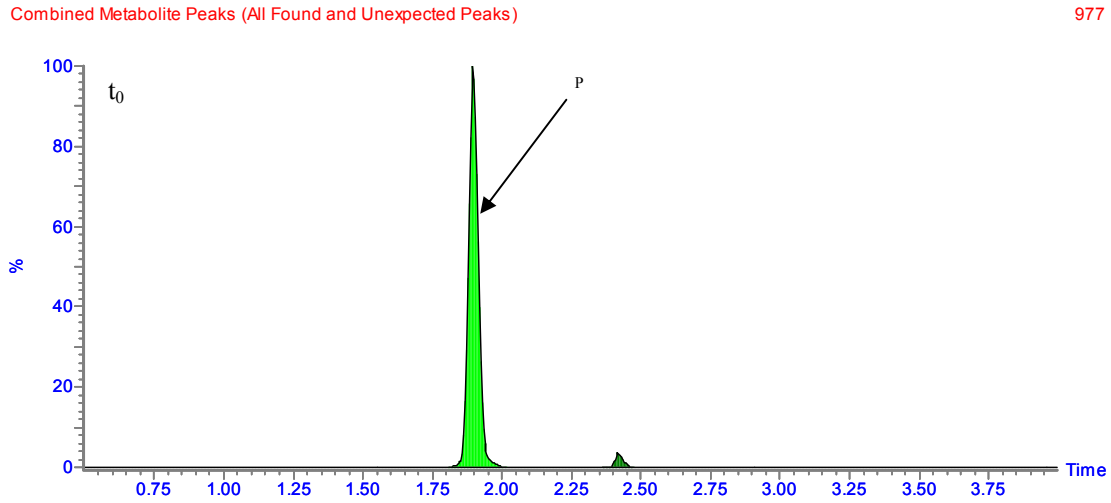


Figure 29. Extracted ion chromatogram of t_0 sample of amodiaquine from the HLM incubation with NADPH present.

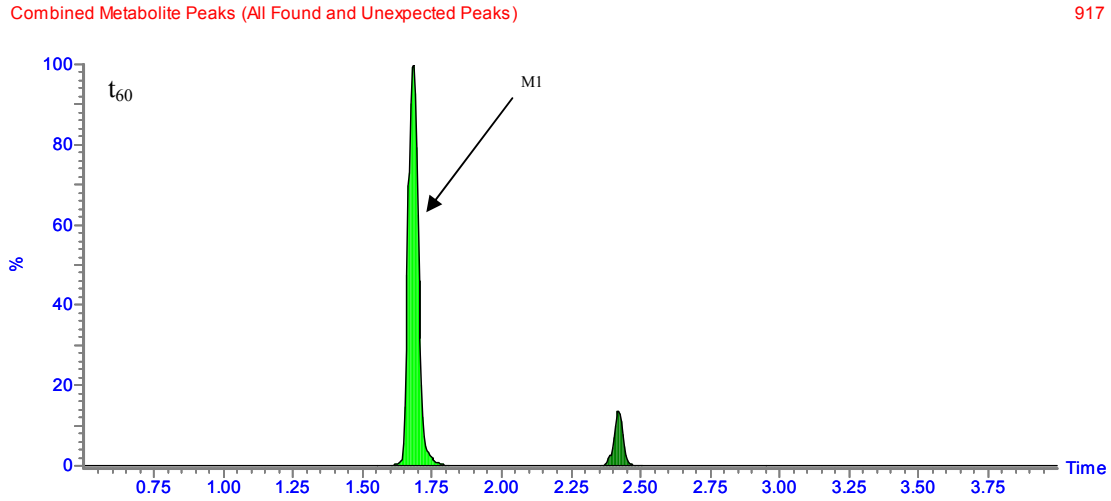


Figure 30. Extracted ion chromatogram of t_{60} sample of amodiaquine from the HLM incubation with NADPH present.

Table 16. Summary of the metabolites of amodiaquine from the HLM incubation with NADPH present.

