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# Simultaneous determination of *in vitro* CYP-activities in human microsomes using hops as a CYP trial inhibitor. A method development study.

Master's Thesis in Molecular Medicine

Trondheim, June 2016

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# Abstract

Cytochrome P450 (CYP) constitutes a superfamily of hemeproteins that plays a key role in the metabolism of drugs and other xenobiotics. Xenobiotic interactions are a major reason for drug-induced toxicity and multiple drug therapy and/or the concomitant use of herbal medicine is common, particularly in patients with several diseases or conditions. A popular assay to evaluate *in vitro* interactions is the cocktail method using pooled microsomes to simultaneous determine several CYP activities. Hops *(Humulus lupulus)* is a herb often recommended for sleep deprivations and menopausal problems in women.

The aim of this thesis was to develop an *in vitro* cocktail method based on Inje's *in vivo* cocktail to investigate the inhibitory potential of natural remedies and other potential CYP inhibitors toward the CYP enzymes 1A2, 2C9, 2C19, 2D6 and 3A4. The final method was tested using hops as a CYP trial inhibitor.

The conducted pilot study gave indications of substrate concentrations, HLM concentration and incubation time for the cocktail method, and the method was further optimized. Addition of $MgCl_2$, ethanol inhibition and substrate specificity was also investigated. The following conditions were chosen; 20 µM phenacetin, 2 µM losartan, 2 µM omeprazole, 10 µM dextromethorphan and 10 µM midazolam, incubated with 0.4 mg proteins/mL HLM for 20 minutes. Maximum ethanol concentration was 1 %. Substrates and metabolites were analyzed by an adapted and validated LC-MSMS method.

The inhibitory potential of two types of hops, dried hops and the dietary supplement Hops Flowers, were investigated. All five CYP enzymes were inhibited by hops, but to a different extent, evaluated by calculated $IC_{50}$-values. Ethanol extracted hops had lower $IC_{50}$-values compared to water extracted hops. The order of inhibition was as following: CYP2C9 was the most affected, then 3A4, 2C19 or 1A2 and 2D6 being the least affected.

In this thesis a cocktail method was developed, validated and showed to be functional. An LC-MSMS method was adapted and validated. *In vitro* «cocktail» inhibition studies are cheap, effective and relatively easy to perform. Even though extrapolation of *in vitro* data to humans have many limitations, *in vitro* cocktail inhibition studies are useful to get increased knowledge of CYP interactions between herbal and conventional medicines.

# Acknowledgements

The experimental work of this thesis has been carried out under the supervision of Bent Hellum and Odd Georg Nilsen at the Institute for Cancer Research and Molecular Medicine. I would like to thank Bent for his enthusiasm and source of knowledge that has taught me how to think and live like a scientist. I would also like to thank Bent for enduring with me as his office mate, keeping my mood up and motivate me to always perform my best, even when the fridge room scared me and the analytical balance hated me.

This thesis could not have been completed without the supervision and inspiration from Odd Georg Nilsen, one day I hope I have the same amount of knowledge that you do.

I would also like to thank everyone that hangs around the lab and attends the lunch room on a regular basis, Turid Nilsen, Sissel Skarra, Ola Dale, Øyvind Glende and Ida Tylleskär for intriguing discussions and contributing to a good social environment.

A special thanks to my boyfriend, Vegard Naustdal, for always cheering on me, ignoring my bad mood and making dinner after long days. I could not have done this without you (especially since testing the method on hops was your idea).

Trondheim, May 2016

Marita Gustavsen

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# Abbreviations

| | |
|---|---|
| ACE | Acetaminophen |
| ACN | Acetonitrile |
| CAF | Caffeine |
| CV | Coefficient of variation |
| CYP | Cytochrome-P450 |
| DDI | Drug-Drug interaction |
| DEX | Dextrorphan |
| DXM | Dextromethorphan |
| EMA | European Medicines Agency |
| EXP | EXP-3174 |
| FDA | US Food and Drug Administration |
| FA | Formic acid |
| HLM | Human liver microsomes |
| HMP | Herbal medicinal products |
| HPLC | High pressure liquid chromatography |
| $IC_{50}$ | Half maximum inhibitory concentration |
| KPO | Potassium phosphate |
| LLOQ | Lower limit of quantitation |
| LOS | Losartan |
| MeOH | Methanol |
| MID | Midazolam |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometer |
| OHM | 1-OH-midazolam |
| OHO | 5-OH-omeprazole |
| OME | Omeprazole |
| PHE | Phenacetin |
| PXH | Paraxhantine |
| QC | Quality control |
| SD | Standard deviation |
| SJW | St. John’s wort |
| STD | Standard solution |

# 1. Introduction

Cytochrome P450 (CYP) constitutes a superfamily of hemeproteins that plays a key role in the metabolism of drugs and other chemicals that do not occur normally in the body, also known as xenobiotics [1]. CYP enzymes consists of several isoforms and their activities can be decreased or increased by xenobiotics. Xenobiotic interactions, like drug-drug interactions (DDIs) or herb-drug interactions, can occur when two or more drugs/herbs compete for the same enzyme [2]. Xenobiotic interactions are a major reason for drug-induced toxicity making the evaluation of potential interactions a key aspect in the development of new drugs and in toxicological studies.

## 1.1 Human Cytochrome P450

Studies on CYP enzymes can be traced back to the 1940s, and in the following years the importance of the CYP system with regard to metabolism of drugs, steroids and carcinogens were discovered [3].

Cytochrome P450 are named so because they are bound to cell membranes (cyto) and contain a heme group (chrome and P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide [4].

CYPs are found in all five biological kingdoms [5]. As of 2006 there were 6422 P450 enzymes identified in different species [6]. Since CYPs are so diverse, a systematic nomenclature system has been made. CYP enzymes with amino acid sequence homology greater than 40% are placed in the same family, designated by an Arabic numeral. Enzymes with greater than 60% homology are placed in the same subfamily, designated by a letter. The individual enzyme or the isoform is represented by an Arabic number which is assigned on an incremental basis [6]. The 6422 identified enzymes are divided into 708 families. There are 57 CYPs described in man, but only the CYPs in family 1, 2 and 3 appear to be responsible for the phase 1 metabolism of exogenous compounds and CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5 metabolizes 70-80 % of all drugs [7]. The approximate fractions of P450 oxidations on drugs catalyzed by individual P450 enzymes can be seen in figure 1.

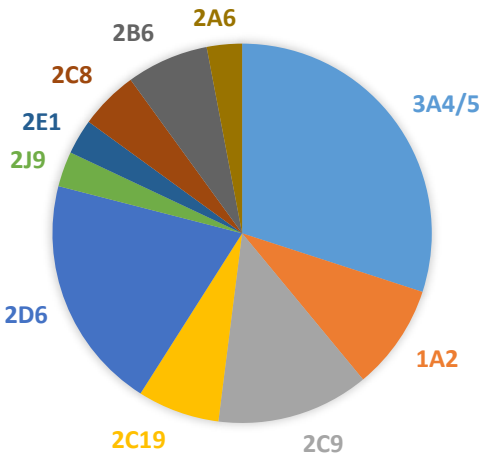


*Figure 1: Diagram showing the approximate fractions of cytochrome P450 oxidations on drugs catalyzed by individual enzymes (adapted from Zanger & Schwab [7]).*

Metabolism of xenobiotics occur many places in the body, including the liver, intestinal wall, lungs, kidneys, and plasma [8]. The liver is the primary site of drug metabolism and function to detoxify and facilitate excretion of xenobiotics by enzymatically converting lipid-soluble compounds, absorbed mainly from the gastrointestinal tract, to more water-soluble compounds, which are excreted into urine or bile [9]. The highest levels of CYP enzymes involved in xenobiotic biotransformation are found in liver endoplasmic reticulum. When eukaryotic cells are crushed in the laboratory, vesicle-like components are formed from the endoplasmic reticulum, called microsomes.

In addition to xenobiotic metabolism, CYPs also contribute to steroid hormone synthesis, vitamin metabolism and the conversion of polyunsaturated fatty acids to biologically active molecules [5, 9]. The concept of conversion of chemicals to reactive products in the body is called bioactivation and might transform a relatively inert chemical to a potential toxic or carcinogenic compound [10]. Examples of this include the oxidation of acetaminophen (paracetamol) by hepatic CYP enzymes to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) that can cause hepatic cell death, or the bioactivation of the procarcinogen benzo[a]pyrene (in cigarette smoke) to the mutagen benzo[a]pyrene diol epoxide by CYP1A1 [11].

Drug metabolism is achieved through phase I reactions, phase II reactions, or both [6, 9]. The most common phase I reaction is mono-oxidation using NADPH (figure 2), which is catalyzed by CYP enzymes and accounts for approximately 75 % of the reactions [3]. Other CYP enzyme oxidations includes hydroxylation, epoxidation (of a double bond), dealkylation,

dehydrogenation and cleavage of esters. CYP enzymes are also capable of catalyzing reduction reactions and isomerizations, and they are thus very versatile [12]. This catalytic versatility gives broad substrate specificity, and explains their involvement in the many biotransformation reactions.

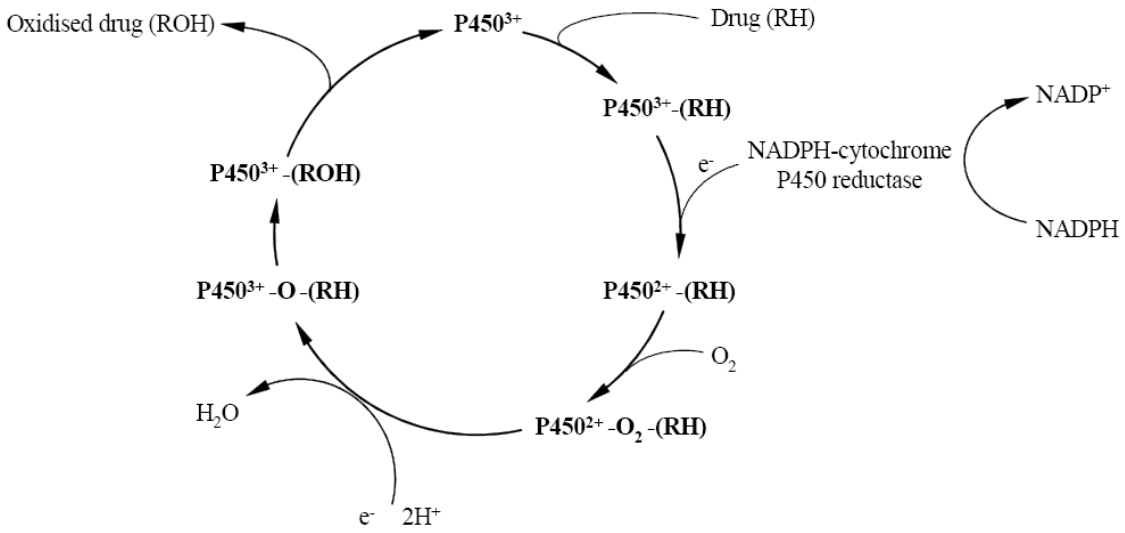


*Figure 2: A simplified catalytic cycle of Cytochrome P450 (adapted from Parkinson [9]).*

The level and activity of each CYP enzyme varies between individuals due to genetic and/or environmental factors and can present a critical issue in medicinal drug therapy [2]. Decreased CYP activity can result from; 1) a genetic mutation that either blocks the synthesis of a CYP enzyme or leads to the synthesis of inactive or unstable enzymes; 2) Exposure to an environmental factor that suppress CYP enzyme expression; or 3) exposure to a xenobiotic that inhibits or inactivates a preexisting CYP enzyme [9]. Increased CYP activity can result from; 1) gene duplication leading to overexpression of the enzyme; 2) Exposure to drugs or other xenobiotics that induce the synthesis of CYP450; or 3) exposure to drugs and other xenobiotics that stimulate the activity of a preexisting enzyme [9].

Inter-individual variation might also be due to genetic polymorphisms, particular for CYP2C9, 2C19 and 2D6. Most members of the CYP families are polymorphic, meaning that the genetic sequence coding in the DNA for a particular enzyme contains stable variations. To be defined as polymorphic, the stable variation must occur in at least 1% of the population. Polymorphisms may alter enzyme activity and can affect drug metabolism [13].

## 1.2 CYP interactions

Drug-drug interactions or, herb-drug interactions, can occur when two or more xenobiotics compete for the same enzyme. Drug interactions can also occur when the CYP responsible for the metabolism of a drug is induced by long-term treatment with a drug or other xenobiotic. Multiple drug therapy and/or the concomitant use of herbal medicine is common, particularly in patients with several diseases or conditions [2]. The likelihood of herb-drug interactions could be higher than drug-drug interactions, as medicinal drugs usually contain single chemical entities, while almost all herbal medicinal products (HMPs) contain mixtures of potentially biological active constituents [14].

The sales of herbs and other natural products have increased dramatically over the last years. With increased acceptance and popularity of HMPs, there is no indications that the trend will stop [15]. As many people buy herbal products on the internet, or in outlets not under governmental control, there is no real overview of either products or sales worldwide. More and more people are taking different herbal medications, believing that the herbs are safe and at least as efficacious as chemical drugs, but without the potential side effects [15, 16].

Herbs can interact with conventional drugs by changing drug absorption, disposition and/or elimination. By inhibiting CYPs, one drug can impair the biotransformation of another, which may lead to an exaggerated pharmacological or toxicological response by the second drug. By induction, one drug can stimulate the metabolism of a second drug and thereby decrease its therapeutic effect [2]. The majority of xenobiotic interactions occurs in the CYP system, the P-glycoprotein mediated efflux pump or through plasma protein binding [9, 14].

The most well-known examples of herb-drug interactions are between St. John's Wort (SJW) and the immunosuppressant cyclosporine, or with oral contraceptives [15, 17]. SJW causes an induction of CYP3A4 *in vivo*, which clinically will lead to faster metabolizing of drugs that are substrates of this enzyme. These drugs might then lose their effect as the increased metabolism might cause sub therapeutic systemic concentrations [16]. For patients combining cyclosporine and SJW, the drug-herb interaction might lead to acute organ rejection episodes, and be fatal [18]. Other herbal remedies reported to modulate drug metabolism *in vivo* (not necessarily in humans) includes garlic, licorice, piperine, and ginseng, among others [14, 16].

## 1.3 Approaches to assess interactions

The cytochrome P450 system plays an important role in the biotransformation and bioactivation of xenobiotics. Recent years have shown that CYPs have an active role in carcinogen metabolism and xenobiotic interactions, as previously described. It is therefore necessary to assess human CYP activity in drug development and/or toxicological studies. Both the American Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have made guidelines for both *in vitro* and *in vivo* interaction studies for development of new drugs and labeling of already existing drugs [19, 20]. However, there are few rules and often little documentation regarding the pharmacokinetics of HMPs.

The assessment of interaction potential employs a variety of models, from basic *in vitro* models to more comprehensive dynamic models, for example physiologically-based pharmacokinetic (PBK) models or clinical studies [19]. Information from studies based on these models help addressing regulatory questions regarding further clinical interaction studies. Negative findings from early *in vitro* and clinical studies can eliminate the need for more extensive clinical interaction studies. If DDIs are discovered in early *in vitro* and *in vivo* studies, further studies should be designed to better quantify the effect and possibly adjust dose regiment or other prescribing modifications. The increased information and discoveries of herb-drug interactions and possible clinical outcomes employs the necessity of more effective methods to assess herb-drug interactions.

### *1.3.1 In vitro models*

*In vitro* approaches are essential because they offer background, as well as anticipatory knowledge, for *in vivo* predictions and are efficient regarding cost and time [21]. Several *in vitro* approaches can be used to assess herb-drug interactions [16]. These approaches may include subcellular fractions, such as liver microsomes, cytosols and homogenates, precision-cut liver slices, isolated and cultured hepatocytes or liver cell lines and cDNA-expressed enzymes [22]. Each of these systems has advantages and limitations. For example, liver microsomes, as used in this study, can be studied long-term, are easily manipulated and optimized, and are ideal for the production of most major metabolites. However, liver microsomes lack certain cofactors like NADPH (nicotinamide adenine dinucleotide phosphate) and UDPGA (urindine diphosphate glucuronic acid) that has to be added to the test system, and

also multiple phase II enzymes that are necessary for studying phase II metabolism reactions [16, 23].

The most appropriate *in vitro* system for biotransformation experiments, is the use of primary hepatocytes. Primary hepatocytes are usually isolated from human liver and therefore contains all the necessary enzymes and cofactors, and is the *in vitro* system that most resembles *in vivo* physiological conditions. The disadvantage with primary hepatocytes is the need for fresh liver and the difficulties of preservation. Cryopreservation techniques have improved, making the use of primary hepatocytes more common and thus more established and characterized as an *in vitro* model, but it is still very expensive and comprehensive to work with [23].

The classical *in vitro* experiment is based on individual incubations and analyses with one CYP enzyme at a time [16, 21], e.g the inhibition study of *Rhodiola rosea* on CYP3A4 [24]. Another *in vitro* method is the cocktail approach, alternatively named n-in-one assay or cassette incubation, which involves the incubation of a substrate mix to monitor several enzyme activities simultaneously [21]. The cocktail approach was initially developed for *in vivo* experiments before being applied to *in vitro* experiments [25, 26]. *In vitro* cocktail approaches are mostly used for inhibition assays using liver microsomes to assess DDI potentials of new chemical entities at early stages of drug development in toxicological and pre-clinical studies [27]. An important aspect of the cocktail approach is that using a high number of substrates increases the difficulty of implementing optimized experimental conditions for all the compounds, and the use of several substrates increase the risk of substrate interactions during incubation [21].

### *1.3.2 In silico methods*

There is an increasing use of *in silico* methods to study CYPs and predict their interactions with xenobiotics [16]. Several examples use a structure-activity relationship analysis to predict how changes in one structure can change the inhibitory effects of a drug. *In silico* methods may be of clinical relevance and significance. For example, knowledge of the substrate specificity and the regulation of CYP is essential for prediction of possible drug interactions.

### *1.3.3 In vivo studies*

*In vivo* interaction studies are usually necessary to provide evidence of findings from *in vitro* studies [16]. Animal studies may give important information on drug interactions, but

interspecies variations when it comes to substrate specificity, catalytic features and amino acid sequences of CYPs, may cause difficulties in extrapolating animal data to humans. Probe substrates and inhibitors can be administered to humans to explore the effects of drugs or herbs on the activity of specific CYP enzymes *in vivo*, e.g. caffeine for CYP1A2. In addition, a cocktail of probe drugs has been used to explore the activities of multiple CYPs, e.g. Inje's cocktail [28]. Inje's cocktail contains caffeine (CYP1A2), losartan (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A4) and were developed as a tool to phenotype *in vivo* enzyme activities of the specific CYPs. These are also the CYPs this thesis will focus on. The biggest advantage of the cocktail approach is the real-time assessment of the activity of various drug-metabolizing enzymes with a single experiment. However, the use of cocktail studies *in vivo* has some limitations, including the mutual interactions between probe drugs, side effects of probe drugs and analytical complexities.

## 1.4 Common CYPs and substrates in interaction studies

The selection of which CYP enzymes and which CYP substrates to use is the first step in any *in vitro* or *in vivo* interaction study. FDA has a list over recommended substrates [19] and an overview of the substrates and the frequencies of use can be studied closer by reading the review article by Spaggiari et al. [21]. A substrate is a good *in vitro* probe when its reaction is highly specific to a particular CYP enzyme and corresponds to a major metabolic pathway. Other important issues regarding cocktail approaches are the enzymatic turnover rate allowing a detectable amount of metabolite, solubility and stability in a cocktail mixture, and interaction potential with other substrates in the mix [27]. This is dependent on substrate and/or enzyme concentration.

### *1.4.1 CYP1A2*

CYP1A2 is involved in the biotransformation of exogenous and endogenous compounds, and in the activation of procarcinogens [6, 29]. The CYP1A subfamily constitutes approximately 15% of the total liver CYP content and metabolizes approximately 20% of all clinically used drugs [7]. CYP1A2 is exclusively expressed in the liver and is essential for the metabolism of various drugs, such as the antipsychotic drug clozapine, the previously used analgesic phenacetin (PHE), the prostate cancer medicine flutamide and the local anesthetic lidocaine.

A common probe substrate for *in vivo* experiments is caffeine (CAF), which is metabolized by N3-demethylation to paraxhantine (PXH) (1,7-dimethylxhantine) by CYP1A2, as illustrated in figure 3 [19, 28]. PHE, on the other hand, is used as probe substrate *in vitro*, but not *in vivo*, because of toxicity in humans [21, 30]. PHE is metabolized by O-deethylation to acetaminophen (ACE), as illustrated in figure 4. Known inhibitors of CYP1A2 include the hepatitis medicine interferon, the antibiotic ciprofloxacin, the histamine receptor antagonist cimetidine and the antiarrhythmic medicine amiodarone. Known inducers of CYP1A2 include broccoli, brussel sprouts, insulin and omeprazole [9]. CYP1A2 is also influenced by both generic and environmental factors, for example are smoking and heavy exercising potent inducers of CYP1A2 [29].

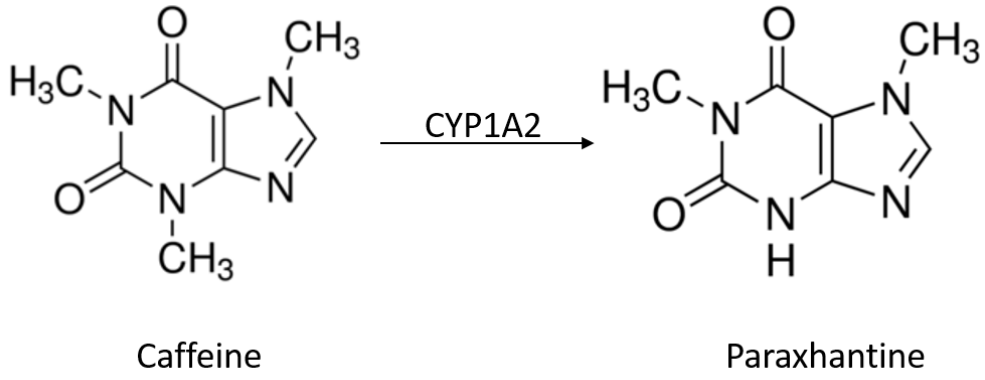


*Figure 3: Molecular structures of the CYP1A2 substrate caffeine and its metabolite paraxhantine.*

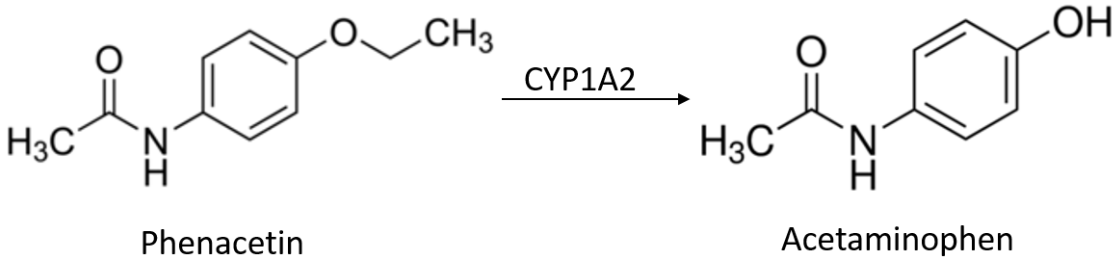


*Figure 4: Molecular structure of the CYP1A2 substrate phenacetin and its metabolite acetaminophen.*

### *1.4.2 CYP2C9*

CYP2C9 is one of the most abundant CYP enzymes in the adult human liver with approximately 20% of the total hepatic CYP content and metabolizes approximately 13 % of all clinical drugs [7, 31], including (S)-warfarin, phenytoin and various nonsteroidal anti-inflammatory drugs (NSAIDs), for example ibuprofen [6, 9]. The anticoagulant warfarin and the anti-epileptic drug phenytoin are of special interest. These two important drugs have a narrow therapeutic window, and a change in CYP2C9 metabolism in humans might influence their systemic concentrations, and lead to therapeutic failure. Phenytoin or the diabetes medicine tolbutamide have traditionally been used for both *in vivo* and *in vitro* experiments [21], but in their development of Inje's cocktail Ryu et al [28] used losartan (LOS) for CYP2C9. LOS is used to treat high blood pressure and is metabolized by CYP2C9 to EXP-3174 (EXP), as illustrated in figure 5. Known inhibitors of CYP2C9 include the antibacterial sulfaphenazole and the antifungal medicine fluconazole. The HMP *Rhodiola rosea* has also been shown to inhibit CYP2C9 in humans [32]. Known inducers include the antiepileptic medicine phenobarbital and the antibiotic rifampin [9].

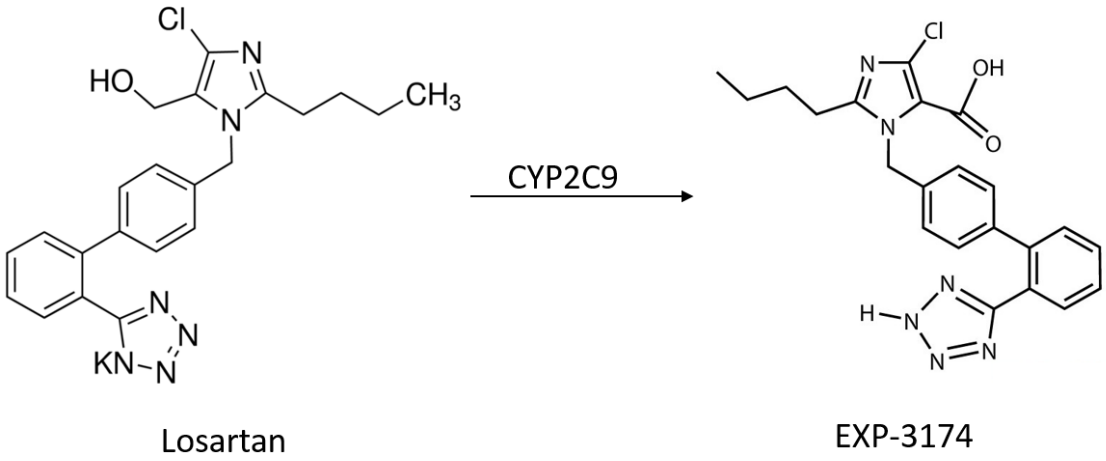


*Figure 5: Molecular structure of the CYP2C9 substrate losartan and its metabolite EXP-3174.*

### *1.4.3 CYP2C19*

CYP2C19 metabolizes approximately 7% of clinical drugs [7], most with a high degree of stereospecificity, like racemic mephenytoin, and proton pump inhibitors, for example omeprazole (OME) (preferably the R-enantiomer) [9]. Probe substrates used both for *in vivo* and *in vitro* experiments include racemic mephenytoin, used as anticonvulsant, the sedative

diazepam and the antidepressant imipramine, however, the most widely used is OME [6]. OME is used to treat gastroesophageal reflux and is metabolized by CYP2C19 to 5-OH-omeprazole (OHO) by a 5-hydroxylation reaction, as illustrated in figure 6. There are relatively few known clinically inhibitors of CYP2C19, the most significant being antidepressants (selective serotonin reuptake inhibitors). Inducers of CYP2C19 include phenobarbital and rifampin [9].

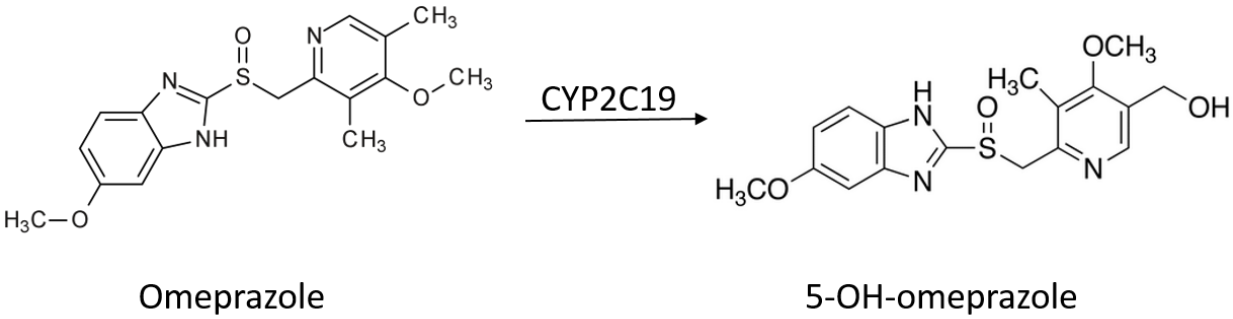


*Figure 6: Molecular structure of the CYP2C19 substrate omeprazole and its metabolite 5-OH-omeprazole.*

### *1.4.4 CYP2D6*

CYP2D6 constitutes approximately 5% of the total liver CYP content, but metabolizes approximately 20% of clinical drugs [7, 33]. Clinically relevant substrates metabolized by CYP2D6 include the analgesics codeine and morphine, the antidepressant imipramine, and the beta-blocker propranolol [9]. The preferred *in vitro* substrates includes the beta blocker bufuralol and dextromethorphan (DXM) [21]. The cough suppressant DXM are metabolized by CYP2D6 to dextrorphan (DEX) by an O-demethylation reaction, as illustrated in figure 7. Known inhibitors of CYP2D6 include the antidepressants buproprion and fluoxetine, the drug addiction detoxifier methadone, and the antiarrhythmic agent quinidine. CYP2D6 is generally regarded as non-inducible [9].

CYP2D6 is highly polymorphic and the enzymatic function is absent in approximately 7% of the Caucasian population. Persons lacking CYP2D6 activity are called poor metabolizers (PM). PMs clear CYP2D6 substrates more slowly and the plasma concentration of these drugs might therefore get increased, giving rise to unwanted adverse effects. For the analgesic opioid codeine and the anti-cancer drug tamoxifen, which CYP2D6 metabolizes to its active metabolites, PMs will not respond to the treatment very well, as the therapeutic response is low. Another CYP2D6 polymorphism have the genotype that leads to ultra-rapid metabolizing (UM)

of CYP2D6 substrates. Approximately 3% of the Caucasian population have the UM-genotype. UM subjects clear the body of the drug more rapidly, leading to sub therapeutic plasma levels of the drug. For codeine metabolism, this means that codeine will be metabolized to morphine more rapidly, and individuals may experience symptoms of morphine overdose [33, 34].

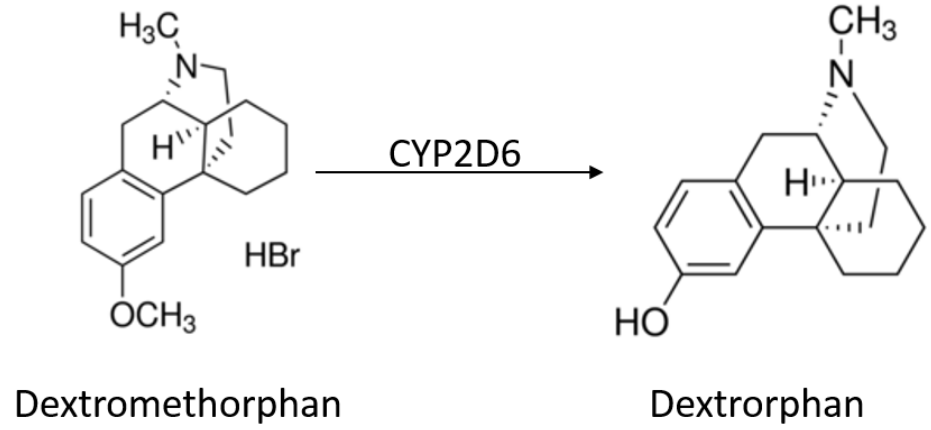


*Figure 7: Molecular structure of the CYP2D6 substrate dextromethorphan and its metabolite dextrorphan.*

### *1.4.5 CYP3A4*

The most abundant CYP enzymes in humans belong to the CYP3A subfamily. CYP3A4 is expressed in the liver and in the small intestines, where it biotransforms an array of steroid hormones and xenobiotics [9]. It has been estimated that CYP3A4 metabolizes about 30-50% of all drugs, giving rise to a large number of clinically relevant drug-interactions [7]. Several CYP3A4 metabolized drugs have been withdrawn from the market because of discovered drug-drug interactions, for example the antihistamine agent terfenadine. Coadministration of terfenadine and the antifungal agent ketoconazole led to fatal ventricular arrhythmias in some patients. Inhibiting agents of CYP3A4, like ketoconazole, resulted in an excessive increase in plasma concentration of terfenadine, that could give fatal outcomes, and thus terfenadine was withdrawn from the market [2].

Other examples of interactions include the inhibition of intestinal CYP3A4 by furanocoumarins in grapefruit juice, which might cause a large increase in absorption of drugs that otherwise undergoes extensive first-pass metabolism [35]. The induction of CYP3A4 by St. John's wort can result in the loss of therapeutic effect of oral contraceptives, anti-HIV medicine and immune suppressants, as described above [17].

The list of clinically relevant substrates for CYP3A4 is long and, in addition to the drugs already mentioned, it also includes the calcium antagonist felodipine, the HIV-medicine indinavir, the anesthesia ketamine and midazolam (MID), quinidine, the antibiotic rifabutin, and testosterone [9]. *In vitro* experiments uses both MID and testosterone frequently [21] while MID is the preferred probe substrate for *in vivo* experiments and is metabolized to 1-OH midazolam (OHM) by a 1'-hydroxylation reaction, as illustrated in figure 8 [6]. Known inhibitors of CYP3A4 include ketoconazole, indinavir and the calcium channel blocker verapamil. Known inducers, in addition to SJW, include rifampin and phenobarbital. [9].

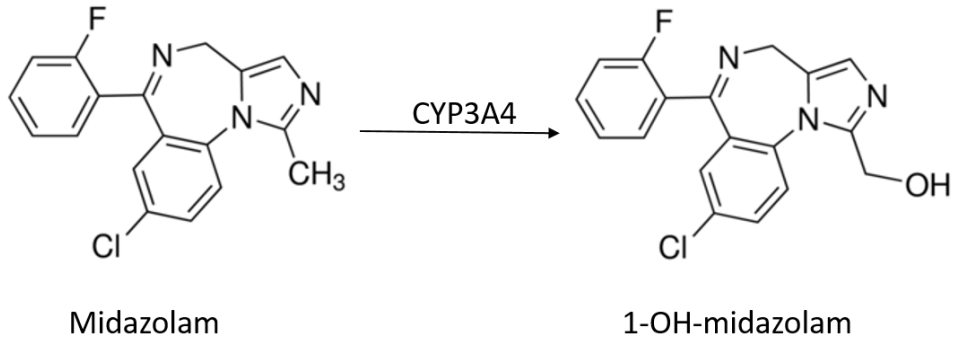


*Figure 8: Molecular structure of the CYP3A4 substrate midazolam and its metabolite 1-OH-midazolam.*

## 1.5 Analytical approaches to interaction studies

The importance of CYP interaction studies has created a need for high throughput analytical methods. Classical *in vitro* analytical methods, investigating one CYP enzyme at a time, use high pressure liquid chromatography (HPLC) with ultraviolet (UV), radiometric, fluorescence and luminescence detections. These classical analytical methods have several limitations including detection of specific substrates, analytical interference, costs and safety issue [21]. The high throughput HPLC coupled with mass spectrometry (LC-MS) offers some advantages over these classical methods, such as the ability to use common drugs as selective CYP substrates, often called probes. Because of the selectivity, specificity and robustness of LC-MS, the cocktail approach has become increasingly popular for monitoring several CYP activities in a single experiment [36, 37].

### *1.5.1 Basic HPLC theory*

Chromatography is an analytical separation technique that separates compounds in time before the analytes can be detected by a detector. HPLC uses high pressure to pump a solution through a packed column containing small stationary phase particles. The HPLC instrument usually consists of pumps, an autosampler, a column, often a column oven and a detector, as illustrated in figure 9. Commonly used detectors includes UV and fluorescent detectors, but electro- and radiochemical detectors are also available [38].

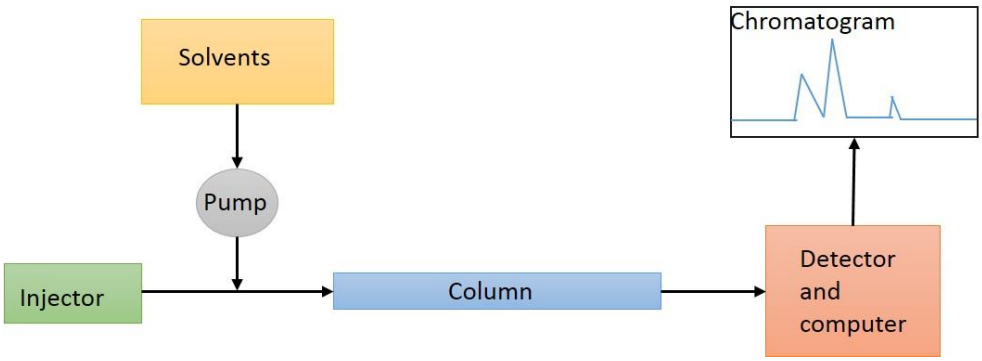


*Figure 9: Schematic drawing of the flow and components of an HPLC-instrument (adapted from Harris [38]).*

HPLC is usually divided in two different separation systems; normal phase chromatography and reverse phase chromatography. Reverse phase chromatography is the most common with a nonpolar (hydrophobic) stationary phase and a polar (hydrophilic) mobile phase. The analytes are separated based on their hydrophobicity. The less polar (hydrophobic) analytes are more attracted to the stationary phase, and the more polar (hydrophilic) analytes are more attracted to the mobile phase and will elute first from the column. Reverse phase chromatography is suitable for separation of nonpolar to partly polar compounds, as long as the analytes are hydrophobic enough to bind to the stationary phase. Metabolic studies usually employ reverse phase chromatography, because it spans most of the analytes, with the metabolites often being more polar than the substrates.

The mobile phase in HPLC flows continually and often combines two solutions, A and B, where A can be water based and B an organic solution. The mobile phase can be optimized, both in regard of what kind of organic solution and the amount, depending on what types of analytes that are to be separated. If the sample material is complex and containing several analytes, it

might be necessary to change the composition of the mobile phase over time, known as gradient elution, to get a better separation of the analytes.

Methanol (MeOH) and acetonitrile (ACN) are often used as the organic mobile phase together with a weak acid, like acetic or formic acid (FA), as proton donor. ACN might have stronger eluting properties, but MeOH is often used because it is cheaper, more accessible and less harmful than acetonitrile.

After separation based on hydrophobicity and elution from the column, the analytes are detected. The detector is set to respond to a physiochemical property of the wanted analyte(s), and the response is digitally amplified and sent to a computer where it is recorded as a peak in a chromatogram.

The retention time of a compound in the chromatogram provide the qualitative aspect of the analyte, and should be identical under identical system conditions. The peak height or area is decided by the quantity of the analyte(s) analyzed. When height/area is compared with standards of known concentrations, the actual amount of analyte can be determined.

### *1.5.2 Basic MS theory*

MS is an analytical technique that can give both quantitative and qualitative information about both organic and nonorganic compounds in complex sample matrixes [39]. The MS consists of four main components: an injection port, an ion source, a mass analyzer and a detector. The main components are illustrated in figure 10. An MS can be preceded by HPLC to implement a high throughput system with increased selectivity and sensitivity for obtaining quantitative data.

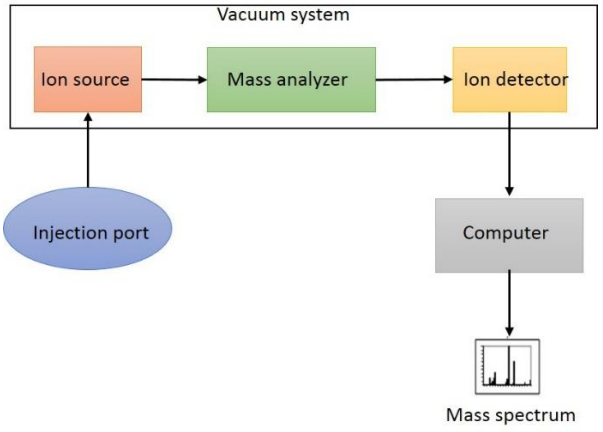


*Figure 10: Schematic drawing of the flow and components of a typical mass spectrometer (adapted from Harris [39])*

When the analytes elute from the HPLC, instead of going through an detector, they are vaporized and ionized in the ion source of the MS [36]. A common ionization technique is electrospray ionization (ESI). After ionization, the ions are fragmented and then accelerated by a magnetic field before separation based on mass-to-charge rato, m/z, in a mass analyzer. The ions are detected qualitatively and quantitatively based on the m/z and the signal intensity. A mass spectrum is generated as a representation of the signal intensity of the ions in the detector and plotted against m/z of the ions.

Mass spectrometry is an increasingly used method for quantitative and qualitative analyses because of its high-throughput with high sensitivity and selectivity. The main components of the MS can be optimized depending on the analytes.

A common MS-methodology when analyzing a few known analytes in a complex matrix is the multiple reaction monitoring (MRM), also called Selected Reaction Monitoring (SRM). MRM is a method used when the MS system consists of a triple quadrupole mass analyzer [39]. A triple quadrupole consists of two identical quadrupoles (Q1 and Q3) that are separated by a collision cell (Q2). With MRM, both quadrupoles are "locked", and only molecules with a predetermined ion mass and fragment mass will be let through the mass analyzer to the detector. This process is illustrated in figure 11. The ions from the ion source will reach the first mass filter Q1 that is locked on a specified ion mass. In the collision cell Q2 the ions are bombarded with a neutral gas and the ions are fragmented to daughter ions. Mass filter Q3 is programmed to only let through the fragmented daughter ions with specific ion mass.

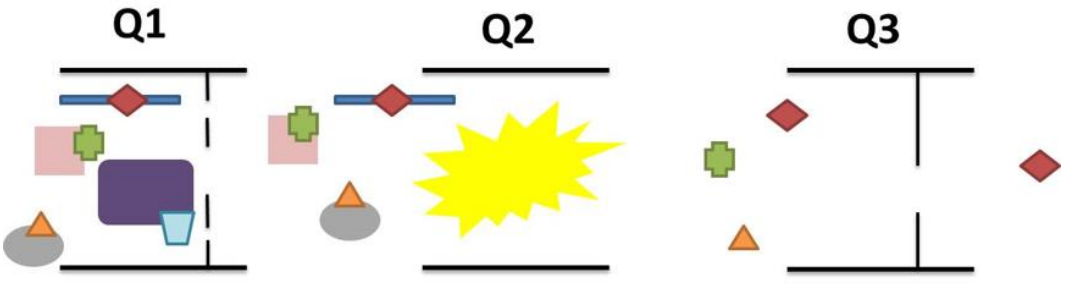


*Figure 11: Simplified illustration of how a triple quadrupole works. Q1 separates ions based on mass. Only ions with preselected m/z will pass on to Q2 were the ions will be fragmented to daughter ions. Only daughter ions with preselected m/z will pass through Q3 to the detector.*

After the analytes have been separated by the HPLC, and only specific analytes are being let through the MS, the result is a highly specific and sensitive method to quantitatively analyze compounds, for example substrates and metabolites from CYP enzyme studies.

## 1.6 Basic enzymatic theory

Enzymes are biological catalysts that increases the rate of chemical reactions at which equilibrium is achieved, without being permanently transformed themselves [40]. The substrate binds the enzyme to form an enzyme-substrate complex where the enzyme provides the means necessary for the substrate to transform to its activated state. The activation happens without addition of external energy, meaning that the enzyme lowers the energy barrier and accelerates the product formation. The key factor affecting the rate of an *in vitro* enzyme reaction is the substrate concentration [S]. Figure 12 shows how the change in [S] affects the initial reaction rate, also called velocity.

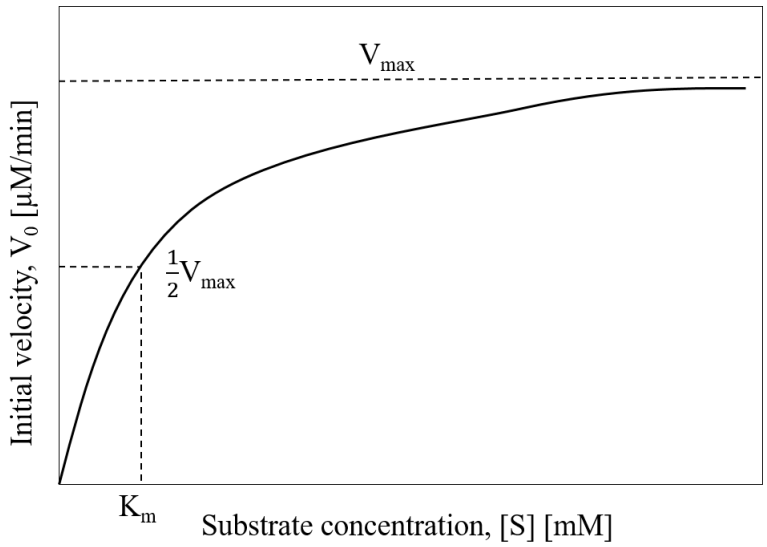


*Figure 12: Effect of substrate concentration on the initial velocity of an enzyme-catalyzed reaction (figure adapted from [40]).*

As illustrated in figure 12, at low [S] there are more enzymes than substrate molecules. When increasing the substrate concentration, the enzymes get saturated and the reaction rate reaches a limit ($V_{max}$) where the number of substrate molecules exceeds the number of active sites. The $K_m$-value represent the substrate concentration at ½ $V_{max}$ and is called the Michalis constant. A low $K_m$ for a specific enzyme indicates that the enzyme has a higher affinity for its substrate.

Enzymes can be activated or inhibited by molecular agents that interfere with the reaction. Activators will increase the rate of reaction, while inhibitors will slow or halt the enzymatic reaction. A common measurement of inhibition is the calculation of an $IC_{50}$-value for the inhibiting agent. The $IC_{50}$-value represent the concentration of inhibitor where the enzyme is

inhibited by 50 %, the half maximum inhibition concentration. An $IC_{50}$-value can be determined by constructing a dose-response curve and examining the effect of increasing concentrations of the proposed inhibiting agent. In general, an increase in inhibitor concentration will decrease the enzyme activity.

## 1.7 Hops (*Humulus lupulus*)

Hops, Latin name *Humulus lupulus*, is a climbing vine belonging to the genus Humulus in the family Cannabeceae [41]. The plant is native to the northern hemisphere and grows vigorously from the end of April to the beginning of July. When hops reach a certain height, and the length of the day is ideal, the plant starts to blossom. Hops are dioecious, meaning that there is a difference between male and female plants. The flowers of the female plants are cone-like and called strobiles (figure 13), and it is this part that is used, both as a HMP and for beer brewing. The male plant is only used for fertilization [42].

*Figure 13: The flowers of the female hops plant, called strobiles.*

### *1.7.1 Traditional use*

Hops are mostly known for its use in beer brewing, but also have a history of being used for medicinal purposes [41, 43]. The therapeutic use of hops in Europe dates back to the ninth century, before that it was only used for making beers, bread and in salads [44]. In the beginning hops were added to beer because of the bitter taste, but over time hops outclassed other alternative beer additives because of its antimicrobial properties. In 1516 the German beer purity law, the *Reinheitsgebot*, was formulated, stating that beer could be made of only malt, hops and water [41]. The benefit of hops in brewing resulted in a more widespread recognition and distribution, and is probably the reason for the increased focus for additional uses, like medicinal, in Europe.