Metabolite	Retention time [min]	$[M+H]^+$	Biotransformation
P	1.90	356.1519	Parent
M1	1.68	283.0656	Deethylation

4 Discussion

4.1 Hepatocyte incubations

From **Table 7** it can be concluded that the covalent binding from the hepatocyte incubations are for the most part well in line with the literature data reported. With the exception of amodiaquine the hepatocyte incubations show an acceptable deviation within the incubated triplicates which could be seen as a first indication that the assay is robust. The major deviations from the literature are observed for the two carboxylic acids diclofenac and zomepirac, which exhibit a significantly higher covalent binding than what has been reported in the literature, and the low covalent binding compounds carbamazepine and acetaminophen. A possible explanation for the deviation observed for the acid compounds could be due to the difference between the activity of the UGTs present in the hepatocytes in-house vs. the ones used in the previously reported studies. A higher UGT activity could lead to more glucuronidation yielding, in the case of carboxylic acid containing test compounds, acyl glucuronides (**1**, **Figure 31**) (see also **Section 1.2.1.2**). The acyl glucuronides can react with nucleophilic moieties of macromolecules and form covalent adducts which has been reported previously for some of the compounds in the introductory part (**Section 1.2.7**). These macromolecular adducts (**2**, **Figure 31**) are likely to be readily hydrolyzed and are therefore not suspected to be involved in pathways leading to toxicity (Bailey & Dickinson, 2003). The acyl glucuronide could also be cleaved off again by glucuronidases resulting in the release of the unaltered carboxylic acid (aglycone) or undergo an intermolecular rearrangement (acyl group migration). Reaction of these isomerized acyl glucuronides with a nucleophilic amine will make the covalent xenobiotic – macromolecule bond irreversible following the Amadori rearrangement (Bailey & Dickinson, 2003), (Stachulski, Harding, Lindon, Maggs, Park, & Wilson, 2006). The different fates of the acyl glucuronide are shown in **Figure 31**.