There are several historic recordings of medicinal use of hops. George III, king of the United Kingdom from 1738-1820, was bedded on pillows filled with hops to calm him [41]. In 1905 the physician Kahnt recommended the use of hop pillows, teas or extracts for sleeping problems associated with nervous disturbances. The medicinal uses of hops were recognized in the Arabic world already in 1015, where hops were described to help in the purification of blood, aid in reducing fever, having anti-inflammatory properties and having calming effects [43]. Hops were also used by several native American tribes and also in Indian-Ayurvedic medicine. In both India and North America hops were used as a sedative, analgesic against toothaches, anti-inflammatory and as a sleep promotor. In traditional Chinese medicine hops have been used to treat insomnia, restlessness, dyspepsia and lack of appetite [44]. Also in China, alcoholic extracts of hops have been used clinically to treat leprosy, pulmonary tuberculosis, acute bacterial dysentery, silicosis and asbestosis [44].

To sum up, hops have been used to treat a wide range of complaints all over the world, mainly as a mild sedative to treat sleeplessness and nervousness, but also to improve appetite and digestion, relieve toothache, earache and neuralgia [43, 45]. Hops have been reported to have diuretic, antispasmodic and anaphrodisiac effects. Hops have also been used as a heated poultice in the treatment of pneumonia, intestinal pains and fevers.

### *1.7.2 Phytochemistry and pharmacological action*

The main structural classes of identified chemical compounds from mature hop cones include terpenes, bitter acids and chalcones. The cones are also rich in flavonol glycosides and catechins [42, 43]. The terpenes are found in the volatile oil, in total 0.3-1.0% of hop strobile weight, primarily β-caryphyllene, farnesene, humulene and myrcene. The bitter acids constitute 5-20% of hop strobile weight and are phloroglucinol derivatives usually classified as α- or β-acids. The bitter acids are a variable and complex mixture in hops. The main α-acids are humulone, cohumulone and adhumulune, the corresponding β-acids are lupulone, colupulon and adlupulon. The α-acids are the crucial compound in beer brewing, contributing to foam stability, as well as the antimicrobial activity.

The most important chalcone found in hops is the prenylflavonoid xantohumol, which is converted to the prenylflavanone isoxhantohumol when thermally treated [46]. There are also several other chalcone prenylflavonoids in hops, with 10-100-fold lower concentration than that of xhantohumol, that isomerizes to its corresponding flavanones. Xhantohumol has been

studied for chemopreventive properties, and it has shown antiproliferative activity in breast, colon and ovarian cancer cell lines [43].

The prenylated flavone 8-prenylnaringenin has been identified as a potent phytoestrogen, and its estrogenic properties have been studied both *in vivo* and *in vitro*, and has shown estrogenic properties in humans in high concentrations. Thus, hop-derived prenylated flavonoids could provide an alternative treatment for the relief of menopausal symptoms [46].

Today hops, or the constituents of hops, are explored for three potential pharmacological actions; The sedative effect, estrogenic activity and as a chemo preventive agent [43]. The chemistry of hops is well documented, although evidence from robust clinical studies are limited [42].

### *1.7.3 Side effects and interactions with drugs*

There is a lack of clinical safety and toxicology data for hops. Respiratory allergy and contact dermatitis have been reported after handling and working with hop cones, while small doses of hops are stated to be non-toxic [42].

Previous studies have shown that flavonoids isolated from hops inhibited cDNA expressed human CYP enzymes that activate carcinogens, including CYP1A1, CYP1A2 and CYP1B1 *in vitro* [47]. Two studies performed by the same research group on different types of alcoholic beverages in microsomes, showed greater CYP inhibition by products containing hops, than products with little or no hops. Most of the products showed a moderate to strong inhibition of CYP2C9 metabolism, but also CYP2C19 and CYP3A metabolism was affected [48, 49]. A recent study on the *in vitro* inhibition effect of hops done by Yuan et al [50] reported that their specially developed hop extract (5 µg/mL) inhibited CYP2C8, CYP2C9, CYP2C19, and CYP1A2 in human liver microsomes.

Hops as herbal remedy is most commonly used in combination with valerian and other herbs as a sleeping and calming aid, but is also sold separately as an herbal product to relief menopausal symptoms [43]. Since hops have the potential to interfere with drug metabolism, more detailed knowledge is necessary.

# 2. Aims of thesis

The cytochrome P450 system plays an important role in the biotransformation and bioactivation of xenobiotics. The recent years have shown that CYPs have an active role in carcinogen metabolism and is a vital part of xenobiotic interactions with clinical importance. It is therefore necessary to assess human CYP activity in drug development or toxicological areas. The *in vitro* cocktail method is used to study inhibition potential of herbs or drugs towards a mix of chosen CYP enzymes with specific substrates. Hops *(Humulus Lupulus)* is a herb often added to calming herbal preparations in combination with other herbs with proposed calming properties. In addition, tea made of hops is recommended for everything from sleep deprivations to menopausal problems in women.

This study will explore the following:

I. Introduce in our laboratory the use of "Inje's cocktail" in an *in vitro* setting using pooled human liver microsomes to simultaneously assess *in vitro* inhibition on the cytochrome P450 enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4.

II. Optimize the *in vitro* cocktail method with regard to substrate concentration, HLM concentration and incubation time.

III. Complete a standard operation procedure (SOP) to the lab archive for the developed *in vitro* cocktail method.

IV. Adaption of a LC-MSMS method for detection of the microsomal CYP substrates and metabolites.

V. Using the developed *in vitro* cocktail method and the adapted LC-MSMS method to identify $IC_{50}$-values for hops towards the different investigated CYP enzymes for identification of inhibition potential.

VI. Comparing the inhibition of CYP enzymes by different extraction of hops using 50% ethanol or water.

# 3. Materials and Methods

## 3.1 Materials

### *3.1.1 Chemicals*

Potassium dihydrogen phosphate, $KH_2PO_4$ (lot#1048731000) and di-potassium hydrogen phosphate, $K_2HPO_4$ (lot#1051041000)) were purchased from Merck, Darmstadt, Germany. Methanol (lot#BCBP20191V) was obtained from Fluka, and ethanol (lot#100022) from Kemetyl Norway. Acetonitrile (lot#1416103801) came from Avantor Performance materials, Poland and formic acid (lot#A018120410) was bought from Acros Organics, Belgium. Water was prepared by a Milli-Q Millipore Advantage A-10 ion-exchanger from Mitron O.R., Melhus, Norway.

Caffeine (lot#0001400932), 1,7-dimethylxhantine (lot#078K4061), losartan potassium (lot#0001417819), omeprazole (lot#098K1189), dextromethorphan hydrobromide monohydrate (lot#013K1428), dextrorphan tartrate (lot#085K4631), phenacetin (lot#S4187644207B11), acetaminophen (lot#116K0124), quinidine (lot#1295350), ketoconazole (lot#121H0524), propranolol hydrochloride (lot#BCBD8251V) and magnesium chloride powder, $MgCl_2$, were purchased from Sigma Aldrich (Norway). EXP-3174 (lot#200013283) was kindly donated from Merck (Germany), 5-OH-omeprazole (lot#62296J08) was kindly donated from AstraZeneca (Sweden), midazolam HCl (Lot#1111B21), was purchased from Lipomed AG (Switzerland) and 1-OH-midazolam was kindly donated from Department of Clinical Pharmacology (St. Olav's University Hospital).

### *3.1.2 Human Liver Microsomes and NADPH*

Pooled human liver microsomes (HLM) was purchased from Gentest BD Biosciences, Woburn, MA, USA (lot number 28831, protein content 20 mg/mL in 250 mM sucrose). The producers' calculated enzyme activity for the relevant enzymes (from datasheet) can be seen in table 1. β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, lot#48H7019) was purchased from Sigma Aldrich, Oslo, Norway.

*Table 1: Enzyme activity from enclosed product datasheet supplied by the producer for the microsomal CYP enzymes relevant for this experiment.*

| **Enzyme** | **Enzyme activity [pmol/(mg×min)]** |
|---|---|
| CYP1A2 | 820 |
| CYP2C9 | 3000 |
| CYP2C19 | 53 |
| CYP2D6 | 99 |
| CYP3A4 | 4900 |

### *3.1.3 Herbal product – Hops (Humulus lupulus)*

Dried hops were purchased from a local herbalist. The dried hops (batch 52702016) was produced by Natur Drogeriet in Hørning, Denmark.

The dietary supplement Hops Flowers (batch 20041199) was produced by Nature's Way Products, Green Bay, USA, and distributed by Urtesenteret, Torvastad, Norway.

## 3.2 HPLC-MSMS System

The liquid chromatography – triple quad system (HPLC/MSMS) was composed of a Shimadzu LC20AD LC system (Shimadzu Scientific Instruments, Columbia, USA) with a controller (CBM-20A), autosampler (SIL-20AC), pumps A and B (LC-20AD), degasser (DGU-20$A_5$) and column oven (CTO-20AC). The column was a 2.1×100 mm XBridge $C_{18}$ column with 3.5 µm packing (Waters, Norway).

The MS system was composed of an Applied Bioscience (AB) SCIEX triplequad 5500 with positive electro spray ionization.

## 3.3 Preparation of experimental solutions

### *3.3.1 Chemicals*

$KH_2PO_4$ and $K_2HPO4$ were dissolved in water and mixed to make a 0.1 M potassium phosphate buffer (KPO, pH 7.4). A washing solution for the HPLC-MSMS system was made by adding 0.1% FA to 50% MeOH. Mobile phase A consisted of milli-Q deionized water with 0.1% FA. Mobile phase B consisted of either MeOH or ACN with 0.1% FA.

Stock solutions of the substrates and metabolites were prepared with the solvents and concentrations described in table 2.

*Table 2: Solvent and stock concentrations of the substrates and metabolites*.

| **Analyte** | **Solvent** | **Stock concentration [mM]** |
|---|---|---|
| CAF* | water | 4.0 |
| PXH* | 50 % MeOH | 1.0 |
| PHE | water | 1.0 |
| ACE | water | 1.0 |
| LOS | 50% MeOH | 0.4 |
| EXP | 50% MeOH | 0.4 |
| OME | 50% MeOH | 0.4 |
| OHO | 50% MeOH | 0.4 |
| DXM | water | 4.0 |
| DEX | water | 4.0 |
| MID | water | 1.0 |
| OHM | water | 1.0 |

*Not included in final setup

Quinidine was dissolved in 0.1 mM KPO buffer to get a 1.0 mM quinidine stock solution. Ketoconazole was dissolved in 50% MeOH to get a 1.0 mM ketoconazole stock solution.

0.48 mg of the internal standard (IS) propranolol was dissolved in 50% MeOH and 50% ACN to make 250 mL of the protein precipitation (stop) solution.

### *3.3.2 Human liver microsomes and NADPH*

The pooled HLM (20 mg microsomal proteins/mL) in 250 mM sucrose (total volume 0.5 mL) were aliquoted in Eppendorf tubes with 50 µL in each to avoid unnecessary freeze and thaw cycles. Before each incubation, the required number of Eppendorf tubes were thawed rapidly and 0.1 M KPO buffer were added to get the desired concentration of microsomal protein. The HLM were stored at -80ºC.

A 5.0 mM NADPH solution was made by dissolving NADPH in 0.1 M KPO buffer.

## 3.4 Preparation of hops extract

### *3.4.1 Ethanol extraction of dried hops*

A teaspoon of dried hops was weighed, crushed and grinded to finer pieces before 15 mL 50% ethanol were added. The solution was heated to 30ºC in a water bath and hops were extracted for one hour with constant stirring. After centrifugation at 3500 rpm for 12 minutes, the supernatant was collected in a pre-weighed beaker, and the pellets were dissolved in 5 mL 50% ethanol and extracted again. The collected supernatant from the two extractions were pooled and evaporated to dryness at 40ºC under a gentle stream of air. The beaker with the dried extracted residue was reweighed and the mass of extracted hops was calculated. The hops were dissolved in a known amount of 50% ethanol to make a stock solution.

### *3.4.2 Tea (water extraction of dried hops)*

A teaspoon of dried hops was weighed, added to boiled water and extracted for 10 minutes. The tea was poured over a filter and the solution was evaporated to dryness at 40ºC under a gentle stream of air. The beaker with the dried extracted residue was reweighed and the mass of the extracted hops was calculated. The hops were dissolved in a known amount of water to make a stock solution.

### *3.4.3 Extraction of dietary supplement hops*

The capsule containing the herbal product was opened and the fine powder weighed in a beaker before 15 mL of water or 50% ethanol were added. The solution was heated to 30ºC in a water bath and hops were extracted for one hour with constant stirring. After centrifugation at 3500 rpm for 12 minutes the supernatant was collected in a pre-weighed beaker, and the pellets were dissolved in 5 mL of the extraction solvent and extracted again. The collected supernatant from the two extractions were pooled and evaporated to dryness at 40ºC under a gentle stream of air. The beaker with the dried extracted residue was reweighed and the mass of the extracted hops was calculated. The hops were dissolved in a known amount of the extraction solvent to make a stock solution.

## 3.5 Analytical method

The substrates and metabolites were analyzed by a validated LC-MSMS method. The LC-MSMS conditions can be seen in table 3 and the assay gradient can be seen in figure 14. MRM parameters for each substrate, metabolite and IS can be seen in appendix A.

*Table 3: LC-MSMS conditions.*

| **LC-MSMS parameters** | **Set conditions** |
|---|---|
| Cooling rack temperature | 15 ºC |
| Sample injection volume | 5 µL |
| Flow rate | 0.3 mL/min |
| Column oven temperature | 30 ºC |
| Mobile phase A | Water w/ 0.1 % FA |
| Mobile phase B | Methanol w/ 0.1 % FA |
| ESI mode | Positive |
| IonSpray voltage | 4500 volts |
| Temperature | 575 ºC |
| Curtain gas flow, nitrogen | 16.0 bar |
| Collision gas flow, nitrogen | 8.0 bar |
| Ion source gas 1 flow, nitrogen | 60 bar |
| Ion source gas 2 flow, nitrogen | 50 bar |
| Run time | 7.5 min |

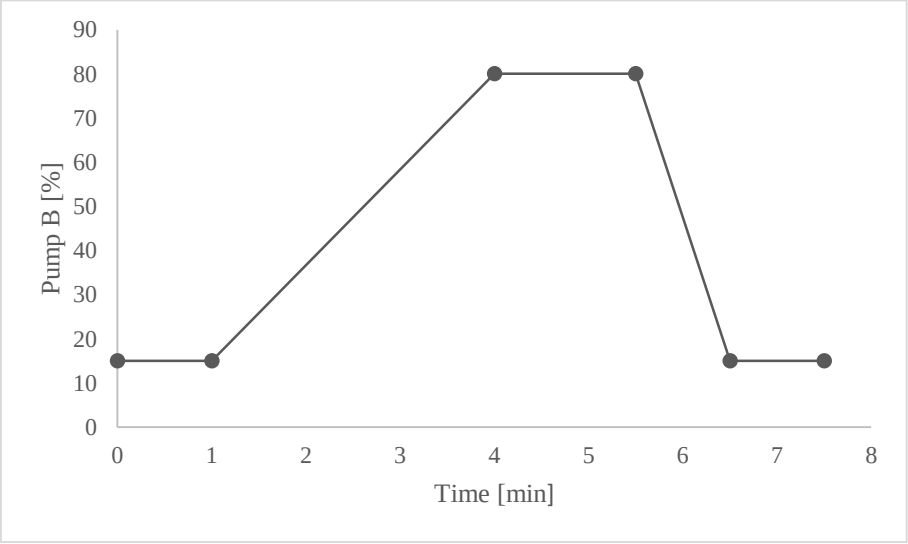


*Figure 14: Gradient of organic phase B, MeOH.*

## 3.6 Quality control of the analytical method

Stock solutions containing all the analytes were made containing 10% 0.1 M KPO-buffer. The stock solutions were further diluted with MeOH to seven different standard solutions (STDs) and four quality controls (QCs) by different laboratory personnel. The STDs and QCs were constructed for the five substrates and the five metabolites with the concentration ranges in table 4.

*Table 4: Concentration range of STDs and QCs.*

| **Analyte** | **Concentration range [nM]** |
|---|---|
| PHE | 2750-22000 |
| ACE | 37.5-300 |
| LOS | 1500-12000 |
| EXP | 7-56 |
| OME | 1500-12000 |
| OHO | 25-200 |
| DXM | 1500-12000 |
| DEX | 37.5-300 |
| MID | 1500-12000 |
| OHM | 25-200 |

Pre-run intra-day accuracy and precision was determined for the LC-MSMS by five replicate determinations per concentration of STDs and QCs in a single analytical run. Pre-run inter-day accuracy and precision was determined by analysis of the same concentrations of STDs and QCs in separate analytical runs on three different days. The precision was calculated based on the percentage coefficient of variation (% CV). The validation assay was performed as recommended the FDA [51]. 100 µL of the stop solution were added to 200 µL of STD or QC before analysis to give the correct concentration of analytes and IS. The area ratios of analyte/IS was plotted against nominal concentration to construct a standard curve for each analyte and the standard curve equation was found using best-of-fit non-linear regression. The lowest limit of quantitation (LLOQ) was based on the lowest concentration of the calibration curve that gave an acceptable accuracy and precision (± 20%). The mean measured concentration of each STDs was required to be within 15% of the nominal concentration. The concentrations of the QCs

were back calculated using the equation for the standard curve and the calculated concentrations had to be within the acceptance criteria for accuracy and precision.

Four non-zero QCs at different concentrations were analyzed together with the samples from each experiment to assure the quality of the analytical series. The actual concentrations of analytes in the QCs were found by back calculations using the standard curve equation. The criteria for acceptance of the analytical series in each individual run was an QC concentration accuracy of 85% - 115% (80%-120% for LLOQ).

## 3.7 Microsomal incubation method

The incubations were conducted in three experimental sessions: (1) A pilot study to determine incubation conditions; (2) Optimization and validation of the conditions determined in (1); (3) The CYP inhibition experiment to determine the inhibition potential of hops.

The incubation system, total volume 200 µL, contained five probe substrates, 1 mM NADPH, phosphate buffer (0.1 M, pH 7.4) and HLM. All samples were incubated in triplicates and the samples were spread randomly in the water bath, which kept a temperature of 37ºC. The reactions were initiated by adding HLM to the incubation system and terminated by adding 100 µL ice-cold stop solution. The samples were centrifuged at 3500 rpm for 12 minutes and supernatants were transferred to MS-vials for LC-MSMS analysis. Details regarding each experimental part are given in the following sections.

### *3.7.1 Pilot study*

The incubation method was tested using substrates corresponding to Inje's cocktail with concentrations previously reported and close to their respectively $K_m$ values [52]. The concentrations of substrates were: 20 µM CAF, 1 µM LOS, 0.2 µM OME, 0.2 µM DXM and 2 µM MID. The incubation method was further explored by testing different concentrations of substrates, different concentrations of HLM (0.2 and 0.4 mg proteins/mL), different incubation times (15 and 25 minutes), and the effect of 3 mM $MgCl_2$ in the incubation solution was also investigated.

Two selective inhibitors, 0.2 µM ketoconazole (CYP3A4) and 0.2 µM quinidine (CYP2D6) were added to selected samples to see if a specific inhibition could be obtained and detected.

CAF was switched with PHE, and new substrate concentrations were tested: 20 µM PHE, 1 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID. These concentrations were chosen for further optimization, together with 1 mM NADPH, 0.4 mg protein/mL HLM and incubation time 20 minutes.

### *3.7.2 Optimization and validation of the incubation method*

Incubation conditions were optimized by incubating the five probe substrates at three different concentrations (table 5), the optimal concentration were further incubated with 0.1, 0.2 and 0.4 mg proteins/mL HLM, and the optimal substrate concentration together with the optimal HLM concentration were incubated for 10, 20 and 30 minutes. The substrates and the CYP produced metabolites in the incubated samples were analyzed using the validated LC-MSMS method described previously.

*Table 5: The five probe substrates and their three different incubation concentrations*

| **Substrate** | **Concentration 1 [µM]** | **Concentration 2 [µM]** | **Concentration 3 [µM]** |
|---|---|---|---|
| PHE | 1 | 20 | 40 |
| LOS | 0.1 | 1 | 10 |
| OME | 0.2 | 2 | 10 |
| DXM | 0.2 | 10 | 25 |
| MID | 1 | 10 | 25 |

The incubation method was validated by adding only one substrate to the incubation mix and comparing the production of the specific metabolite to a control incubation with all substrates present.

The CYP inhibition potential of ethanol was tested by adding ethanol in increasing concentrations; 0, 0.5, 1, 2 and 5 % to see if this affected enzyme activity in the microsomes.

The optimized and validated method consisted of: 20 µM PHE, 2 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID incubated with 0.4 mg proteins/mL HLM for 20 minutes.

### *3.7.3 Incubation with hops*

Increasing concentrations of ethanol extracted hops were incubated with HLM using the optimized method from section 3.7.2. The hops stock solution was diluted with KPO buffer to 7 or 8 working solutions with increasing hops concentration, of which 40 µL were added to the microsomal incubations. All samples contained a final concentration of 1% ethanol.

Water extracted hops was diluted to 7 or 8 different working solutions with increasing hops concentrations, of which 40 µL were added to the microsomal incubations, before being incubated using the optimized method from section 3.7.2.

## 3.8 Calculations and statistics

The raw data were based on AUC (area under the curve) of peaks representing each analyte obtained from the LC-MSMS. Each analyte AUC was divided by AUC for the IS to area ratio (analyte/IS) by AB Sciex Analyst® software. In the inhibition study, the results are based on area ratio of metabolite peak area ratio (met/IS) divided by substrate peak area ratio (sub/IS), from here on referred to as area ratio of met/sub.

Calculations and statistics were performed in Excel 2016 (Microsoft). Standard curves, $IC_{50}$-curves and regression calculations were done in SigmaPlot 13.0 (Systat Software 2014).

Standard curve regressions were based on a quadratic fit model and were back calculated with equation 1.

$$f = y_0 + ax + bx^2 \qquad (1)$$

Best-of-fit non-linear regression was used for $IC_{50}$-curves, and $IC_{50}$-values were calculated based on the derived equation (equation 2-4 were used; two parameter exponential decay, three parameter exponential decay and four parameter logistic curve, respectively). All parameters in the equations were calculated by SigmaPlot.

$$f = ae^{-bx} \qquad (2)$$

$$f = y_0 + ae^{-bx} \qquad (3)$$

$$f = \frac{\min + (max - \min)}{1 + \left(\frac{x}{EC50}\right)^{-hillslope}} \qquad (4)$$

# 4. Results

The aim of this thesis was to develop an *in vitro* cocktail method based on Inje's *in vivo* cocktail to investigate the inhibitory potential of natural remedies and other potential CYP inhibitors. The method was further used to investigate the inhibitory potential of hops (*Humulus lupulus*).

## 4.1 Quality control of the analytical method

The STDs and QCs were constructed for the five substrates and the five metabolites in the concentration range previously reported in table 4.

### *4.1.1 Decomposing of omeprazole*

When making the STDs it was discovered that OME would decompose when mixed in the standard solution with the other analytes; this could be observed when extracting OME from the chromatogram as shown in figure 15. The same effect could not be noticed when analyzing dilutions of the stock solution alone.

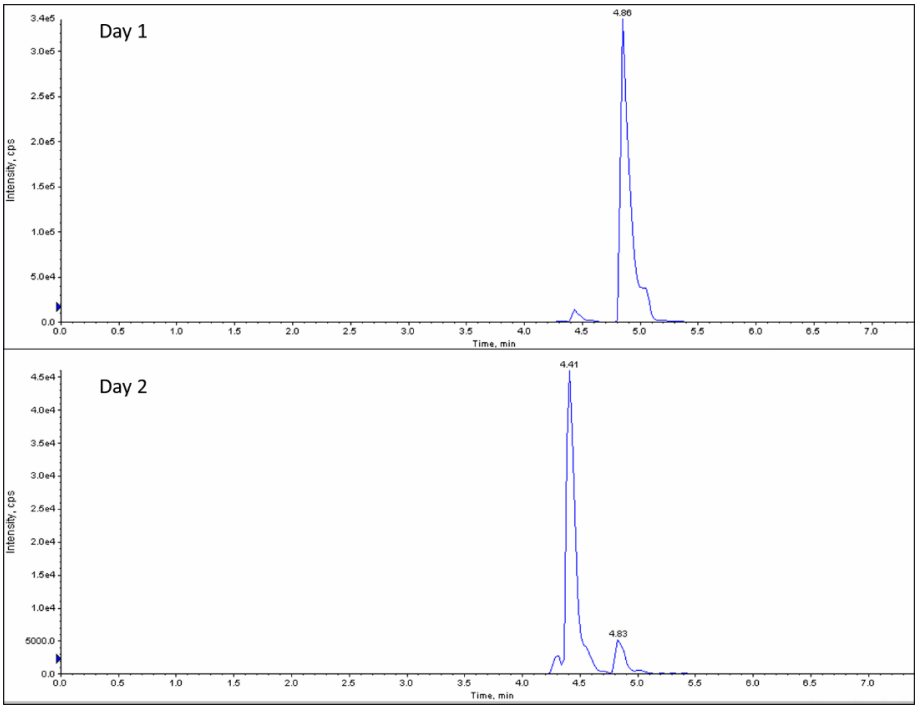


*Figure 15: Extracted peak chromatogram of OME from an early attempt to make STDs showing how the OME-peak changed from day 1 to day 2.*

### *4.1.2 Validation of LC-MSMS performance*

Pre-run intra- and inter-day validation of the LC-MSMS method was performed as described in section 3.6. The next sections will address QCs and STDs for each individual analyte and report the pre-run intra- and inter-day accuracy and precision (% CV).

**Phenacetin**

A representative standard curve for PHE can be seen in figure 16, concentration range 2750-22000 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 6. Non-linear regression gave the equation $f = -29.3+0.020x+(8.08\times10^{-7})x^2$.

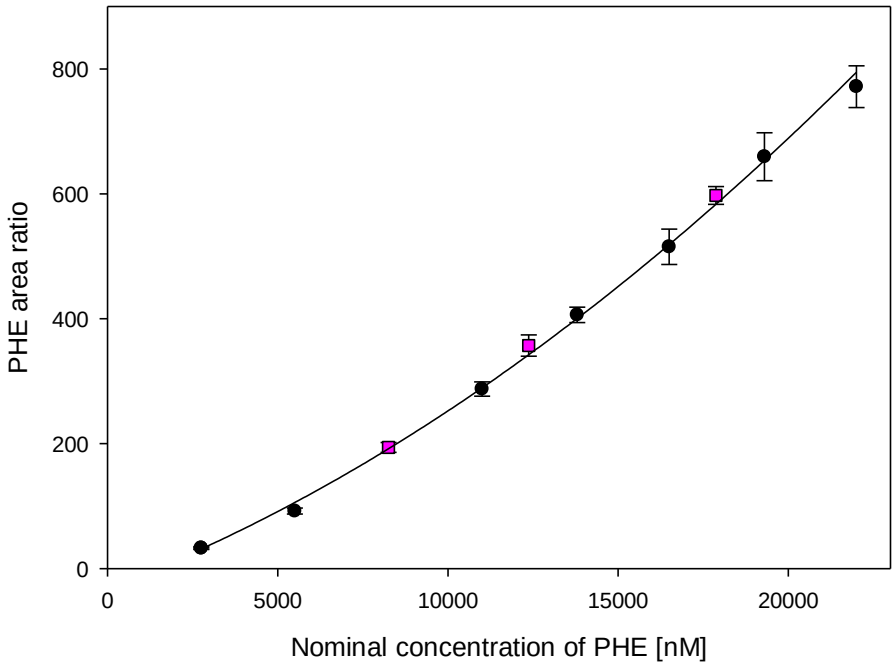


*Figure 16: A representative standard curve for PHE showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 2750-22000 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 2250 nM is both a STD and a QC.*

*Table 6: Calculated intra- and inter-day accuracy and precision (% CV) for PHE.*

| | | **Intra-day (n=5)** | | **Inter-day (n=15)** | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 2750 | 0.44 | 104 | 6.8 | 103 |
| **QC1** | 2750 | 1.7 | 103 | 4.3 | 100 |
| **STD2** | 5500 | 3.6 | 93.2 | 5.2 | 94.7 |
| **QC2** | 8250 | 0.45 | 103 | 4.0 | 100 |
| **STD3** | 11000 | 1.2 | 101 | 4.0 | 99.6 |
| **QC3** | 12375 | 1.5 | 104 | 4.8 | 101 |
| **STD4** | 13750 | 2.0 | 102 | 3.1 | 102 |
| **STD5** | 16500 | 1.5 | 100 | 5.5 | 101 |
| **QC4** | 17875 | 0.94 | 102 | 2.4 | 101 |
| **STD6** | 19250 | 0.26 | 101 | 5.8 | 100 |
| **STD7** | 22000 | 0.50 | 98.3 | 4.3 | 98.5 |

**Acetaminophen**

A representative standard curve for ACE can be seen in figure 17, concentration range 37.5-300 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 7. Non-linear regression gave the equation $f=-0.079+0.0091x+(2.3\times10^{-5})x^2$.

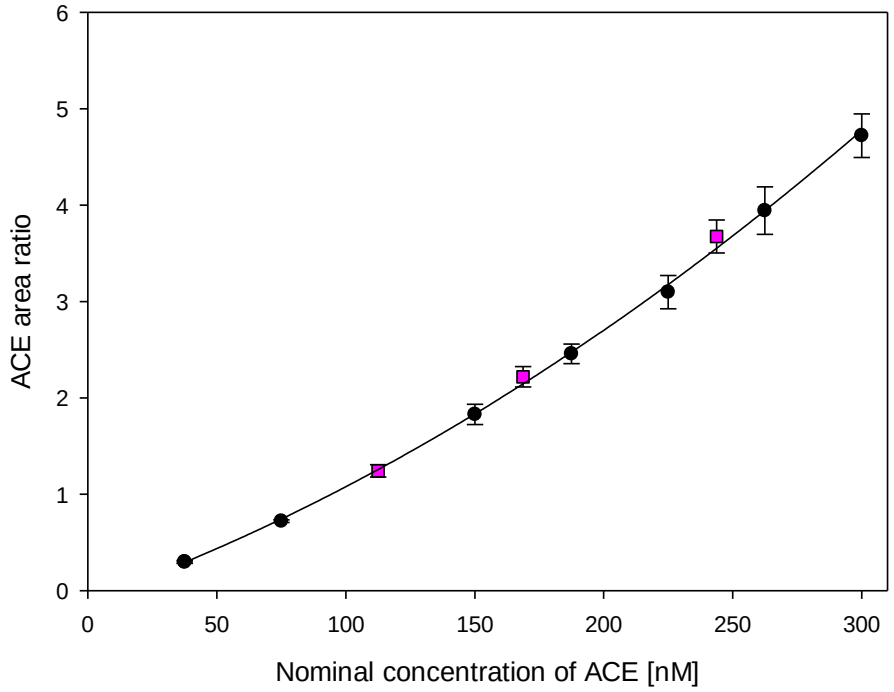


*Figure 17: A representative standard curve for ACE showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 37.5-300 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 37.5 nM is both a STD and a QC.*

*Table 7: Calculated intra- and inter-day accuracy and precision (% CV) for ACE.*

| | | Intra-day (n=5) | | Inter-day (n=15) | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 37.5 | 0.80 | 101 | 4.8 | 101 |
| **QC1** | 37.5 | 1.1 | 100 | 4.2 | 100 |
| **STD2** | 75.0 | 0.66 | 98.6 | 1.9 | 97.7 |
| **QC2** | 112.5 | 1.2 | 100 | 5.2 | 99.8 |
| **STD3** | 150.0 | 1.4 | 101 | 5.8 | 99.9 |
| **QC3** | 168.8 | 1.5 | 104 | 4.8 | 101 |
| **STD4** | 187.5 | 0.57 | 101 | 4.1 | 99.6 |
| **STD5** | 225.0 | 2.1 | 99.0 | 5.6 | 100 |
| **QC4** | 243.8 | 1.1 | 103 | 4.6 | 102 |
| **STD6** | 262.5 | 0.32 | 101 | 6.2 | 100 |
| **STD7** | 300.0 | 0.13 | 99.8 | 4.8 | 99.0 |

**Losartan**

A representative standard curve for LOS can be seen in figure 18, concentration range 1500-12000 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 8. Non-linear regression gave the equation $f = -49.7+0.140x+(-1.43\times10^{-6})x^2$.

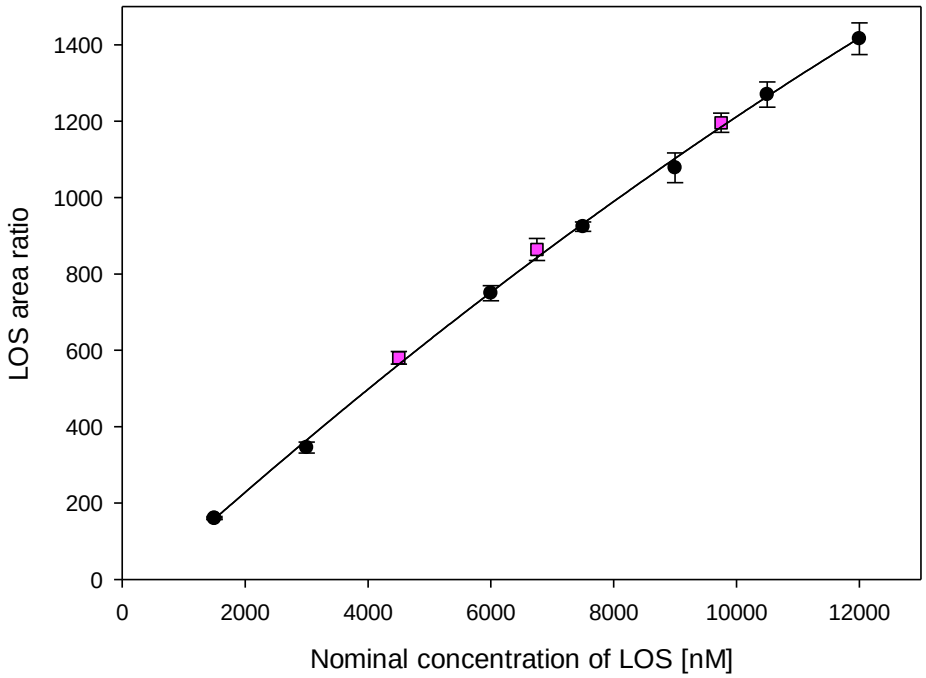


*Figure 18: A representative standard curve for LOS showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 1500-12000 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 1500 nM is both a STD and a QC.*

*Table 8: Calculated intra- and inter-day accuracy and precision (% CV) for LOS.*

| | | Intra-day (n=5) | | Inter-day (n=15) | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy(%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 1500 | 1.2 | 101 | 3.2 | 99.6 |
| **QC1** | 1500 | 1.9 | 102 | 2.5 | 100 |
| **STD2** | 3000 | 4.2 | 96.9 | 5.2 | 98.8 |
| **QC2** | 4500 | 2.1 | 105 | 2.8 | 103 |
| **STD3** | 6000 | 0.22 | 101 | 2.6 | 99.9 |
| **QC3** | 6750 | 2.1 | 104 | 3.3 | 100 |
| **STD4** | 7500 | 1.3 | 100 | 4.0 | 98.4 |
| **STD5** | 9000 | 1.7 | 98.4 | 3.6 | 98.6 |
| **QC4** | 9750 | 0.75 | 102 | 2.1 | 101 |
| **STD6** | 10500 | 0.97 | 101 | 2.6 | 101 |
| **STD7** | 12000 | 2.0 | 99.3 | 2.9 | 100 |

**EXP-3174**

A representative standard curve for EXP can be seen in figure 19, concentration range 7-56 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 9. Non-linear regression gave the equation $f= -0.096+0.095x+(9.0\times10^{-4})x^2$.

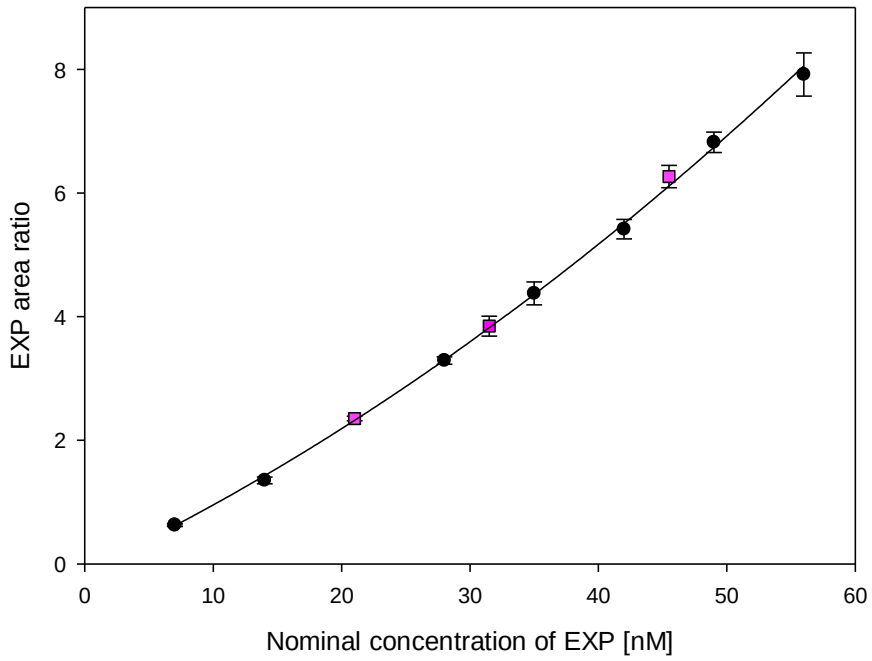


*Figure 19: A representative standard curve for EXP showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 7-56 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 7 nM is both a STD and a QC.*

*Table 9: Calculated intra- and inter-day accuracy and precision (% CV) for EXP.*

| | | Intra-day (n=5) | | Inter-day (n=15) | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 7.0 | 2.0 | 102 | 3.4 | 100 |
| **QC1** | 7.0 | 1.9 | 102 | 3.1 | 101 |
| **STD2** | 14.0 | 1.5 | 96.5 | 4.0 | 96.8 |
| **QC2** | 21.0 | 0.78 | 102 | 1.8 | 100 |
| **STD3** | 28.0 | 0.53 | 101 | 1.8 | 98.9 |
| **QC3** | 31.5 | 1.5 | 101 | 4.0 | 101 |
| **STD4** | 35.0 | 1.5 | 101 | 4.3 | 99.4 |
| **STD5** | 42.0 | 2.2 | 99.3 | 2.9 | 99.5 |
| **QC4** | 45.5 | 1.3 | 102 | 2.6 | 101 |
| **STD6** | 49.0 | 0.30 | 101 | 2.4 | 100 |
| **STD7** | 56.0 | 0.68 | 99.1 | 4.4 | 98.5 |

**Omeprazole**

A representative standard curve for OME can be seen in figure 20, concentration range 1500-12000 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 10. Non-linear regression gave the equation $f= -1.9+0.0053x+(1.1\times10^{-6})x^2$.

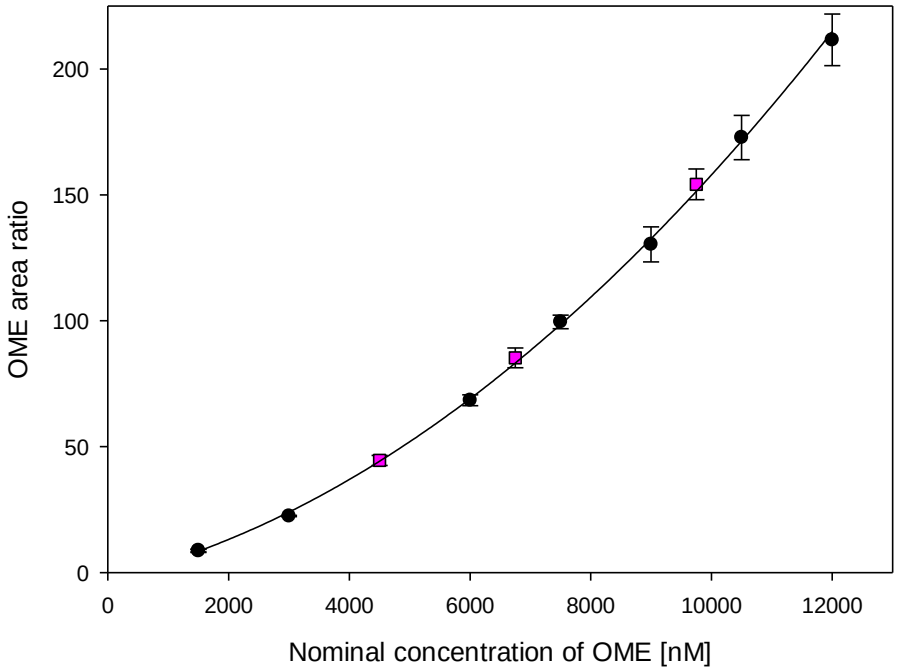


*Figure 20: A representative standard curve for OME showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 1500-12000 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 1500 nM is both a STD and a QC.*

*Table 10: Calculated intra- and inter-day accuracy and precision (% CV) for OME.*

| | | Intra-day (n=5) | | Inter-day (n=15) | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 1500 | 1.3 | 102 | 6.0 | 102 |
| **QC1** | 1500 | 1.6 | 101 | 7.3 | 101 |
| **STD2** | 3000 | 1.5 | 96.7 | 5.2 | 97.0 |
| **QC2** | 4500 | 1.6 | 102 | 4.5 | 99.9 |
| **STD3** | 6000 | 1.1 | 100 | 3.1 | 99.0 |
| **QC3** | 6750 | 1.0 | 102 | 4.6 | 101 |
| **STD4** | 7500 | 1.4 | 101 | 2.7 | 100 |
| **STD5** | 9000 | 1.7 | 99.4 | 5.3 | 101 |
| **QC4** | 9750 | 1.1 | 101 | 4.0 | 101 |
| **STD6** | 10500 | 0.40 | 101 | 5.1 | 101 |
| **STD7** | 12000 | 0.20 | 99.3 | 4.8 | 98.9 |

**5-OH-omeprazole**

A representative standard curve for OHO can be seen in figure 21, concentrations range 25-200 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 11. Non-linear regression gave the equation $f = -0.19+0.0035x+(1.0\times10^{-4})x^2$.

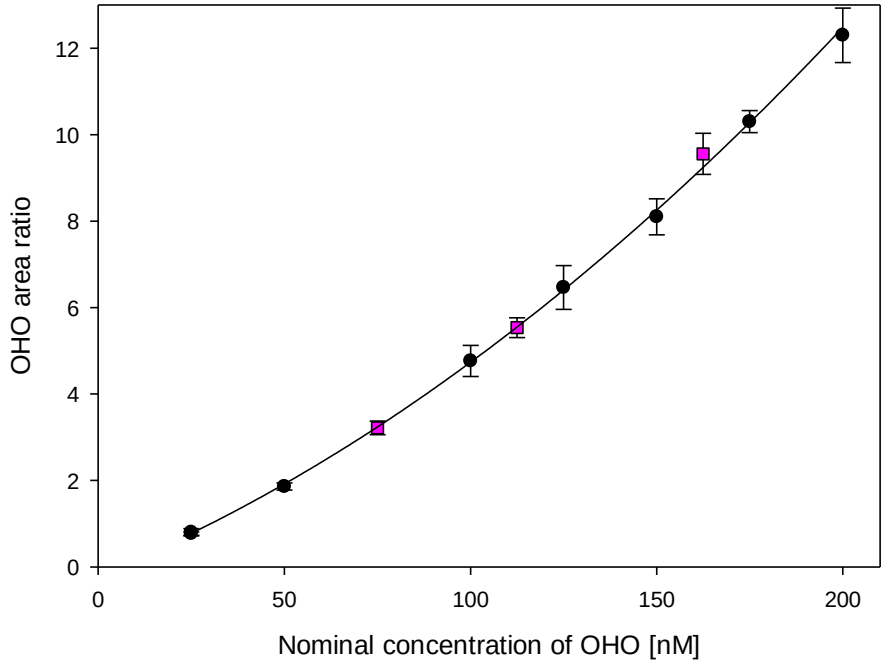


*Figure 21: A representative standard curve for OHO showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 25-200 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 25 nM is both a STD and a QC.*

*Table 11: Calculated intra- and inter-day accuracy and precision (% CV) for OHO.*

| | | **Intra-day (n=5)** | | **Inter-day (n=15)** | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 25.0 | 2.3 | 102 | 11 | 102 |
| **QC1** | 25.0 | 1.1 | 98.8 | 4.9 | 101 |
| **STD2** | 50.0 | 3.2 | 97.4 | 4.5 | 98.7 |
| **QC2** | 75.0 | 3.5 | 99.5 | 4.9 | 103 |
| **STD3** | 100.0 | 5.5 | 101 | 7.5 | 102 |
| **QC3** | 112.5 | 4.4 | 100 | 5.1 | 104 |
| **STD4** | 125.0 | 5.8 | 101 | 7.8 | 103 |
| **STD5** | 150.0 | 4.9 | 99.1 | 5.1 | 102 |
| **QC4** | 162.5 | 6.4 | 103 | 7.0 | 106 |
| **STD6** | 175.0 | 6.5 | 101 | 7.5 | 103 |
| **STD7** | 200.0 | 6.7 | 99.6 | 8.1 | 102 |

**Dextromethorphan**

A representative standard curve for DXM can be seen in figure 22, concentration range 1500-12000 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 12. Non-linear regression gave the equation $f = 21.6+0.0805x+(1.39\times10^{-6})x^2$.

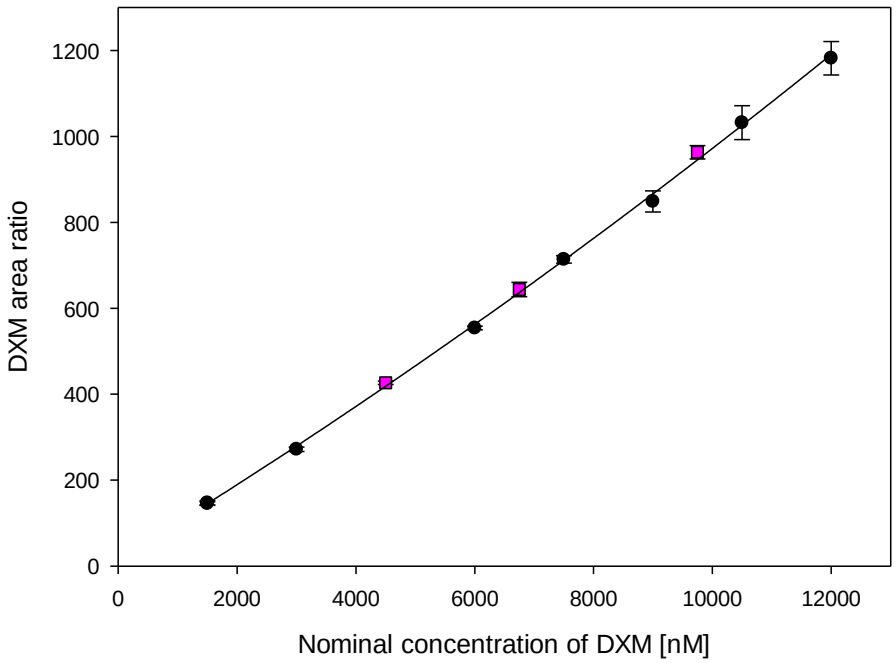


*Figure 22: A representative standard curve for DXM showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 1500-12000 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 1500 nM is both a STD and a QC.*

*Table 12: Calculated intra- and inter-day accuracy and precision (% CV) for DXM.*

| | | **Intra-day (n=5)** | | **Inter-day (n=15)** | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 1500 | 1.3 | 101 | 3.4 | 100 |
| **QC1** | 1500 | 0.84 | 100 | 2.8 | 101 |
| **STD2** | 3000 | 1.7 | 98.5 | 1.9 | 99.0 |
| **QC2** | 4500 | 0.90 | 103 | 2.0 | 102 |
| **STD3** | 6000 | 0.73 | 99.8 | 1.8 | 98.6 |
| **QC3** | 6750 | 1.0 | 102 | 2.6 | 101 |
| **STD4** | 7500 | 2.1 | 101 | 3.0 | 99.0 |
| **STD5** | 9000 | 1.9 | 98.9 | 2.9 | 100 |
| **QC4** | 9750 | 1.2 | 102 | 1.6 | 102 |
| **STD6** | 10500 | 0.24 | 101 | 3.8 | 100 |
| **STD7** | 12000 | 0.11 | 99.5 | 3.3 | 99.3 |

**Dextrorphan**

A representative standard curve for DEX can be seen in figure 23, concentration range 37.5-300 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 13. Non-linear regression gave the equation $f = -0.52+0.096x+(3.0\times10^{-4})x^2$.