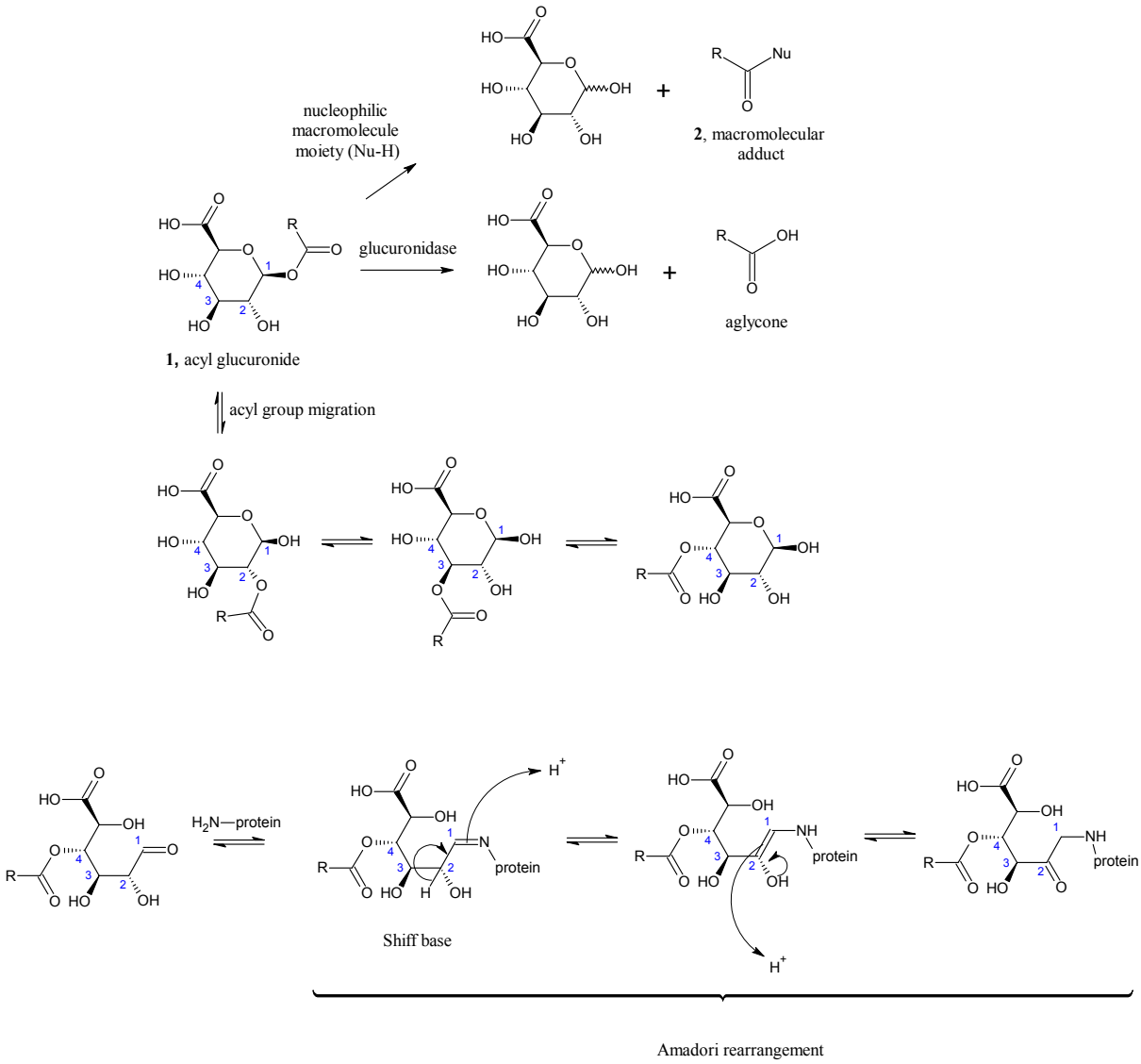


Figure 31. Acyl glucuronide reaction pathways. For the acyl migrated glucuronides, the reaction of the 4' isomer with a biological nucleophile is shown.

The latter pathway referred to as the glycation, would manifest as higher covalent binding in this assay. Carboxylic acid compounds are known to be particularly susceptive to glucuronidation (Benet, et al., 1993). Since all hepatocyte batches will have varying UGT activities it will be important to take into account how this will shift especially carboxylic acid compounds in the zone classification system. One way of achieving this could be to incubate a standard set of compounds known to be efficiently glucuronidated by UGTs and correct the cut off values according to the placement of these compounds. Another approach could be to do activity testing of certain UGTs and evaluate the zones based on these results.

A major difference between the assays presented in the literature (Nakayama, et al., 2009), (Usui, Mise, Hashizume, Yabuki, & Komuro, 2009) and the assay in this project is the washing procedure. For this project a more automated way of washing has been used in the

form of the cell harvester in an attempt to minimize the non-specific binding component. This procedure differs significantly from the manual washing used by Nakayama, et al., 2009 and Usui et al., 2009. The automated washing performed with the cell harvester was satisfactory since the back added values were consistenly low. Some points that remain uncertain regarding the precision of the hepatocyte incubations after this project is the day to day variability, the variability between laborants and the impact of the variation of the hepatocyte batches.

Another factor which is considered important is the incubation time. For carbamazepine it can be concluded from the chromatograms (**Figure 16** and **Figure 17**) that the compound is turned over to a small extent. This raises a question about which incubation time is suitable for which compound. It may be critical to predetermine the turnover for the compounds in the current hepatocyte batch and performing the covalent binding assay with a compound specific incubation time, for instance one half life which may be time and resource demanding.

Based on the produced zone system (**Figure 13**), it seems difficult to distinguish classification zones, however a larger data set (i.e. more compounds tested) may help to separate the zones and assign accurate cut off values. In the zone classification system produced by Nakayama, et al., 2009, diagonal cut off lines have been introduced in the log-log covalent binding–daily dose plot, conceptually presented in **Figure 2.** To construct statistically significant cut off values from these assay results more incubated compounds are needed. One parameter that stands out as particularly unspecific is the daily dose. A quick look at the therapeutic daily doses (**Table 1**) reveals a large variation for most drugs. Acetaminophen for example has a recommended daily dose of 900–4000 mg/day. When an accurate and more complete zone system is in place it should not be viewed as only a classification system for categorizing candidate drugs, but also as a potential tool for approximating safe dosage with respect to covalent binding. If for instance a compound generates a certain amount of covalent binding in the assay and is placed in the zone classification system a maximum dose limit can be determined if the treatment is to remain in the safe zone. This breathes new life in Paracelsus thesis "… the right dose differentiates a poison".

4.2 Metabolic profiles from hepatocyte incubations

The metabolic profiles of the compounds from the hepatocyte incubations (**Figure 14-26** and **Table 9-14**) reveal metabolism of the parent molecule to some extent in all cases except carbamazepine. The results are discussed below.

4.2.1 Amodiaquine

The chromatography of the amodiaquine sample (**Figure 14**, and **Figure 15**) was not optimal. This is likely to be caused by saturation of the column as a result of a substantially higher vol% ACN in the injected sample compared to the mobile phase. This may have disrupted the binding equilibrium between the polar amodiaquine and the C18 column hence causing a fraction of the amodiaquine to elute faster. The two hour incubation resulted in the formation of deethylated amodiaquine as the main metabolite. This is consistent with the literature presented in **Section 1.2.7.2** where desethylamodiaquine is presented as the active component of the drug.

4.2.2 Carbamazepine

From the LC-MS data of carbamazepine (**Figure 16** and **Figure 17**) it is obvious that carbamazepine is turned over to a very little extent which may explain the low covalent binding. Carbamazepine should be attempted in a longer incubation, adjusted for the clearance of the drug.

4.2.3 Clozapine

The chromatograms of clozapine (**Figure 18** and **Figure 19**) reveal some, but far from complete turnover of the parent compound since a substantial amount parent is left after the incubation. The major metabolite found is, consistent with the literature (see **Section 1.2.7.4**), the demethylated clozapine. The second largest metabolite has a $[M+H]^+$ of 345.1345, based on the retention time it is proposed to be an *N*-oxide, which is characteristically eluting after the parent.

4.2.4 Diclofenac

The chromatographic system used for diclofenac works satisfactorily. Diclofenac elutes as a sharp peak with a retention time of 4.38 minutes and is completely turned over during the two hour incubation period. One of the metabolites formed is a hydroxylated diclofenac which is

a finding consistent with the literature (see **Section 1.2.7.5)**. A noteworthy observation from the chromatograms (**Figure 20** and **Figure 21**) is that the largest fraction of the metabolites is glucuronidated diclofenac. This is noteworthy considering the initial discussion (see **section 4.1**) regarding high UGT activity potentially leading to a large amount glucuronides.

4.2.5 Troglitazone

The two hour hepatocyte incubation resulted in high turnover for troglitazone, although parent is still present in the final sample (see **Figure 22** and **Figure 23**). The major metabolite of troglitazone was a glutathione adduct, which was expected as a trapped CYP mediated metabolite (see **Section 1.2.7.6**). Other metabolites were hydroxylated troglitazone, which constituted the largest fraction of the observed metabolites. There is also a suspected secondary metabolite present as the di-hydroxylated troglitazone.

4.2.6 Zomepirac

The chromatograms of zomepirac (**Figure 24-26**) reveal a substantial but not complete parent turnover in the implemented four hour incubation. Assuming equal mass spectrometric response, the major fraction of the metabolites formed is glucuronidated zomepirac, which was expected based on the previous literature findings (see **Section 1.2.7.7**) and from the points made regarding acyl glucuronides in the initial discussion (see **Section 4.1**). The most striking observation with respect to the analysis results of zomepirac is the closely eluting glucuronides showed in **Figure 25** and **Figure 26** which is consistent with the glucuronide isomerization (acyl group migration) discussed in **Section 4.1** and previously reported by Benet, et al., 1993. The metabolites **M1, M2, M3, M5** and **M6** showed in **Figure 26** are likely to be the isomers resulting from the acyl group migration, which would further support that UGT mediated glucuronidation of zomepirac is taking place. The structures of the proposed acyl glucuronide isomers are shown as **1-4**, **Figure 32**. One of the five metabolites shown in **Figure 26** is likely to be an epimer of the isomers in **Figure 32**.