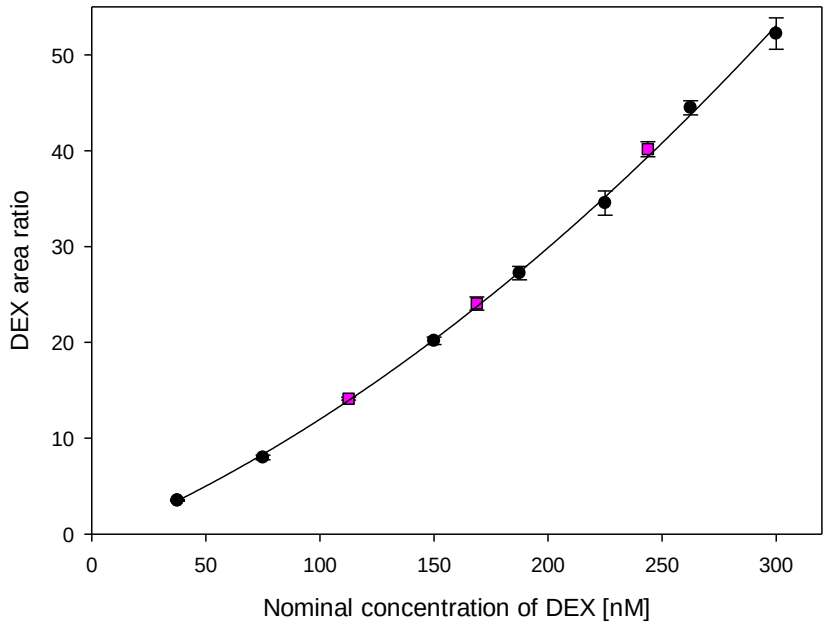


*Figure 23: A representative standard curve for DEX showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 37.5-300 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 37.5 nM is both a STD and a QC.*

*Table 13: Calculated intra- and inter-day accuracy and precision (% CV) for DEX.*

| | | **Intra-day (n=5)** | | **Inter-day (n=15)** | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 37.5 | 1.4 | 101 | 2.7 | 101 |
| **QC1** | 37.5 | 2.3 | 101 | 4.1 | 101 |
| **STD2** | 75.0 | 1.0 | 97.8 | 3.0 | 96.0 |
| **QC2** | 112.5 | 2.5 | 102 | 3.1 | 99.6 |
| **STD3** | 150.0 | 1.7 | 101 | 1.9 | 98.4 |
| **QC3** | 168.8 | 3.1 | 102 | 4.9 | 99.2 |
| **STD4** | 187.5 | 2.3 | 100 | 2.6 | 98.7 |
| **STD5** | 225.0 | 3.8 | 99.2 | 4.6 | 99.5 |
| **QC4** | 243.8 | 3.7 | 102 | 3.9 | 99.2 |
| **STD6** | 262.5 | 2.0 | 102 | 3.7 | 99.6 |
| **STD7** | 300.0 | 2.6 | 99.1 | 3.1 | 97.9 |

**Midazolam**

A representative standard curve for MID can be seen in figure 24, concentration range 1500-12000 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 14. Non-linear regression gave the equation $f = -7.6+0.014x+(1.3\times10^{-6})x^2$.

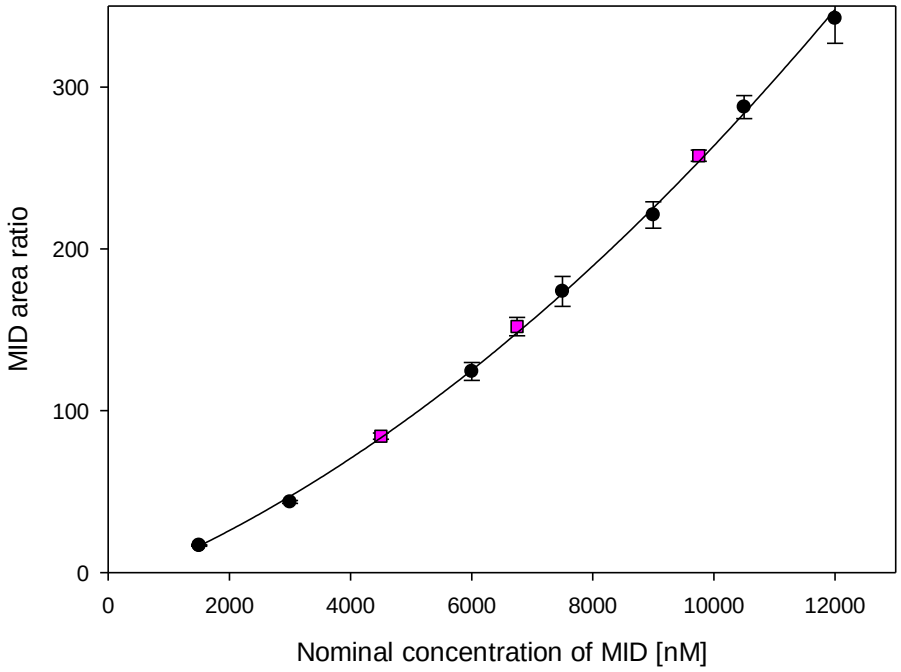


*Figure 24: A representative standard curve for MID showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 1500-12000 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 1500 nM is both a STD and a QC.*

*Table 14: Calculated intra- and inter-day accuracy and precision (%CV) for MID.*

| | | Intra-day (n=5) | | Inter-day (n=15) | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 1500 | 1.4 | 102 | 1.8 | 101 |
| **QC1** | 1500 | 1.7 | 101 | 2.4 | 101 |
| **STD2** | 3000 | 1.6 | 96.2 | 2.0 | 95.4 |
| **QC2** | 4500 | 0.93 | 102 | 2.3 | 99.9 |
| **STD3** | 6000 | 0.72 | 101 | 4.5 | 99.6 |
| **QC3** | 6750 | 0.54 | 103 | 3.8 | 102 |
| **STD4** | 7500 | 1.4 | 101 | 5.3 | 99.9 |
| **STD5** | 9000 | 1.8 | 99.2 | 3.7 | 100 |
| **QC4** | 9750 | 1.5 | 101 | 1.3 | 101 |
| **STD6** | 10500 | 0.42 | 101 | 2.5 | 100 |
| **STD7** | 12000 | 0.47 | 99.1 | 4.5 | 98.6 |

**1-OH-midazolam**

A representative standard curve for OHM can be seen in figure 25, concentration range 25-200 nM. Calculation of intra- and inter-day accuracy and precision (% CV) can be seen in table 15. Non-linear regression gave the equation $f = -0.036+0.014x+(6.9\times10^{-5})x^2$.

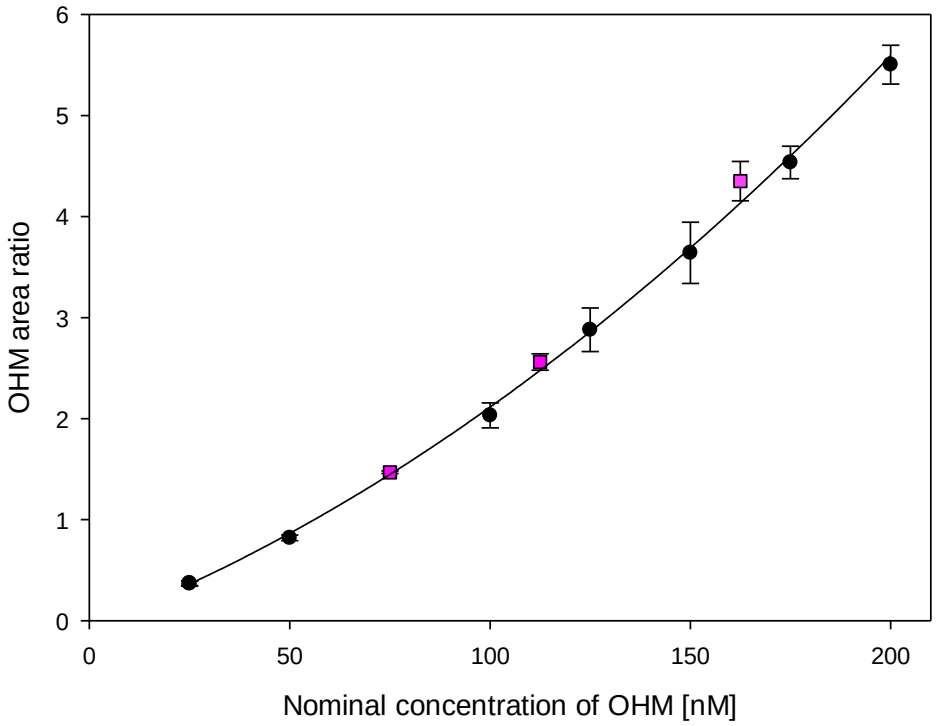


*Figure 25: A representative standard curve for OHM showing mean area ratio with standard deviation from 5 replicate determination of the same concentration (n=5) against nominal concentrations from 25-200 nM. The circles represent the STDs and the pink squares represent the QCs. The lowest concentration at LLOQ 25 nM is both a STD and a QC.*

*Table 15: Calculated intra- and inter-day accuracy and precision (% CV) for OHM.*

| | | **Intra-day (n=5)** | | **Inter-day (n=15)** | |
|---|---|---|---|---|---|
| **Sample** | **Concentration (nM)** | **CV (%)** | **Accuracy (%)** | **CV (%)** | **Accuracy (%)** |
| **STD1** | 25.0 | 1.5 | 102 | 7.2 | 101 |
| **QC1** | 25.0 | 1.1 | 103 | 6.2 | 101 |
| **STD2** | 50.0 | 1.2 | 97.9 | 3.4 | 96.5 |
| **QC2** | 75.0 | 1.3 | 103 | 1.9 | 101 |
| **STD3** | 100.0 | 1.4 | 98.9 | 6.1 | 98.5 |
| **QC3** | 112.5 | 1.4 | 104 | 3.2 | 101 |
| **STD4** | 125.0 | 1.4 | 102 | 7.5 | 99.7 |
| **STD5** | 150.0 | 1.7 | 100 | 8.3 | 101 |
| **QC4** | 162.5 | 1.4 | 105 | 4.5 | 102 |
| **STD6** | 175.0 | 0.34 | 99.9 | 3.6 | 99.3 |
| **STD7** | 200.0 | 0.45 | 99.6 | 3.5 | 99.0 |

## 4.2 Pilot study

The pilot study was performed to test the substrates, the concentration of the liver microsomes and the incubation time. The conditions were as mentioned in chapter 3.7.1. The results are illustrated by area ratio of CYP produced metabolite (met/IS).

The chromatogram for a test solution containing 0.2 µM of each analyte with their retention time can be seen in figure 26.

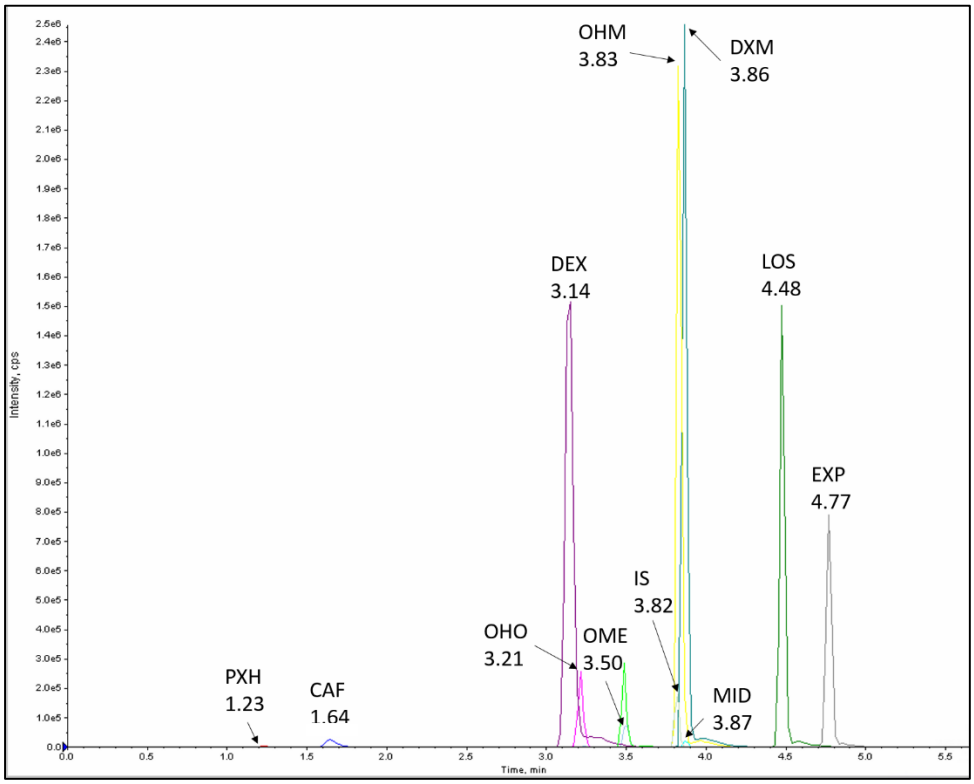


*Figure 26: Chromatogram of the substrates CAF*, LOS, OME, DXM and MID, and the metabolites PXH*, EXP, OHO, DEX and OHM with retention time [min].*

*Not included in the final setup.

### *4.2.1 HLM concentration*

Table 16 shows area ratio of CYP produced metabolites compared for two HLM concentrations (0.2 and 0.4 mg proteins/mL) incubated for 15 minutes.

*Table 16: Area ratio (metabolite/IS) of CYP produced metabolite after incubation with 20 µM CAF*, 1 µM LOS, 0.2 µM OME, 0.2 µM DXM and 2 µM MID for 15 minutes with 0.2 and 0.4 mg proteins/mL HLM. Data shown as mean ± SD (n=3).*

| | **Area ratio** | |
|---|---|---|
| **HLM conc. [mg/mL]** | **0.2** | **0.4** |
| **PXH*** | 0 | 0 |
| **EXP** | 0.26 ± 0.06 | 0.58 ± 0.07 |
| **OHO** | 0.12 ± 0.08 | 0.14 ± 0.04 |
| **DEX** | 0.74 ± 0.1 | 1.7 ± 0.2 |
| **OHM** | 1.3 ± 0.4 | 1.3 ± 0.5 |

*Not included in the final setup.

### *4.2.2 Incubation time*

The incubation time was tested by comparing the area ratio of CYP produced metabolites for an incubation time of 15 and 25 minutes with 0.4 mg proteins/mL HLM, the results can be seen in table 17.

*Table 17: Area ratio (metabolite/IS) of CYP produced metabolite after incubation with 20 µM CAF*, 1 µM LOS, 0.2 µM OME, 0.2 µM DXM and 2 µM MID for 15 and 25 minutes with 0.4 mg proteins/mL HLM. Data shown as mean ± SD (n=3).*

| | **Area ratio** | |
|---|---|---|
| **Time [min]** | **15** | **25** |
| **PXH*** | 0 | 0 |
| **EXP** | 0.58 ± 0.07 | 0.97 ± 0.1 |
| **OHO** | 0.14 ± 0.04 | 0.32 ± 0.02 |
| **DEX** | 1.7 ± 0.2 | 1.9 ± 0.1 |
| **OHM** | 1.3 ± 0.5 | 1.2 ± 0.1 |

*Not included in the final setup.

The decrease in CYP produced OHM is not significant ($p = 0.7$, n.s), this will be discussed further in section 5.2.

### *4.2.3 Effect of $MgCl_2$*

$MgCl_2$ (3 mM) were added to the incubation mixture, the area ratio of CYP produced metabolites after 25 minutes of incubation with 0.4 mg proteins/mL HLM can be seen in table 18.

*Table 18: Area ratio (metabolite/IS) of CYP produced metabolite after incubation with 20 µM CAF*, 1 µM LOS, 0.2 µM OME, 0.2 µM DXM and 2 µM MID for 25 minutes with 0.4 mg proteins/mL HLM without and with 3mM $MgCl_2$ added. Data shown as mean ± SD (n=3).*

| | **Area ratio** | |
|---|---|---|
| | **Control** | **$MgCl_2$** |
| **PXH*** | 0 | 0 |
| **EXP** | 0.92 ± 0.2 | 1.2 ± 0.06 |
| **OHO** | 0.12 ± 0.01 | 0.12 ± 0.03 |
| **DEX** | 2.5 ± 0.4 | 2.3 ± 0.2 |
| **OHM** | 1.1 ± 0.08 | 1.1 ± 0.04 |

*Not included in the final setup.

### *4.2.4 Increasing substrate concentration*

The concentrations of OME, DXM, MID and LOS were increased to 10 µM in the incubation mix. The concentration of CAF remained the same at 20 µM. The area ratio of CYP produced metabolite after 20 minutes of incubation with 0.4 mg proteins/mL HLM can be seen in table 19.

*Table 19: Area ratio (metabolite/IS) of CYP produced metabolite after incubation with 20 µM CAF*, 10 µM OME, DXM, MID and LOS for 20 minutes with 0.4 mg proteins/mL HLM. Data shown as mean ± SD (n=3).*

| | **Area ratio** |
|---|---|
| **PXH*** | 0 |
| **EXP** | 1.0 ± 0.03 |
| **OHO** | 0.95 ± 0.09 |
| **DEX** | 20 ± 1 |
| **OHM** | 16 ± 1 |

*Not included in the final setup.

Since the experiment with increased substrate concentrations were incubated for 20 minutes, the area ratio of CYP produced metabolite could not be directly compared with previous experiments. To assure that there was an increase in metabolite production, enzyme activity was compared by using the standard curve equation for each respective metabolite to calculate the concentrations. The calculated enzyme activity can be seen in table 20. Calculations were not performed for PXH.

*Table 20: Calculated enzyme activity (nmol metabolite/(mg proteins×min)) based on the experiment with low substrate concentration; 1 µM LOS, 0.2 µM OME, 0.2 µM DXM and 2 µM MID for 25 minutes with 0.4 mg proteins/mL HLM, and with increased substrate concentrations; 10 µM OME, DXM, MID and LOS for 20 minutes with 0.4 mg proteins/mL HLM.*

| **Enzyme activity [nm/(mg proteins×min)]** | **Low substrate conc.** | **Increased substrate conc.** |
|---|---|---|
| **CYP2C9** | 1.0 | 1.3 |
| **CYP2C19** | 1.4 | 3.6 |
| **CYP2D6** | 2.3 | 18 |
| **CYP3A4** | 6.5 | 49 |

### *4.2.5 Replacing caffeine with phenacetin*

CAF was replaced with 20 µM PHE, that metabolizes to ACE. 20 µM PHE was incubated with 10 µM of OME, DXM, MID and LOS for 20 minutes with 0.2 and 0.4 mg proteins/mL, respectively. The MS classified two peaks as ACE; one eluting after 1.28 minutes and the other after 3.9 minutes, as can be seen in figure 27. The first peak at 1.28 minutes was identified to be representative for ACE. The area of CYP produced metabolites from the incubations can be seen in table 21.

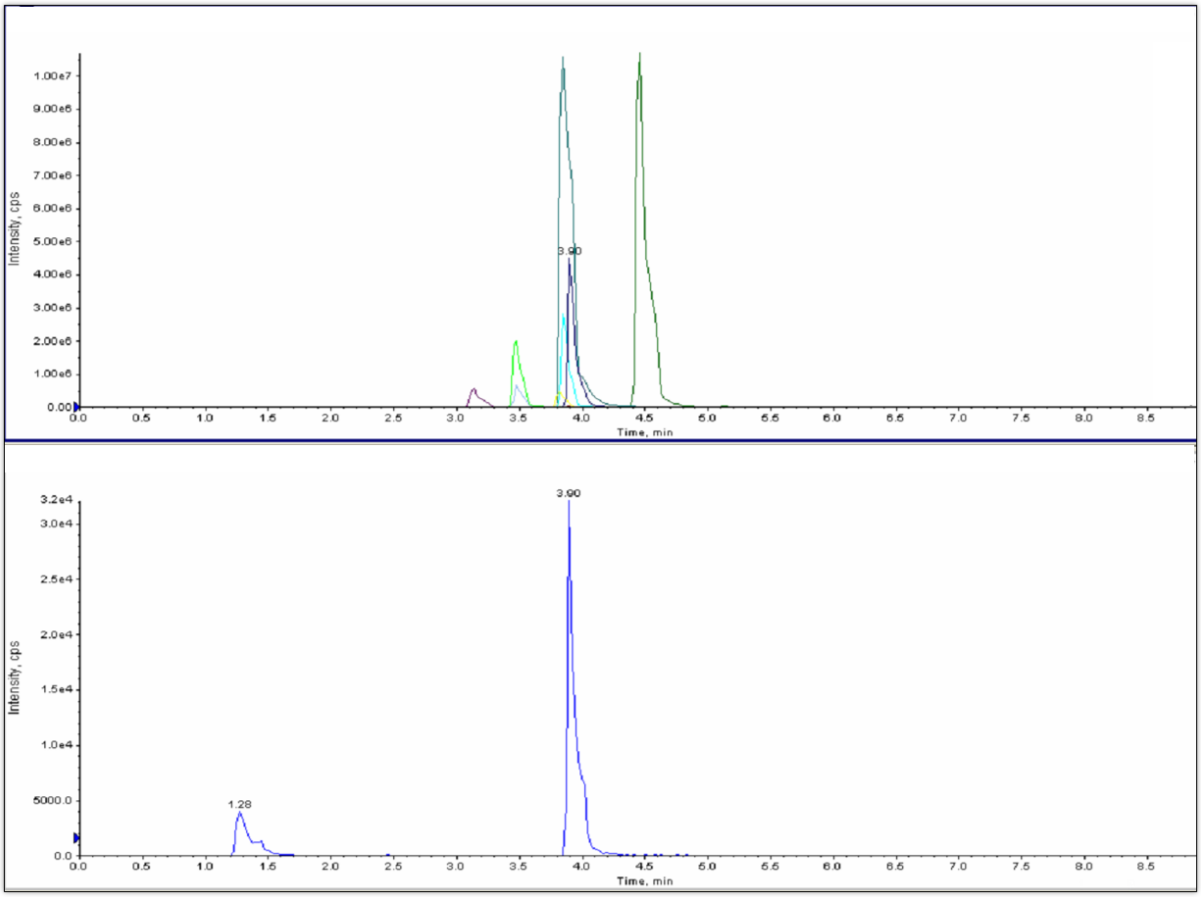


*Figure 27: The upper chromatogram from the incubation with 20 µM PHE, incubated with 10 µM of OME, DXM, MID and LOS for 20 minutes with 0.4 mg proteins/mL. The lower chromatogram shows peak extraction of the two ACE peaks.*

*Table 21. Area ratio (metabolite/IS) of CYP produced metabolite after incubation with 20 µM PHE, 10 µM OME, DXM, MID and LOS for 20 minutes with 0.2 and 0.4 mg proteins/mL HLM. Data shown as mean ± SD (n=3).*

| | **Area ratio** | |
|---|---|---|
| **HLM conc. [mg/ml]** | **0.2** | **0.4** |
| **ACE** | 0.11 ± 0.003 | 0.19 ± 0.03 |
| **EXP** | 0.35 ± 0.02 | 0.75 ± 0.1 |
| **OHO** | 0.40 ± 0.02 | 0.90 ± 0.04 |
| **DEX** | 12 ± 0.7 | 20 ± 1 |
| **OHM** | 8.8 ± 0.6 | 18 ± 2 |

### *4.2.6 Substrate consumption*

The amount of substrate (sub/IS) left in the incubation solutions after metabolism were compared to a sample without NADPH, and the percentage substrate used was calculated, as can be seen in table 22.