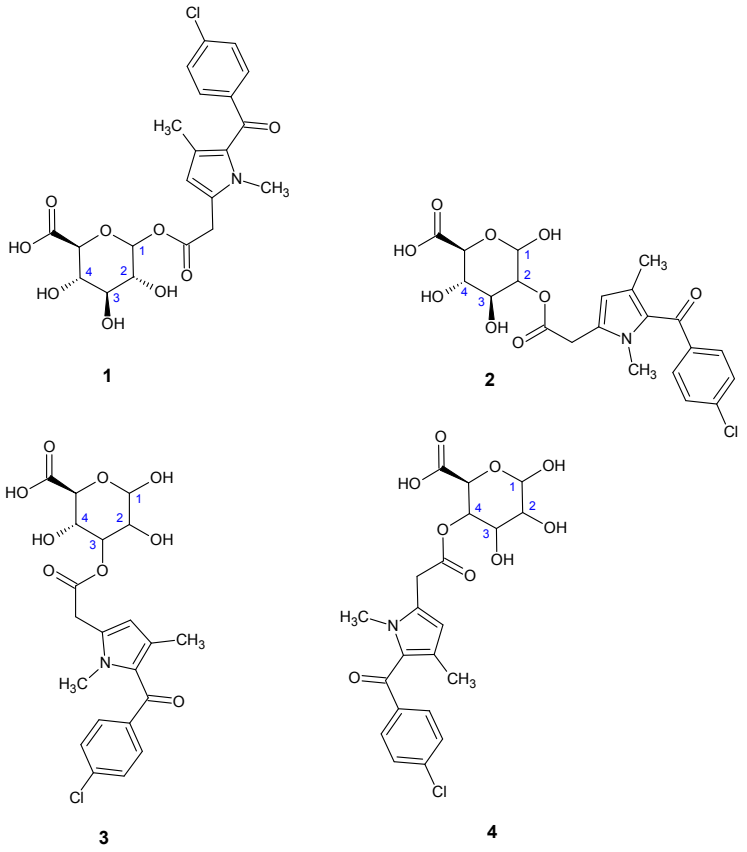


Figure 32. Proposed acyl glucuronide isomers of zomepirac from the hepatocyte incubation.

4.3 Metabolic profiles from amodiaquine HLM incubations

The covalent binding data from the amodiaquine HLM incubation (**Table 8**) shows that the incubation without NADPH yielded a higher amount of covalent binding than the NADPH incubation. Since NADPH is a cofactor to CYPs (see **Section 1.2.1.1**) it is clear that a non-CYP mediated reaction of amodiaquine in the HLM incubation results in more covalent binding than the corresponding incubation with NADPH present. This may be a result of the auto oxidation of amodiaquine to a reactive quinoneimine as reported by Maggs, Kitteringham, Breckenridge, & Park, 1987, shown in **Figure 33**.

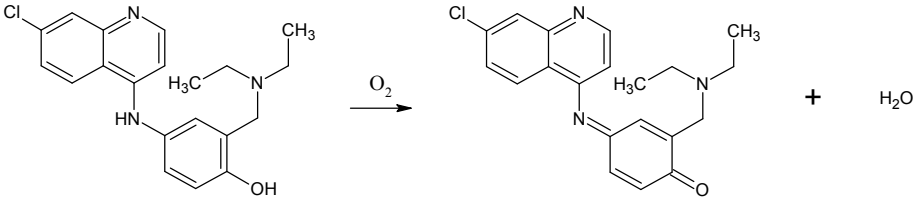


Figure 33. Auto oxidation of amodiaquine to a quinoneimine in aqueous media.

This implies that the parent amodiaquine in the presence of NADPH is being converted by a CYP mediated biotransformation pathway to presumably stable metabolite leading to less covalent binding than the auto oxidation pathway. By visual inspection of the chromatograms of the HLM incubated amodiaquine (**Figure 27-30**) it can be concluded that formation of deethylated amodiaquine does not occur in the incubation without NADPH. In the NADPH incubation however parent amodiaquine has been completely turned over and deethylated amodiaquine is the only observed metabolite. This points to the fact that biotransformation of amodiaquine to deethylated amodiaquine is indeed CYP catalyzed as mentioned in **Section 1.2.7.2.** The low parent turnover in the non-NADPH incubation compared to the complete turnover in the incubation with NADPH present may look surprising when considering the covalent binding data for amodiaquine (see **Table 8**); however a small amount of the auto oxidation product is sufficient to produce the exhibited covalent binding.

Considering that both hepatocyte incubations and HLM incubations seems reproducible with respect to previously reported data it would be interesting to see how a 3-dimensional combined HLM covalent binding vs. hepatocyte covalent binding vs. daily dose zone classification system would turn out. This would essentially create volumes instead of areas in which the compounds can be placed in. With this in mind one can picture additional dimensions added to the system, for instance other *in vitro* models or other species.

5 Conclusions

Several conclusions can be drawn from this project. Since the incubations resulted in obvious parent turnover for a majority of the compounds and the metabolic pattern was in accordance with previously reported metabolites, it is concluded that the hepatocytes and HLMs used were of good vigor and the preparation procedure resulted in well functioning incubations. It is also concluded that the developed *in vitro* covalent binding assay produced results that are in coherence with previously reported covalent binding data for both hepatocyte and HLM incubations. In the case of deviating covalent binding, which was most apparent for the carboxylic acids diclofenac and zomepirac, UGT mediated acyl glucuronide formation is proposed to be a likely cause. This is supported by the metabolic profile of zomepirac which indicates acyl glucuronide formation and isomerization. Although no quantitative acyl glucuronide formation comparison has been (or could be) done with the literature data incubations, the fact that the two carboxylic acids are the only major deviants and that they deviate in the same way (i.e. has higher covalent binding) enhances the suspicion of higher UGT activity in the hepatocytes used in comparison to the hepatocytes used in the studies described in the literature. Regarding the zone classification system it is concluded that cut off values distinguishing the safe from non-safe zones were not assignable. Upon expansion of the incubated compound data set, cut off values may prove possible to assign.

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8 Appendix 1 – Thawing and preparation of cryopreserved hepatocytes.

The following procedure describes how two complementary hepatocyte batches were prepared. Since each hepatocyte tube contained approximately 4 000 000 - 5 000 000 cells and each compound incubation required (6 × 360 + 3 × 45) µL = 2295 µL (1 000 000 cells/mL) hepatocyte solution, one pair of hepatocyte tubes was sufficient to incubate 3-4 compounds.

The two complementary hepatocyte batches IKA and FPF were stored in -80°C in DMSO. They were thawed from -80°C to 37°C in a 37°C water bath and immediately poured into 50 mL HSM. After gentle mixing the solution was centrifuged for 6 minutes at 80 *g*, 22°C. The supernatant was discarded using a vacuum suction device and the pellet was resuspended in 500 µL HSM. 50 mL Easycoll (32 vol-%), prepared by mixing 32 mL HSM, 16.2 mL Easycoll and 1.8 mL 10 x HBSS, was added to the resuspended pellet. The solution was gently mixed and then centrifuged for 15 minutes at 100 *g*, 22°C. Again the supernatant was discarded using a vacuum suction device and the pellet was again suspended in 500 µL HSM. Another 2 mL of HSM was added to achieve an approximated concentration of 3 000 000 – 4 000 000 cells/mL. The amount of cells and cell viability was determined and the concentration was adjusted to 1 000 000 cells/mL with an appropriate addition of HSM.

9 Appendix 2 – Preparation of ^{3}H-labeled compounds for hepatocyte incubations

For ^{3}H-labeled compounds a 100 µM, 200 000 DPM/µL solution was required for the hepatocyte incubations. To achieve this a 500 µM, 1 000 000 DPM/µL solution was prepared by mixing a 10 mM non-labeled compound solution with a labeled compound solution followed by a 20 fold dilution with 50 vol-% ACN in H_2O. This 500 µM 1 000 000 DPM/µL solution was then diluted 5 times with HSM to yield the desired conditions. Therefore the 10 mM solution needed a radioactive concentration of $20 \times 1\,000\,000$ DPM/µL = 20 000 000 DPM/µL. The preparation of ^{3}H-amodiaquine will serve as an example of how all ^{3}H-labeled compounds were prepared.

The received amount ^{3}H-amodiaquine was 100 µL dissolved in ethanol (RC: 41.43 MBq/mL; C: 98.409 nmol/mL) ≅ 4,143 MBq = 2.483×10^{8} DPM. The ethanol was evaporated under a gentle flow of nitrogen gas.

2.483×10^{8} DPM / 20 000 000 DPM/µL = 12.42 µL DMSO was added to yield the correct RC in the 10 mM solution.

n_{tot} = 12.42 µL × 10 mM (= 124.2 nmol) + 98.409 nmol/mL × 0.1 mL (= 9.8 nmol) = 134 nmol total in DMSO solution.

$V = n_{tot} / C$ = 134 nmol / 10 mM = 13.4 µL ⇒ (13.4 – 12.42) µL = 1 µL additional DMSO was added to compensate radiolabeled addition (the small change this makes in the RC is neglected). This gave a 13.4 µL (10 mM, ≈ 20 000 000 DPM/µL) DMSO solution.