*Table 22: Calculated percentage consumption of substrate based on (substrate/IS) from samples without (n=1) and with NADPH (n=3). Data shown as mean ± SD.*

| | **Without NADPH** | **With NADPH** | **Consumed [%]** |
|---|---|---|---|
| **PHE** | 59 | 53 ± 4 | 10 |
| **LOS** | 250 | 214 ± 7.4 | 14 |
| **OME** | 9.4 | 8.1 ± 0.2 | 13 |
| **DXM** | 282 | 261 ± 22 | 7.6 |
| **MID** | 48 | 23 ± 4 | 52 |

### *4.2.7 Inhibition test*

Specific inhibitors of CYP2D6 and CYP3A4, quinidine and ketoconazole, were added to the incubation mixture with concentrations of 0.2 µM to test if an inhibition could be obtained and detected using the developed method. The area ratio of CYP produced metabolites can be seen in table 23.

*Table 23: Area ratio of metabolite/IS after incubation with 20 µM PHE 10 µM of OME, DXM, MID and LOS for 20 minutes with 0.4 mg proteins/mL HLM. 0.2 µM quinidine and ketoconazole were added as specific inhibitors of CYP2D6 and CYP3A4 respectively. The gray boxes show the inhibited enzymes. Data shown as mean ± SD (n=3).*

| | **Area ratio (met/IS)** | | |
|---|---|---|---|
| | **Control** | **Quinidine** | **Ketoconazole** |
| **ACE** | 0.19 ± 0.03 | 0.19 ± 0.02 | 0.21 ± 0.04 |
| **EXP** | 0.75 ± 0.1 | 0.88 ± 0.1 | 0.43 ± 0.07 |
| **OHO** | 0.90 ± 0.04 | 0.90 ± 0.1 | 0.47 ± 0.03 |
| **DEX** | 20 ± 1 | 8.9 ± 0.6 | 20 ± 1 |
| **OHM** | 18 ± 2 | 18 ± 1 | 6.8 ± 2 |

An inhibition of CYP2C9 and CYP2C19, in addition to CYP3A4, in the ketoconazole samples can be observed. This inhibition is probably due to MeOH, since the ketoconazole stock solution was prepared with MeOH, thus having a higher concentration of MeOH than the quinidine and control samples, this will be discussed further in section 5.2.1.

### *4.2.8 HPLC mobile phase*

Both ACN and MeOH were tested as the organic solvent of the mobile phase for the LC-MSMS method. The area ratios of metabolites were compared for four STD solutions (table 24). The chromatogram for the test solution with concentration 200 nM for each analyte, showing all substrates and metabolites eluted with MeOH as organic phase can be seen in figure 28. Extracted peaks for all analytes can be seen in appendix B.

*Table 24: Comparing detected metabolite for four standard solutions using ACN and MeOH respectively as organic solvent in the mobile phase (n=1).*

| | **ACE** | | **EXP** | | **OHO** | | **DEX** | | **OHM** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **conc. (nM)** | **ACN** | **MeOH** | **ACN** | **MeOH** | **ACN** | **MeOH** | **ACN** | **MeOH** | **ACN** | **MeOH** |
| **200** | 0.16 | 0.25 | 5.1 | 7.0 | 0.85 | 2.1 | 16 | 13 | 19 | 41 |
| **100** | 0.10 | 0.13 | 2.6 | 3.5 | 0.43 | 1.1 | 8.6 | 6.7 | 8.4 | 17 |
| **20** | 0.020 | 0.030 | 0.52 | 0.68 | 0.090 | 0.23 | 1.9 | 1.5 | 1.5 | 2.8 |
| **10** | 0.010 | 0.020 | 0.25 | 0.34 | 0.040 | 0.11 | 0.97 | 0.76 | 0.78 | 1.4 |

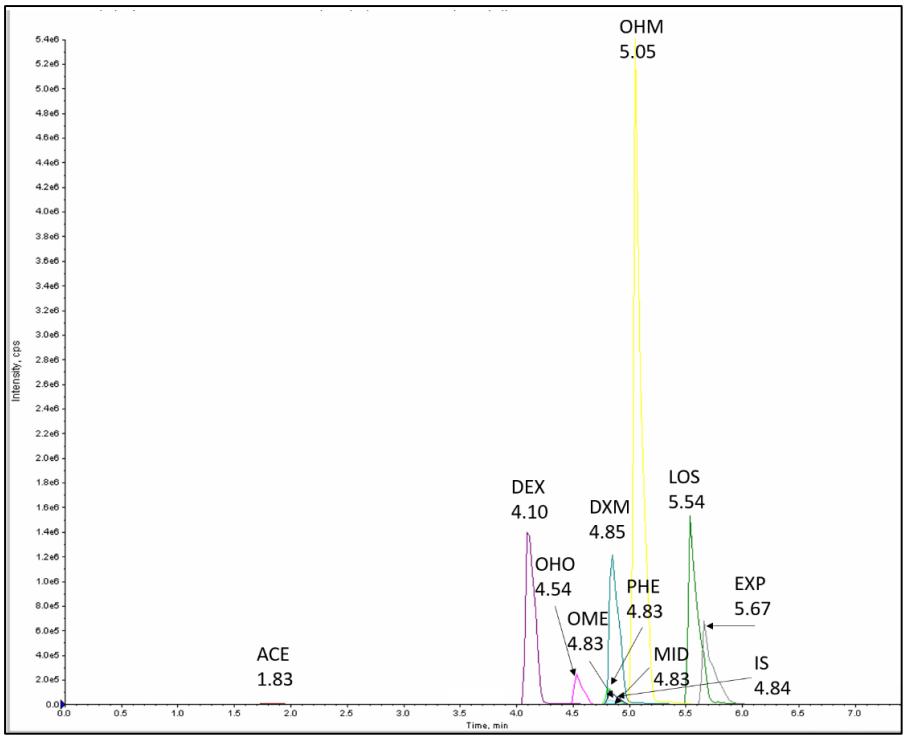


*Figure 28: Chromatogram of substrates and metabolites with their retention time [min] after elution with MeOH as organic solvent in the mobile phase.*

The incubation conditions chosen for further optimization and validation were: 20 µM PHE, 10 µM LOS, 10 µM OME, 10 µM DXM and 10 µM MID, incubation for 20 minutes with 0.4 mg proteins/mL HLM. The incubation mixture also contained 1 mM NADPH, and KPO-buffer to a total volume of 200 µL. It was decided to use MeOH as organic solvent in the HPLC mobile phase.

## 4.3 Optimization and validation of the incubation method

The incubation method was optimized by incubating three concentrations of substrates and HLM for three different lengths of time. The incubation conditions, as described in chapter 3.7.2 were used, changing one parameter at a time. To further validate the method one substrate at a time were incubated and compared to a control that was incubated with all five substrates. The inhibitory potential of ethanol in the incubation mix was also investigated.

### *4.3.1 Substrate concentration*

The incubation was performed with three different concentrations of each substrate, as written in table 5. The incubation lasted for 20 minutes with 0.4 mg proteins/mL HLM. The area ratios of met/IS are plotted against the substrate concentration in figure 29.

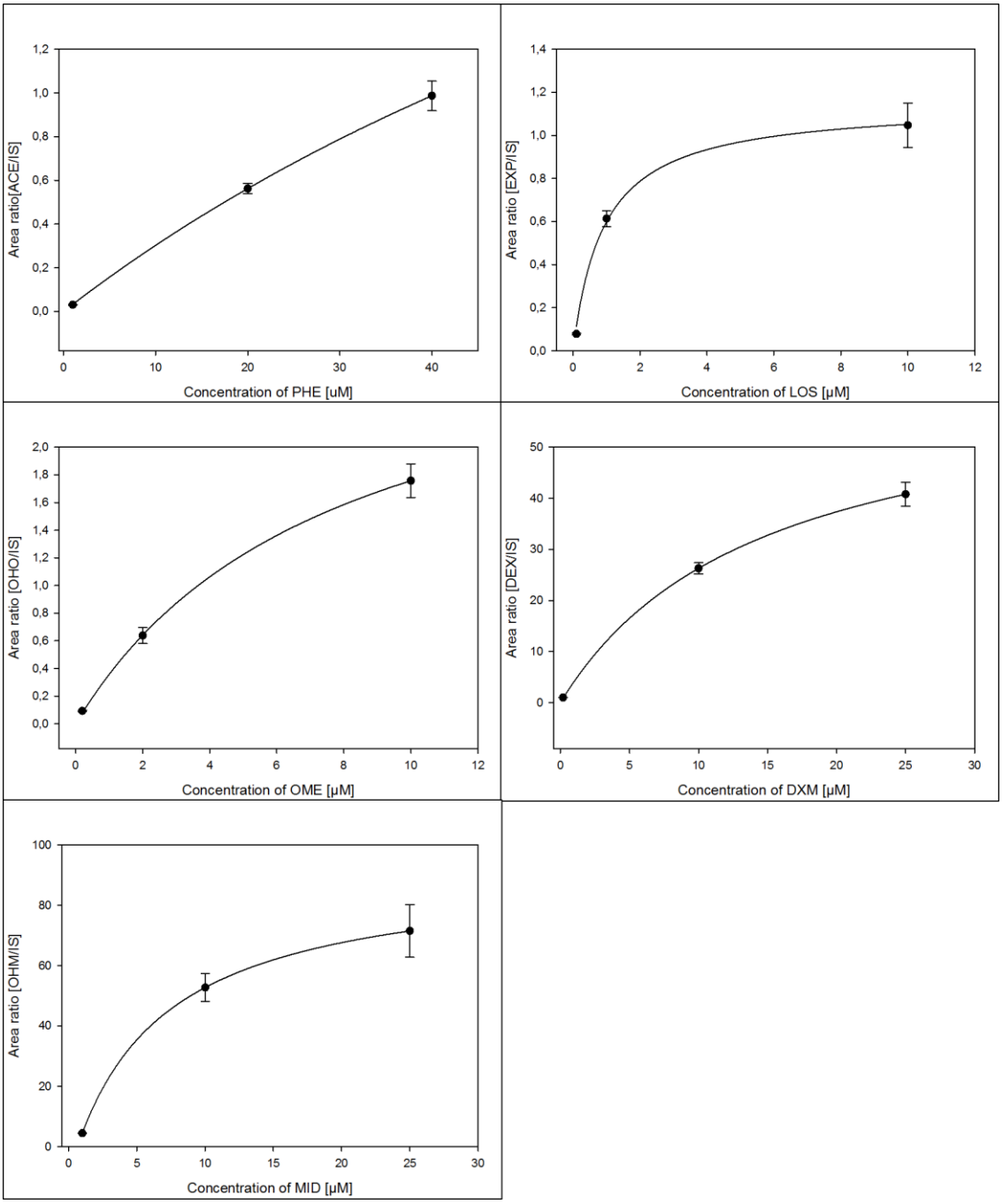


*Figure 29: 0.4 mg proteins/mL HLM were incubated for 20 minutes with three concentrations of each substrate: 1, 20 and 40 µM PHE, 0.1, 1 and 10 µM LOS, 0.2, 2 and 10 µM OME, 0.2, 10 and 25 µM DXM, 1, 10 and 25 µM MID. The graphs are showing mean ± SD (n=3) of area ratio (metabolite/IS) against substrate concentration.*

Based on the substrate concentration experiments an approximate Michalis constant, $K_m$, was calculated for each enzyme. The calculations were done by plotting the invers of enzyme activity against the invers of substrate concentration in a Lineweaver-Burke plot, and the $K_m$ vas represented by the value where the linear line crossed the x-axis (y = 0). The Lineweaver-Burke plots can be seen in appendix C. The calculated $K_m$-values can be seen in table 25.

*Table 25: Experimental $K_m$-values for each substrate in the cocktail.*

| | **$K_m$** |
|---|---|
| **PHE** | 6.7 |
| **LOS** | 0.51 |
| **OME** | 0.76 |
| **DXM** | 3.1 |
| **MID** | 5.9 |

### *4.3.2 HLM concentration*

The incubation was performed with three concentrations of HLM: 0.1, 0.2 and 0.4 mg proteins/mL. The incubation lasted for 20 minutes with 20 µM PHE, 2 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID. The area ratio of met/IS for the different metabolites are plotted against the concentration of HLM in figure 30. Each plot had a $r^2$-value above 0.99.

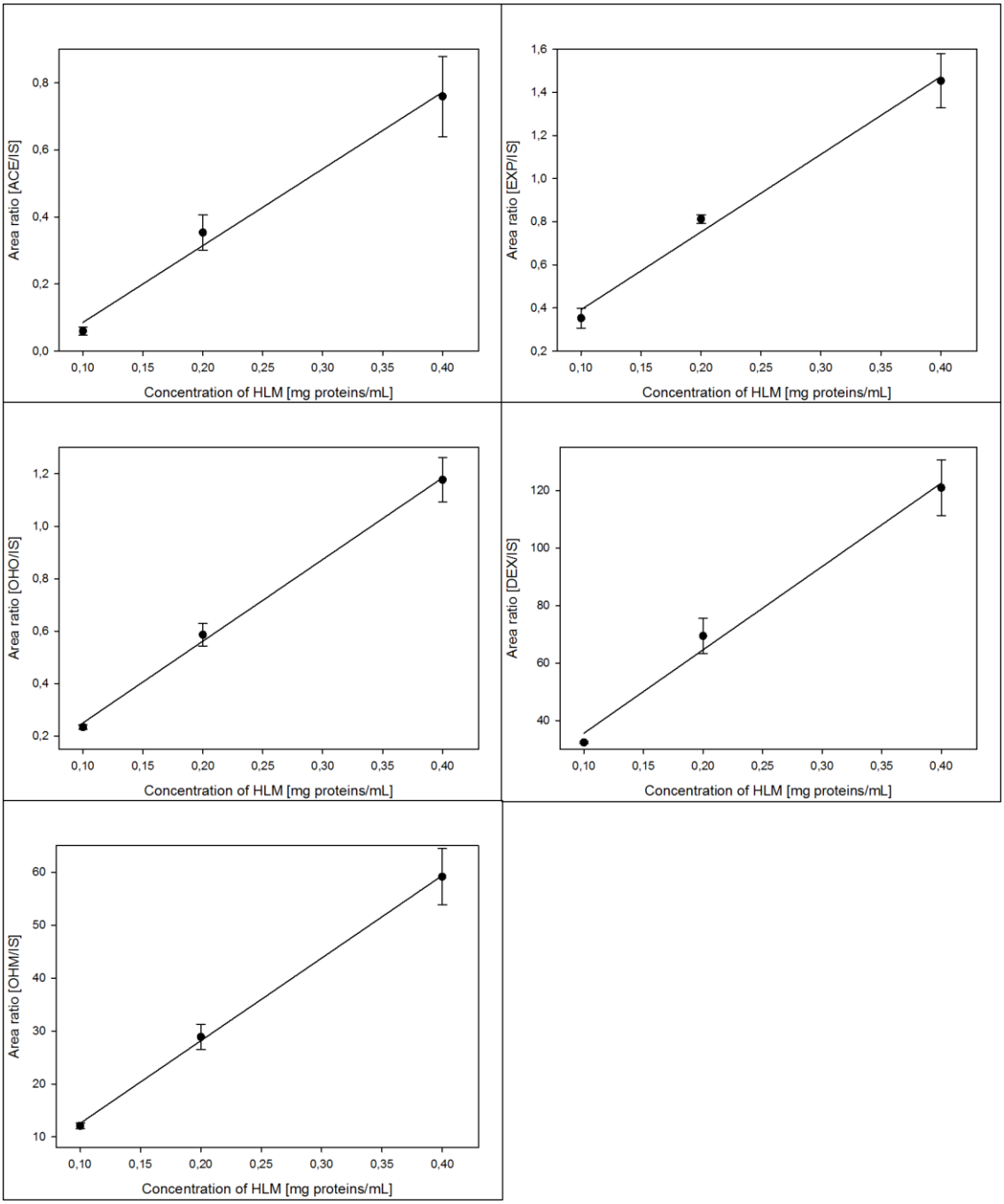


*Figure 30: 20 µM PHE, 2 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID were incubated for 20 minutes with three concentrations of HLM: 0.1, 0.2 and 0.4 mg proteins/mL. The graphs are showing mean ± SD (n=3) of area ratio (metabolite/IS) against concentration of HLM.*

### *4.3.3 Incubation time*

The incubation was performed at three different time intervals: 10, 20 and 30 minutes with 0.4 mg proteins/mL and 20 µM PHE, 2 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID. The area ratio of met/IS of the different metabolites are plotted against the time in figure 31. The $r^2$-value was above 0.985 for all metabolites. The CV was below 15% for all calculated means.

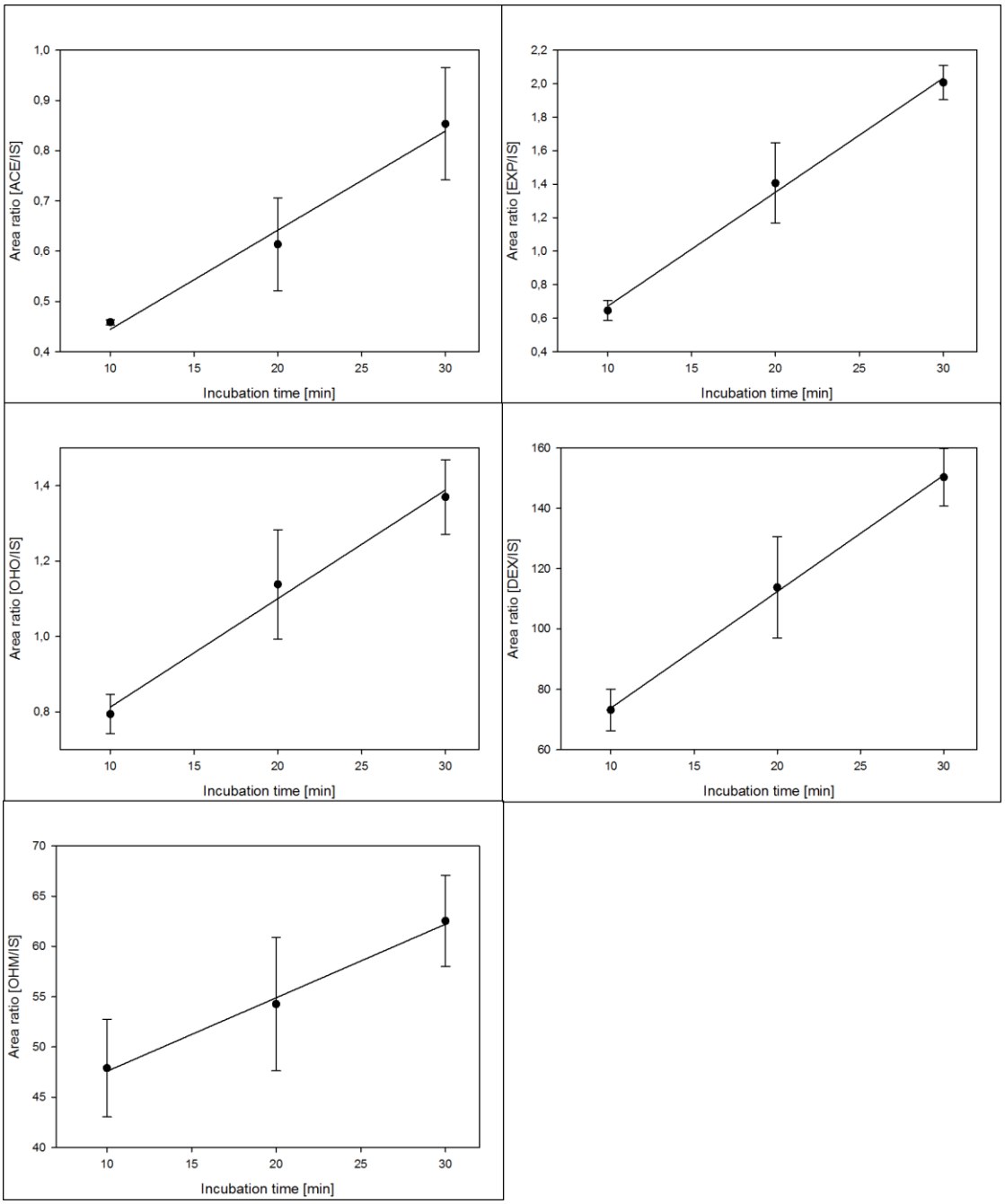


*Figure 31: 20 µM PHE, 2 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID were incubated with 0.4 mg proteins/mL HLM with three time intervals: 10, 20 and 30 minutes. The graphs are showing mean ± SD (n=3) of area ratio (metabolite/IS) against incubation time.*

### *4.3.4 Substrate specificity*

One substrate at a time was incubated alone and the amount of CYP produced metabolite was compared to a control containing all substrates in a mix shown in figure 32.

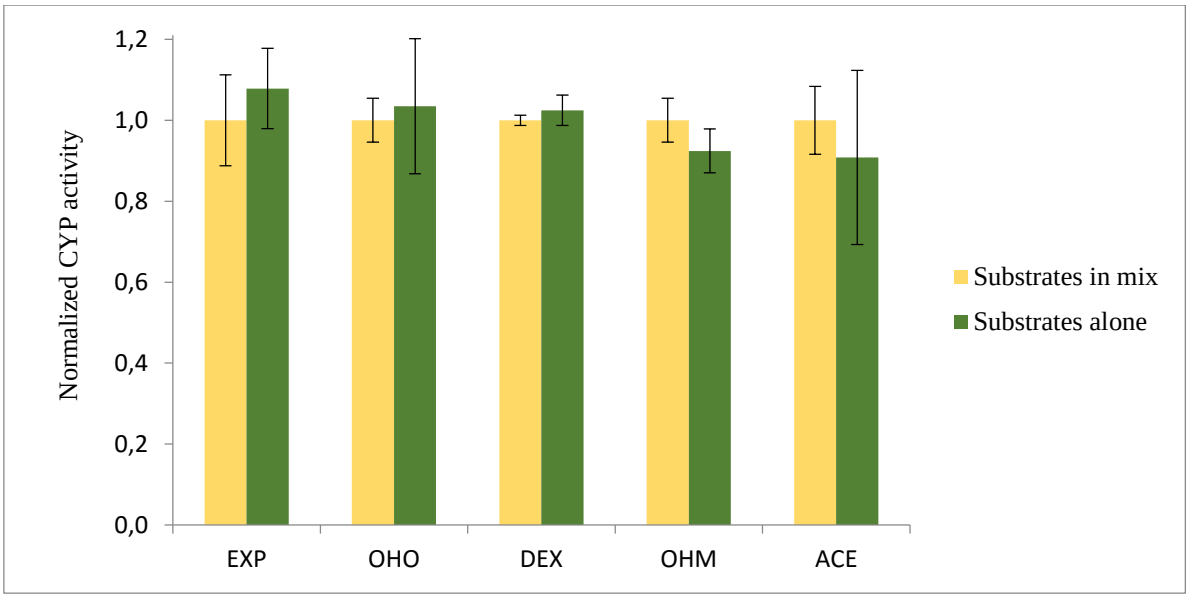


*Figure 32: Comparing the normalized values of CYP production of metabolites when substrates were incubated alone with substrates in a mix. Data shown as means ± SD (n=3).*

A two-sided paired student's t-test showed that the CYP production of metabolites in the control and alone did not differ significantly ($p \geq 0.05$, n.s)

### *4.3.5 Influence of ethanol*

The influence of ethanol on the different CYP-enzymes were investigated. The enzyme activity (% of control) for each CYP was plotted against amount of ethanol (%) in the incubation and presented as a 3D-bar chart (figure 33). Error bars are not illustrated in the figure to make it more orderly. The CV was below 15 % for all calculated means.

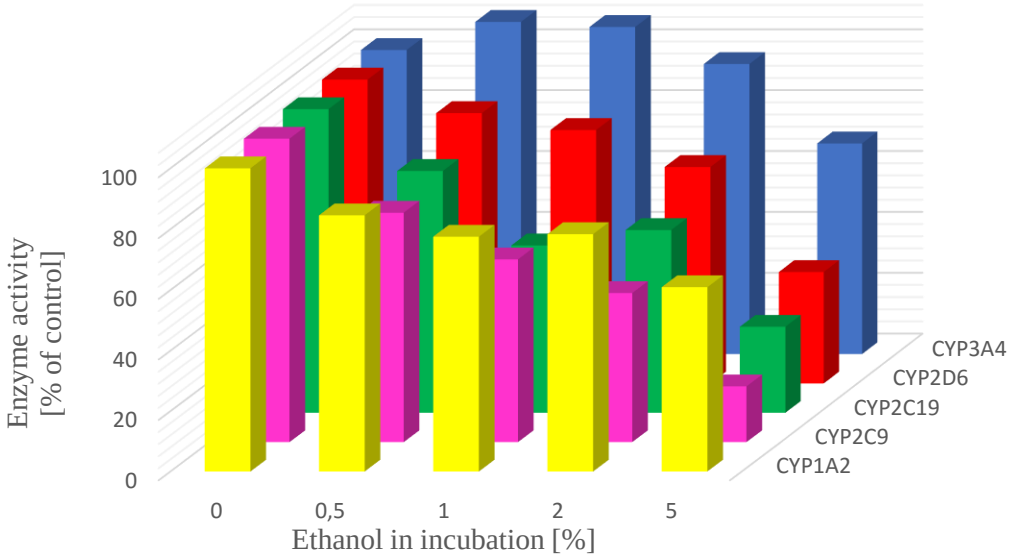


*Figure 33: The inhibitory effect of ethanol on the different CYP-enzymes investigated. The enzyme activity is based on area of mean CYP produced met/IS (n=3).*

Based on the results from the optimization and validation, the following conditions were chosen for the cocktail method: 20 µM PHE, 2 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID incubated with 0.4 mg proteins/mL for 20 minutes. When adding a herbal extract, the ethanol concentration in the cocktail should be less than 1%. Total incubation volume 200 µL.

## 4.4 Incubation with hops

Two types of hops were investigated, dried hops and the dietary supplement Hops Flowers as described in section 3.4 and 3.7.3.

### *4.4.1 Dried hops*

The weight of the dried hops, the amount of dried substance after extraction, the calculated recovery rate, the stock concentration and the diluted working concentrations for both the tea (water) extract and the ethanol extract of dried hops can be seen in table 26.

*Table 26:Data for the extraction and preparation of the dried hops stock and working solutions.*

| **Dried hops** | **Tea (water extract)** | **Ethanol extract** |
|---|---|---|
| Amount [mg] | 128.4 | 176.4 |
| Recovered [mg] | 36.0 | 52.3 |
| Recovery rate [%] | 28 | 30 |
| Stock concentration [mg/mL] | 7.20 | 26.2 |
| Working solutions [mg/mL] | 0.18-1.4 | 0.0080-0.52 |

The $IC_{50}$-curves from experiments dried hops made as tea and ethanol extracted dried hops were compared for each CYP-enzyme and the result can be seen in figure 34. The calculated mean $IC_{50}$-values for each CYP-enzyme for both tea and ethanol extract can be seen in table 27.

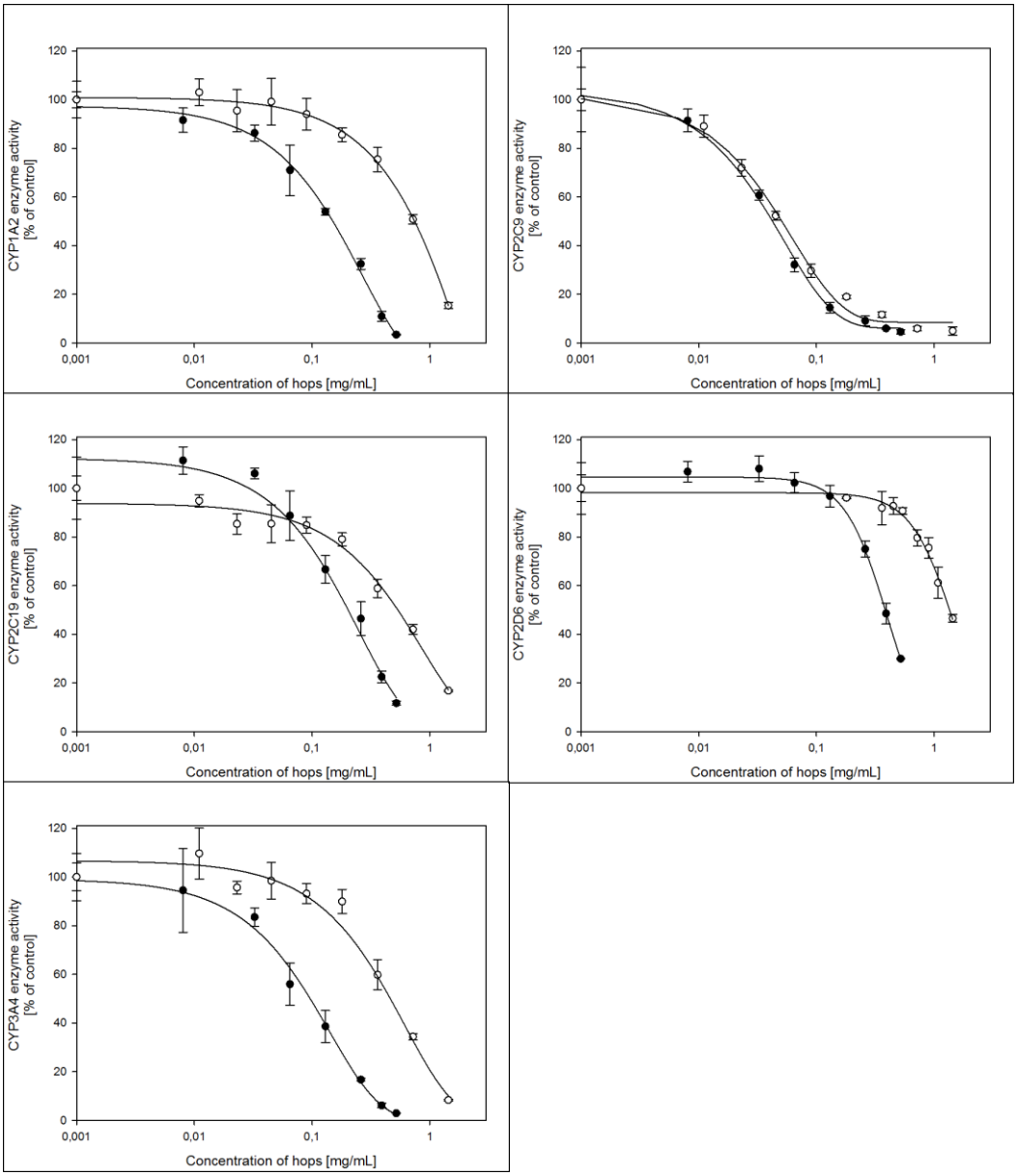


*Figure 34: Comparison of dried hops prepared as tea (white dots) and dried hops extracted with 50% ethanol (black dots) for each CYP-enzyme investigated presented as $IC_{50}$-curves from two representative experiments. Data shown as mean ± SD (n=3).*

*Table 27: $IC_{50}$-values presented as means of replicates of dried hops prepared as tea (from two experiments with three parallels of each concentration) and dried hops extracted with 50% ethanol (from three experiments with three parallels of each concentration) for each CYP-enzyme investigated.*

| | | **Tea** | **50% ethanol** |
|---|---|---|---|
| **Hops $IC_{50}$ [mg/mL]** | CYP1A2 | 0.75 ± 0.008 | 0.18 ± 0.03 |
| | CYP2C9 | 0.060 ± 0.01 | 0.046 ± 0.005 |
| | CYP2C19 | 0.66 ± 0.2 | 0.21 ± 0.06 |
| | CYP2D6 | 1.4 ± 0.01 | 0.37 ± 0.09 |
| | CYP3A4 | 0.51 ± 0.07 | 0.089 ± 0.008 |

### *4.4.2 Dietary supplement Hops Flowers*

The weight of the dietary supplement Hops Flowers, the amount of dried substance after extraction, the calculated recovery rate, the stock concentration and the diluted working concentrations for the water extract and the ethanol extract of the dietary supplement can be seen in table 28.

*Table 28: Data for the extraction and preparation of the dietary supplement Hops Flowers stock and working solutions*

| **Dietary supplement hops** | **Water extract** | **Ethanol extract** |
|---|---|---|
| Amount [mg] | 313.1 | 310.4 |
| Recovered [mg] | 76.6 | 87.7 |
| Recovery rate [%] | 25 | 28 |
| Stock concentration [mg/mL] | 25.5 | 43.9 |
| Working solutions [mg/mL] | 0.0070-2.5 | 0.0020-0.88 |

The $IC_{50}$-curves for water and ethanol extracted dietary supplement hops were compared for each CYP-enzyme, and the result can be seen in figure 35. The calculated mean $IC_{50}$-values for each CYP-enzyme for both water and ethanol extract can be seen in table 29.

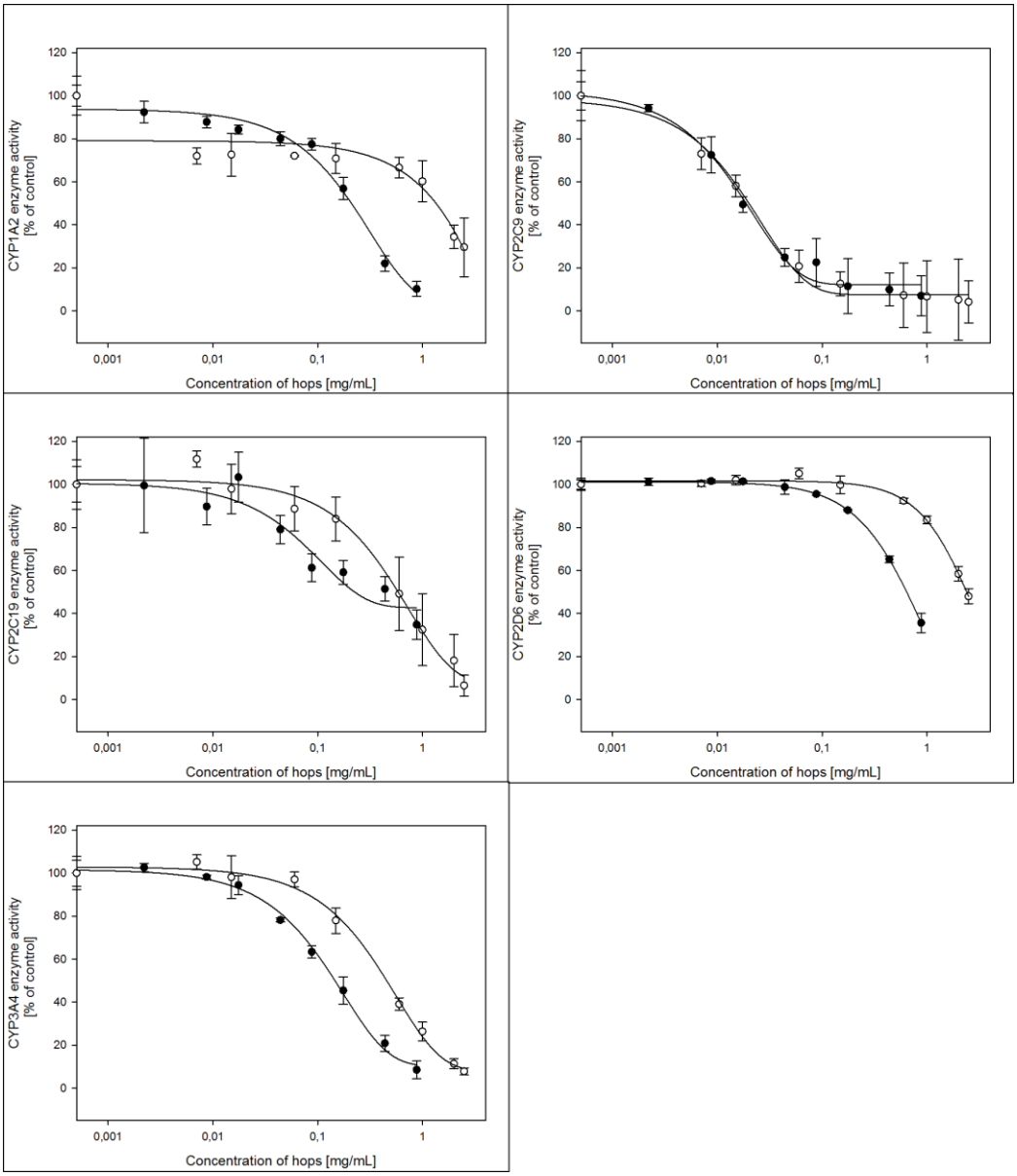


*Figure 35: Comparison of dietary supplement hops extracted with water (white dots) and 50% ethanol (black dots) for each CYP-enzyme investigated presented as $IC_{50}$-curves from two representative experiments. Data shown as mean ± SD (n=3).*

*Table 29: $IC_{50}$-values presented as means of replicates of dietary supplement hops extracted with water (from two experiments with three parallels of each concentration) and 50% ethanol (from three experiments with three parallels of each concentration) respectively for each CYP-enzyme investigated.*

| | | **water** | **50% ethanol** |
|---|---|---|---|
| **Hops $IC_{50}$ [mg/mL]** | CYP1A2 | 1.7 ± 0.5 | 0.27 ± 0.06 |
| | CYP2C9 | 0.025 ± 0.006 | 0.022 ± 0.004 |
| | CYP2C19 | 0.61 ± 0.03 | 0.22 ± 0.03 |
| | CYP2D6 | 2.4 ± 0.06 | 0.78 ± 0.2 |
| | CYP3A4 | 0.48 ± 0.04 | 0.12 ± 0.02 |

### *4.4.3 Comparison of ethanol extracted dried hops and dietary supplement hops*

$IC_{50}$-curves of ethanol extracted dried hops and ethanol extracted dietary supplement hops were compared, and shown in figure 36.

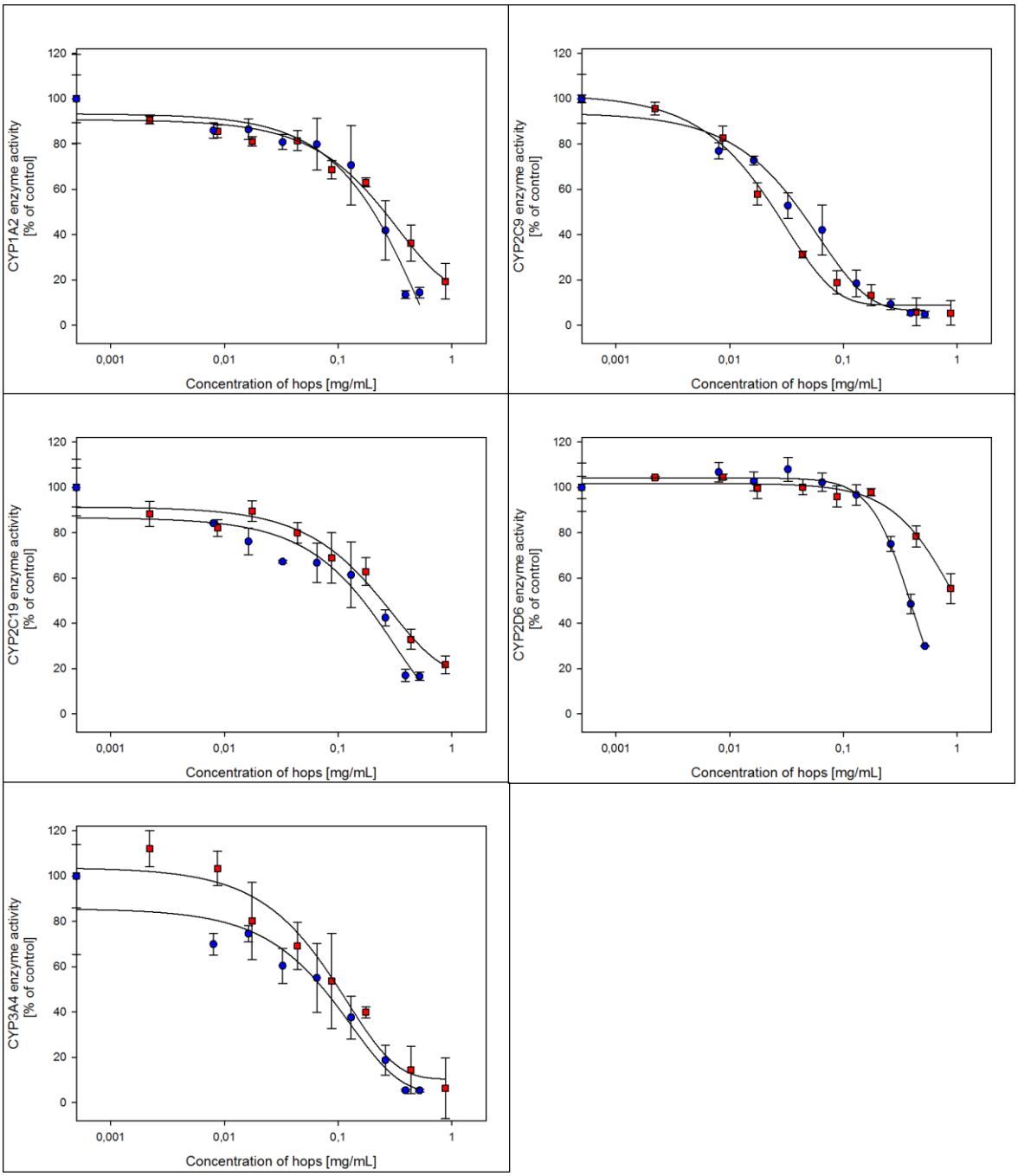


*Figure 36: $IC_{50}$-curves comparing ethanol extracted dried hops (blue circle dots) and dietary supplement hops (red square dots) for each of the investigated CYP-enzymes. Data shown as mean ± SD (n=3).*

## 4.5 Comparing $IC_{50}$-values based on met/sub with met/IS

In section 4.4 results have been reported based on area ratio of met/sub, while in section 4.1 - 4.3 the results are reported based on area ratio of met/IS. A comparison of the $IC_{50}$-values from two representative experiments with result based on met/sub and met/IS respectively, can be seen in table 30.

*Table 30: A comparison of the $IC_{50}$-values with result based on met/sub and met/IS respectively, from two representative experiment on dietary supplement hops, both water (n=1) and ethanol extracted (n=1).*

| | | **Water extracted** | | **Ethanol extracted** | |
|---|---|---|---|---|---|
| | | **met/sub** | **met/IS** | **met/sub** | **met/IS** |
| **Hops $IC_{50}$ [mg/mL]** | CYP1A2 | 2.1 | 1.8 | 0.26 | 0.24 |
| | CYP2C9 | 0.029 | 0.037 | 0.026 | 0.026 |
| | CYP2C19 | 0.63 | 0.44 | 0.24 | 0.11 |
| | CYP2D6 | 2.5 | 2.3 | 1.0 | >1.9 |
| | CYP3A4 | 0.50 | 0.95 | 0.10 | 0.29 |

# 5. Discussion

In this thesis an *in vitro* cocktail method based on Inje's *in vivo* cocktail, was developed to simultaneously investigate the inhibitory potential of a compound on five different CYP enzymes. The finished method was tested using the herb hops (*Humulus lupulus*).

## 5.1 Quality control of the analytical method

### *5.1.1 Decomposition of omeprazole*

When analyzing the STDs, a probable decomposition of OME was discovered (figure 15). OME would decompose when mixed in the standard solution, but not when kept alone in the stock solution. Previous studies report that OME is unstable at lower pH-values and that the rate of degradation was accelerated when a phosphate buffer was used [53]. In a series of tests it was discovered that adding a small amount of KPO-buffer to the standard solution actually decreased the decomposition of OME, this might be because it resulted in a better pH or changed the ionic strength of the compounds. More experimental research could have been performed on the subject of decomposition, for example close monitoring of pH, but since the problem was solved, it was deemed unnecessary.

Since phosphate buffer was recorded to accelerate the degradation of OME, and phosphate buffer was used in the incubation experiments, it emphasizes the necessity of analyzing the incubate samples immediately after the incubation.

### *5.1.2 Validation of LC-MSMS performance*

For all substrates and metabolites, the pre-run intra- and inter-day accuracy and precision was within the acceptance criteria suggested by the FDA [51] (table 6-15). It can be noticed that the standard curves illustrated for all the analytes are not all linear, best of fit regression was tested and the quadratic fit gave best fit for all analytes in the specific concentration range chosen (figure 16-25). The standard curves might employ a linear fit if the range of concentrations had been narrower.

### *5.1.3 Stability*

The experiments were conducted over several months, the STDs and QCs were made in October, the experiments with dried hops were done in November/December, and experiments

on the dietary supplement hops were performed in January. From the measured area ratio of the QCs analyzed with each experimental series, some variation was noticed (data not included). The variation in area ratio was probably due to an instability of the mixed substrates over time. A stability test of the analytes could have been a part of this thesis, and should be performed before implementing the procedure in research.

It could also be noticed that there was more variation in area ratios of ACE and PHE than the other analytes. This could be due to low Q1 and Q3 masses for the MRM-transitions, since both molecules have low masses compared to the others, which might imply a beginning instability of the MS. The instability of the MS raises some questions about the validity of the experimental data, but the main aim in this thesis was the development of an *in vitro* cocktail method and some variations in detected range are not necessarily important, but might be a reason for the high standard deviation in some of the $IC_{50}$-hops values.

A change in the chromatogram could also be observed over time, the peaks were less separated, had increased tailing and some changes in peak size that might imply abrasion of the column over time. The quantitative data from the MS is based on counts per second and the method used IS, meaning that the column abrasion would affect the IS as well, and by dividing the analyte area on IS area the effect of the abrasion would be cancelled out, although it visually does not look good.

## 5.2 Pilot study

The first experiments were performed to test the substrates, the concentration of the liver microsomes and the incubation time to get an understanding of the experimental conditions. The initial concentrations of the substrates were chosen to be 20 µM CAF, 1 µM LOS, 0.2 µM OME, 0.2 µM DXM and 2 µM MID, based on previously reported concentrations from literature and reported $K_m$-values [52].

To get an impression of the chromatogram with retention time and peak heights, a test solution containing 0.2 µM of each analyte was analyzed (figure 26). The peaks are all visible above the background noise, but some of the peaks are not separated very well and have some minor tailing. The separation and tailing is only affecting the visual outcome, and does not affect the quantitation data which is based on counts per second detected by the MS.

Incubations with two different concentrations of HLMs (0.2 and 0.4 mg proteins/mL), were compared after incubation for 15 minutes. As expected, more CYP produced metabolite could

be detected with higher concentration of HLM, with the exception of OHM and OHO that barely had an increase (table 16). The lack of increase in concentration is probably due to too low concentrations of their respective substrates (MID and OME) and a high turnover rate for their enzymes, which can be confirmed by the low area ratio of detected substrate (data not included). There was no detected PXH.

In the incubation time test (15 and 25 minutes) more metabolites were produced after 25 minutes of incubation, as expected, with the exception of OHM, where a small, but not significant, decrease could be observed (table 17). An increase in production of OHM might have been observed with a higher substrate concentration, as more substrate would be available for the enzyme. There was no detected PXH.