Dilution 20 times with 50 vol-% ACN in H_2O (i.e. addition of 255 µL) ⇒ 268 µL (500 µM, ≈ 1 000 000 DPM/µL) solution.

Dilution 5 times with HSM (i.e. addition of 1072 µL) ⇒ 1340 µL (100 µM, ≈ 200 000 DPM/µL) solution

This preparation procedure rendered a 100 µM, ≈ 200 000 DPM/µL solution with 1 vol-% DMSO, 9.5 vol-% H_2O, 9.5 vol-% ACN and 80 vol-% Williams hepatocyte medium. The molar fraction of ^{3}H-amodiaquine was 9.841 / (9.841 + 124.2) = 7.3 mol-%.

Each compound had a different fraction labeled compound depending on its SA.

^{3}H-amodiaquine, 7.3 mol-% labeled; ^{3}H-acetaminophen, 4.2 mol-% labeled and ^{3}H-zomepirac, 1.4 mol-% labeled.

10 Appendix 3 – Preparation of ^{14}C-labeled compounds for hepatocyte incubations

For the ^{14}C-labeled compounds a 100 µM solution was required for the hepatocyte incubations. To achieve this a 500 µM solution was prepared from a 10 mM DMSO solution, by diluting with 50 vol-% ACN in H_2O. This 500 µM solution was then diluted 5 times with HSM to produce the stock conditions.

The volume needed for the incubation was 40 µL × 3 (incubation) + 20 µL × 3 (back added) + 5 µL × 3 (t_0 LC-analysis) = 195 µL. To avoid pipetting small volumes of DMSO the compounds were prepared in excess. The preparation of ^{14}C-diclofenac will serve as an example of how all ^{14}C-labeled compounds were prepared.

The received amount ^{14}C-diclofenac was 71 µL (RC: 4.2 MBq/mL; C: 893.6 nmol/mL) = 63.44 nmol (= n_{tot}) dissolved in ethanol. The ethanol was evaporated under a gentle flow of nitrogen gas.

$V = n_{tot} / C$ = 63.44 nmol / 10 mM = 6.34 µL DMSO was added ⇒ 6.34 µL (10 mM) DMSO was solution.

Dilution 20 times with 50 vol-% ACN in H_2O (i.e. addition of 120 µL) ⇒ 126.34 µL (500 µM) solution.

Dilution 5 times with HSM (i.e. addition of 505 µL) ⇒ 631.34 µL (100 µM) solution

This preparation procedure rendered a 100 µM solution with 1 vol-% DMSO, 9.5 vol-% H_2O, 9.5 vol-% ACN and 80 vol-% HSM.

11 Appendix 4 – Preparation of ^{3}H-amodiquine for HLM incubations

For the HLM incubation of ^{3}H-amodiquine a 500 µM, 1 000 000 DPM/µL solution was required. This was achieved by mixing a 10 mM non-labeled amodiquine solution in DMSO with ^{3}H-amodiquine followed by a 20 × dilution with 50 vol-% ACN in H_2O.

The received amount ^{3}H-amodiaquine was 140 µL dissolved in ethanol (RC: 41.43 MBq/mL; C: 98.409 nmol/mL) ≅ 5.80 MBq = 3.477 $\times 10^{8}$ DPM. The ethanol was evaporated under a gentle flow of nitrogen gas. Non-labeled amodiaquine (29 µL, 10 mM) in DMSO was added.
n_{tot} = 29 µL × 10 mM (= 290 nmol) + 98.409 nmol/mL × 0.14 mL (= 13.78 nmol) = 303.78 nmol total in DMSO solution.
V = n_{tot} / C = 303.78 nmol / 10 mM = 30.4 µL ⇒ (30.4 – 29) µL = 1.4 µL additional DMSO was added to compensate radiolabeled addition (the small change this makes in the RC is neglected). This gave a 30.4 µL (10 mM, ≈ 20 000 000 DPM/µL) DMSO solution.
Dilution 20 times with 50 vol-% ACN in H_2O (i.e. addition of 578 µL) ⇒ 608 µL (500 µM ≈ 1 000 000 DPM/µL) solution.

This preparation rendered a 500 µM, ≈ 1 000 000 DPM/µL solution with 5 vol-% DMSO, 45 vol-% ACN and 45 vol-% H_2O. The molar fraction ^{3}H-amodiaquine was 13.78 / 303.78 = 4.5%.


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