Several articles report cocktail-methods with a NADPH-regenerating system containing $MgCl_2$, for example Wang et al [54], where 3 mM $MgCl_2$ was used in their cocktail incubations. Since the cocktail developed in this thesis used NADPH and not an NADPH-regenerating solution, 3 Mm $MgCl_2$ were added to the cocktail-mix to see if this increased the metabolite production. As can be seen in table 18, the amount of CYP produced metabolites did not increase significantly and there was still no detected PXH, so it was decided that this was not a necessary addition to the reaction mix.

Since the amount of CYP produced metabolites did not double with double HLM concentration or increased with prolonged incubation, concentrations of OME, DXM, MID and LOS were increased to 10 µM in the incubation mix. The concentration of CAF remained the same at 20 µM. The incubations contained 0.4 mg/mL HLM, and total incubation time was 20 minutes. As expected, more CYP produced metabolites were detected (table 19). Enzyme activity was calculated to ensure increased activity with increased amount of substrate. Table 20 shows that increased substrate availability also increased enzyme activity, with the most noticeable increase being for CYP3A4, which substantiates the assumptions regarding why OHM area ratio did not increase at lower MID concentrations.

The concentration of CAF could also have been increased, but since there were no detected PXH at all, it was decided unnecessary.

### *5.2.1 Replacing caffeine with phenacetin*

None of the previous experiments gave any detectable amount of PXH. The CAF N3-demethylation to PXH is very sensitive to solvent effects from MeOH and ethanol, and

CYP1A2 has also shown a low *in vitro* affinity for CAF. These are the main reasons why CAF is not a preferred *in vitro* probe substrate [27]. CAF was replaced with 20 µM PHE. PHE is the most used CYP1A2 probe substrate *in vitro* because of its high affinity for CYP1A2 and low interaction potential, but cannot be used *in vivo* because of toxicity in humans [30].

When studying the chromatogram after incubation with PHE instead of CAF (figure 27), two peaks could be observed identified as ACE based on MRM, one eluting after 1.28 minutes and another after 3.90 minutes. Comparing the peaks with a chromatogram of a standard solution, it could be seen that the first peak at 1.28 minutes was identical to ACE, and the latter peak at 3.90 minutes was identical to PHE. Such interference has been reported previously and are presumed to be caused by in source fragmentation of PHE from electrospray ionization that yields an ion structurally identical to ACE [55]. Since the two peaks were well separated, the in-source fragmentation does not affect the detection of ACE.

Incubations with PHE were performed for 20 minutes with 0.2 and 0.4 mg/mL HLM. The incubation results in table 21 show that the CYP produced metabolites approximately doubles with double HLM concentration for all added substrates, indicating that enough substrate were available for each of the enzymes to get a satisfying turnover.

The amount of remaining substrates (sub/IS) in the incubation after metabolism were compared to a sample without NADPH, and the percentage of used substrate were calculated (table 22). For all substrates, the consumption was below 15% except MID, which had an end concentration of 52 %. Guidelines developed by Pharmaceutical Research and Manufacturers of America (PhRMA) for *in vitro* drug-drug interaction studies using HLM suggests that less than 20 % of the substrate should be consumed to ensure enough turnover [23].

The consumption of MID was a lot higher than the other substrates indicating that the concentration of MID could have been increased. Based on previous experiments, where the amount of metabolite of OHM doubled with double HLM concentration and the increased enzyme activity, it seemed that the amount of MID was sufficient. It should be noted that the sample without NADPH was not analyzed in triplicate, but a sample without NADPH was included in several experiments and similar consumption percentages were observed (data not included).

To test if an inhibition was possible to detect using this system and method, the known specific inhibitors quinidine (CYP2D6) and ketoconazole (CYP3A4) were added to the incubation mixture in concentrations of 0.2 µM. The samples containing quinidine showed a noticeable

lower amount of DEX than in the control samples without quinidine, it could also be noted that the rest of the metabolites were approximately the same (table 23). For the sample containing ketoconazole it could be seen a decline in amount of OHM, as expected, but also a smaller decrease in amount of EXP and OHO. Ketoconazole is not known to inhibit CYP2C9 or CYP2C19 significantly, but the decrease in CYP produced EXP and OHO might be caused by an additional inhibition by MeOH. Ketoconazole was dissolved in MeOH while quinidine was dissolved in water. The ketoconazole samples should have been compared with control samples with the same amount of MeOH added.

Previous experiments have shown that CYP2C19, with mephenytoin as substrate, was not very susceptible for MeOH inhibition [56, 57], but the result varies for CYP2C9. Using tolbutamide as CYP2C9 substrate a strong inhibition at low MeOH concentrations was shown, while using diclofenac as substrate only low levels of inhibition was observed, emphasizing that the inhibition results are very dependent on what substrate used. The differences in substrates might explain why CYP2C9 and CYP2C19 seem to be the enzymes most affected by MeOH in this study. From section 4.3.5 it can also be observed that CYP2C9 and CYP2C19 was the enzymes most affected by ethanol, this will be discussed more in section 5.3.5. When comparing the samples containing ketoconazole with the control samples and studying CYP1A2 and 2D6, no significant difference can be observed in this experiment. Previous studies agree that CYP1A2 is not affected by MeOH (≤ 3%), while at 1% MeOH concentration, CYP2D6 was 25% inhibited using bufuralol [56], and slightly inhibited using DEX as substrate [57]. The study by Busby et al [56] discovered that CYP3A4 was not affected by MeOH concentrations below 3%.

This experiment shows the importance of alcohol influence and keeping the matrix equal in incubations.

### *5.2.2 HPLC mobile phase*

Both MeOH and ACN are common organic solvents used as mobile phases for chromatographic assays. It was investigated whether the choice of organic solvent in the mobile phase could influence the peak size in the analysis. Table 24 gives a brief comparison of amount of CYP produced metabolite eluted first with ACN, then with MeOH. As mentioned in section 1.5.1; ACN might have stronger eluting properties, but MeOH is often used because it is cheaper, more accessible and less harmful than acetonitrile. In this experiment, using MeOH as mobile phase generally gave an increase in detected metabolite with the exception of DEX where a

small decrease could be observed. It should be noted that the results are only based on one sample of each standard solution, so the results only indicate that MeOH is the better organic solvent in this experiment, and no real conclusion could be determined without further experiments.

By comparing the chromatograms eluted with ACN and MeOH in figure 26 and 28 respectively, some differences can be noticed. In general, MeOH eluted analytes has longer retention time and the peaks cluster more. Since the analytes are detected based on MRM transitions, the visual separation is not that important and it was decided to use MeOH as organic phase based on the increased signal of most of the metabolites.

## 5.3 Optimization and validation of the incubation method

The incubation method was optimized and validated during a set of experiments by changing the concentrations of HLM, substrates and the incubation time, one parameter at a time. To further validate the method, one substrate at a time were incubated and compared with a control containing all substrates, and the influence of ethanol in the incubation mix was tested.

### *5.3.1 Substrate concentration*

The incubation was performed with three different concentrations of each substrate, as explained in section 3.7.2. Figure 29 shows that none of the plots are linear which might indicate that the reactions are moving towards steady state ($V_{max}$). At $V_{max}$ all of the active sites on the enzyme are busy at all time, and the production of metabolite remains constant as long as enough substrate is available.

The guidelines developed by PhRMA suggest that the concentration of the substrates should be at or below its Michaelis constant ($K_m$) [23]. Based on previously reported values by FDA the substrate concentrations used in this experiment for PHE, OME and MID were below reported $K_m$, the concentration of DXM were barely above the reported $K_m$, and there were no reported values for LOS [52]. A crude $K_m$-value was calculated for each enzyme (table 25), based on three substrate concentrations in the experiment, and a comparison of the experimental $K_m$-values with the FDA reported $K_m$-values can be seen in table 31. According to the experimental $K_m$ the chosen substrate concentrations are too high, but comparing the experimental with the FDA reported $K_m$-values show that they are all in accordance, except for CYP2C19 which is significantly lower. However, the calculations of the experimental $K_m$-values are only based on

a plot with three concentrations and not very reliable. The experiment could have been repeated with more concentrations of the substrate to get a reliable result.

*Table 31: Comparison of experimental $K_m$ and FDA reported $K_m$*

| | **Experimental $K_m$** | **Reported $K_m$** |
|---|---|---|
| **CYP1A2** | 6.7 | 1.7-152 |
| **CYP2C9** | 0.51 | - |
| **CYP2C19** | 0.76 | 17-26 |
| **CYP2D6** | 3.1 | 0.44-8.5 |
| **CYP3A4** | 5.9 | 1.0-14 |

There are several reports of increasing probability of interactions with increased substrate availability. PHE strongly reduces CYP2C9 activity in HLMs at concentrations above 50 µM, the interaction potential is negligible for OME concentrations below 40 µM, DXM concentrations less than 25 µM and MID concentrations below 10 µM [21, 27]. At high concentrations of MID the 1-hydroxylation reaction by CYP3A4 changes from Michalis-Menten kinetics to substrate-inhibition kinetics, meaning that at high concentrations MID will inhibit CYP3A4, and the Michalis-Menten plot in figure 12 will decrease after reaching $V_{max}$. Both CYP3A and CYP2C9 are involved in the metabolism of LOS to EXP in cDNA expressed enzymes, but the CYP3A reaction is a minor pathway and is proposed to only be involved in *in vivo* LOS clearance at high concentrations [58]. LOS is a relatively new compound, and to the author's knowledge has not been used previously in *in vitro* CYP cocktails, therefore it is a lack of available interaction data. This is further explored in section 5.3.4.

Choosing the correct substrate concentration is a difficult step, it cannot be too high because it increases the chance of interactions with other enzymes, and it cannot be too low because then it will affect the production and detection of metabolites. Based on the curved concentration plots, and an overall assessment, 20 µM PHE, 2 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID was decided appropriate for further development of the method.

### *5.3.2 HLM concentration*

The incubation method was performed with three concentrations of HLM. Figure 30 show a linear relationship between the amount of CYP produced metabolite and HLM concentration.

PhRMA suggests that the concentration of HLM should be below 0.5 mg/mL to avoid unnecessary protein bindings, and that the formation of metabolite should be linear with HLM concentration and time [23]. Since the chosen HLM concentration of 0.4 mg proteins/mL is less than 0.5 mg/mL and linear with HLM concentration for all substrates it was assumed to be an optimal concentration. One higher concentration than 0.4 mg proteins/mL could have been added to the experiment to assure linearity above the chosen concentration as well.

### *5.3.3 Incubation time*

HLMs were incubated for 10, 20 and 30 minutes. Figure 31 shows that the CYP production of metabolite is linear with incubation time for all metabolites. The area ratios for the time experiments have higher standard deviations than for the substrate and HLM concentration, but the CV is below 15% for all measurements. The high SD implies a variation between the samples and the experiment could have been repeated to ensure linearity, but with an $r^2$-value above 0.985 and with CVs below 15% it was decided to be valid, and that incubating for 20 minutes was appropriate for all substrates.

### *5.3.4 Substrate specificity*

A common problem with cocktail methods is the interaction between substrates, meaning that one substrate specific for one enzyme may inhibit other enzymes or be metabolized by several enzymes and hence the metabolite formation will not be representative for the specific enzyme activity. To ensure that the substrates in this cocktail do not interact with each other, one substrate at a time were incubated with HLM alone and the CYP produced metabolite were compared to an incubation control containing all substrates (figure 32). A paired t-test revealed that the production of metabolites in the control and alone were not significantly different implying that there were no interactions between the substrates, and the substrates were not metabolized by other enzymes. An inhibition test could have been performed by adding one specific inhibitor at a time to the incubation mix and comparing the results, but since the t-test indicated that there were no interactions, and the effect of only one enzyme at a time was not of interest in this study, further experiments were not performed.

### *5.3.5 Influence of ethanol*

Organic solvents inhibit CYP enzymes [56]. Since this method was developed primarily for the investigation of herbs, the inhibitory effect of ethanol had to be investigated, as ethanol often is used for extraction of herbal constituents. The effect of ethanol on CYP-enzymes varies, and figure 33 shows that CYP2C9 and CYP2C19 are more affected than CYP3A4 and CYP1A2. Busby et al [56] reports that 1% ethanol did not inhibit CYP1A2, 2C9 and CYP3A in cDNA expressed enzymes, but the results from this experiment shows that microsomes might be affected differently than cDNA expressed enzymes, in addition to using different substrates that might be affected differently.

The amount of ethanol present in the incubations should be kept as low as possible. From the obtained data and figure 33, an ethanol concentration of 0.5 % (giving roughly a 20 % inhibition of the most affected enzymes) would be appropriate. However, in order to gain high enough concentrations of hops in the incubations, it was decided that 1% ethanol is acceptable, as long as all the samples contain the same amount, also including control samples. When adding the same amount of ethanol to all samples and calculating percentage enzyme activity of the control sample, with no added herbal extract, the inhibitory effect of ethanol cancels out.

### *5.3.6 The optimized method – concluding remarks*

There are several cocktail methods developed for studying *in vitro* inhibition potential of herbs or drugs, and all offers its advantages and disadvantages. In this study, a cocktail method has been developed to study *in vitro* CYP inhibition using HLM and substrates based on the *in vivo* Inje's cocktail [28]. The pilot study gave indications of substrate concentrations, HLM concentration and the incubation time. Since the CYP1A2 metabolite of CAF, PXH, could not be detected in any pilot incubations, it was decided to change the CYP1A2 substrate to the FDA recommended PHE. After increasing concentrations, 20 µM PHE, 10 µM LOS, 10 µM OME, 10 µM DXM and 10 µM MID and incubation for 20 minutes with 0.4 mg proteins/mL HLM were chosen for further optimization. An inhibition test confirmed that this method could be used to detect inhibitions and also specified the importance of alcohol influence.

After optimization and validation, the chosen conditions were: 20 µM PHE, 2 µM LOS, 2 µM OME, 10 µM DXM and 10 µM MID, incubation for 20 minutes with 0.4 mg proteins/mL HLM. The formation of metabolite was linear with both HLM concentration and time as recommended, and a paired t-test confirmed that the substrates interacted minimal by testing

that the amount of produced metabolites in the incubation mixture with all substrates available were the same, as when only one substrate at a time were available.

## 5.4 Incubation with hops

To test the developed method two types of hops, dried hops and the dietary supplement Hops Flowers, were investigated for their inhibitory effect towards the five CYP enzymes. Dried hops were extracted using boiling water, as would have been done when making tea, and also extracted with 50 % ethanol, as would have been done when making a tincture. The dietary supplement Hops Flowers were extracted with water and 50 % ethanol (see section 3.4 for more details).

### *5.4.1 Dried hops*

Figure 34 shows $IC_{50}$-curves of dried hops prepared as tea and dried hops extracted with 50% ethanol. The order of inhibition is approximately the same for both extracts, with CYP2D6 being the least affected with $IC_{50}$-values of 1.4 and 0.37 mg/mL respectively, then CYP2C19 or CYP1A2 with approximately the same $IC_{50}$-values, CYP3A4, and CYP2C9 being most affected with $IC_{50}$-values of 0.060 and 0.046 mg/mL respectively (complete list of $IC_{50}$-values can be seen in table 27).

CYP2C9 has an $IC_{50}$-curve that is almost identical between the tea and ethanol extract, implying that the constituent(s) of hops that inhibit CYP2C9 might be present in both extracts. The other enzymes show greater differences in $IC_{50}$-values, for example CYP3A4 that has an $IC_{50}$-value of 0.51 and 0.088 mg/mL for the tea and ethanol extract, respectively. The greater differences might imply that some non-polar constituents present in the ethanol extract contributes to the inhibition of some enzymes, but since the differences in $IC_{50}$-values between the extracts varies from enzyme to enzyme, it can be deduced that different constituents in hops inhibits different enzymes.

The calculated recovery percentage shows that almost the same amount gets extracted in the tea extract and the ethanol extract, but since the ethanol extract has a lower $IC_{50}$-value for all enzymes there might be some inhibitory non-polar constituents that can be found in the ethanol extract and not in the tea extract. For example, hops contain some volatile oils that would not have been extracted with water [43].

CYP2C9 was the enzyme most easily inhibited by hops with the lowest $IC_{50}$-values for all the different extracts. As mentioned in section 1.7.3, several studies reported CYP2C9 as the most affected enzyme. The study by Yyan et al [50] of inhibition by hops, performed using microsomes, found that CYP2C9 was the enzyme most inhibited with an $IC_{50}$-value of 0.9 µg/mL, which is a lot lower than the lowest in this study at 0.02 mg/mL (20 µg/mL). Yyan et al. also claimed that there was not any significant inhibition of CYP3A4, which does not agree with this study where CYP3A4 is one of the most affected enzymes. Other studies performed by Foster et al [48, 49] revealed that several of the hop-containing beverages inhibited CYP2C9 and CYP3A4 with 76-100%, agreeing with the result obtained in this study.

The discrepancies in inhibition results might be caused by using different substrates in addition to differences in the herbal extraction method. Previous studies have also reported an *in vitro* inhibitory effect of the prenylphenols isoxanhumol, 8-prenylnaningenin and xantohumol, which are known compounds of hops [50, 59].

### *5.4.2 Dietary supplement Hops flowers*

Hops are also available as a dietary supplement, finely grinded hops flowers in capsules. The dietary supplement was extracted with water and 50 % ethanol. The two extracts of the dietary supplement had somewhat lower recovery rate than the dried hops, probably because of added magnesium stereate as an anti-adherent that would not be extracted.

The $IC_{50}$-calculations (table 29) for the dietary supplement revealed almost the same as for the dried hops, with the ethanol extracts having lower $IC_{50}$-values than the water extracts, and CYP2D6 being the least affected with $IC_{50}$-values 2.4 and 0.78 mg/mL for water and ethanol extracts respectively, then CYP1A2, CYP2C19, CYP3A4 and CYP2C9 being the most affected with $IC_{50}$-values 0.025 and 0.022 mg/mL respectively. Again, CYP2C9 has the $IC_{50}$-values that are almost identical between the water and ethanol extract.

### *5.4.3 Comparison of dried hops and dietary supplement hops*

Since the two water-based extracts were extracted differently; the dried hops were extracted as tea and the dietary supplement extracted using the same method as the ethanol extracts, it was found more relevant to compare $IC_{50}$-curves of ethanol extracts of dried hops and the dietary supplement hops. From figure 36 it can be seen that the shape of the plots is similar for the same enzymes, as should be expected using the same extraction and incubation method on the

same herbal product. When comparing the $IC_{50}$-values for the ethanol extractions, it can be observed that CYP2C19 and CYP3A4 have almost identical $IC_{50}$-values for the two types of hops, while CYP1A2 and CYP2C9 have a higher $IC_{50}$-value for the dried hops than the dietary supplement, and CYP2D6 has a lower $IC_{50}$-value for the ethanol extracted dried hops than the dietary supplement.

When comparing the $IC_{50}$-values of the tea (water extracted dried hops) with the water extracted dietary supplement, a difference in values could be expected. It might be expected that the water extracted dietary supplement would affect the enzymes more, with lower $IC_{50}$-values, because of a more extensive extraction process and finer grinded powder, thus leading to more compounds being extracted. This is not the case in this study, where CYP1A2 and CYP2D6 have almost twice as high $IC_{50}$-values for the water-extracted dietary supplement than the dried hops tea, while CYP2C19 and CYP3A4 have almost the same $IC_{50}$-values for the two types of hops, and only the water extracted dietary supplement shows an almost half $IC_{50}$-value for CYP2C19 compared to the tea extract.

From the comparisons above, some trends can be noticed for the two types of hops investigated. Even though the ethanol extracts in general have lower $IC_{50}$-values than the water extracts, CYP2C19 and CYP3A4 have almost the same $IC_{50}$-values for extracts with identical solvent, but independent of type of hops. CYP2C9 are more affected by the dietary supplement than the dried hops, CYP2D6 are more affected by the dried hops than the dietary supplement, and inhibition of CYP1A2 varies depending on extraction solvent.

The difference in inhibitory potential can be caused by the use of different cultivations of hops which are not stated by the supplier. The differences in inhibition potential of the two types of hops investigated might also be because of different growth conditions in terms of nutrition, soil condition and temperature or different time of harvest, that have been shown to influence the composition of constituents in hops [60].

### *5.4.4 Inhibition by hops – concluding remarks*

CYP2C9 was the enzyme most easily inhibited by hops with the lowest $IC_{50}$-values for all of the different extracts, while CYP2D6 was the least affected. When comparing the results from this study with other reports, even though there are some differences in method and results, the different studies performed on hops and constituents of hops, indicates that hops has an inhibitory effect on CYP enzymes *in vitro.*

The *in vitro* $IC_{50}$-values from this study are comparable with results from other herbal interaction studies, for example crude extracts of SJW, raspberry leaf and ginger was shown to inhibit cDNA expressed CYP3A4 with an $IC_{50}$-value of 40, 81 and 565 µg/mL respectively. SJW was found to inhibit cDNA expressed CYP2C9 with an $IC_{50}$-value of 19 µg/mL [61, 62].

Another herbal interaction study by Fasinu et al [63] investigated the inhibition potential of three popular African medicinal herbs on CYP1A2, 2C9, 2C19 and 3A4 using HLMs. Crude extracts of Bowiea volubilis inhibited the metabolic activity of CYP1A2 and CYP3A4 with $IC_{50}$-values of 92.3 µg/mL and 8.1 µg/mL respectively, Spirostachys africana showed inhibitory activity against CYP1A2 and CYP3A4 with respective $IC_{50}$-values of 14.3 µg/mL and 47.4 µg/mL, and Tulbaghia violacea with inhibitory activity against CYP1A2 at 767.4 µg/mL and CYP2C9 at 921 µg/mL. The latter $IC_{50}$-values were commented as a mild inhibition. The range of these $IC_{50}$-values are in accordance to the range of values obtained in this experiment with hops, indicating that the method developed in this study might be functional for detecting a wide range of $IC_{50}$-values.

Since the ethanol-extracts seem to have lower $IC_{50}$-values, ethanol extraction is recommended to get the "worst-case" scenario for possible interactions.

## 5.5 Use of in vitro methods to predict in vivo interactions

Investigating xenobiotic drug interaction potential using high throughput *in vitro* screening methods are standard and accepted in research by the scientific community and the pharmaceutical industry, but a number of limitations are recognized both in regard to methodology and interpretation [64]. As mentioned in section 1.3.1, several systems are available for *in vitro* assessment of drug-drug interactions, with HLM being the most popular *in vitro* model. The major advantages of using HLM includes low cost, simplicity in use and being one of the best-characterized systems. However, microsomes contain only phase I enriched CYPs and lacks some phase II enzymes and cofactors. The high concentration of added NADPH and substrates in microsomes together with a lack of phase II reactions might result in higher biotransformation rates compared to *in vivo* results, and thus gives unrealistic $IC_{50}$-values. HLMs are often chosen as experimental system because it gives more information than cDNA-expressed CYPs and are simpler, cheaper to perform and more available than primary hepatocytes and liver slices. Limitations and strengths of the different experimental system are further reviewed in [22].

When studying herbal products, several limitations must be assessed. Most HMPs are made up of several naturally occurring compounds making it virtually impossible to perform clinical studies with each individual constituent, thus *in vitro* screening methods, as developed here, gives indications of what HMPs might be involved in interactions and whether clinical studies are relevant or not [64]. A methodological consideration is the production of unknown metabolites from the herbal extracts that are poorly characterized and may contribute to the net inhibitory effect observed *in vitro* that would not be observed *in vivo.* Another well-known problem when assessing herbal products is the selection of *in vitro* concentrations, because most often the *in vitro* concentrations are not related to *in vivo* situations, because it is dependent on disposition of the inhibitor, plasma protein binding, uptake in liver and rate of clearance. As opposed to medicinal drugs, information about absorption and bioavailability of herbal constituents are often limited or unknown, so assessing bioavailability is almost impossible without performing clinical studies. These limitations make it difficult to extrapolate *in vitro* data to humans.

Several incidents of misinterpretation of *in vitro* studies have been reported, for example were SJW first thought to inhibit CYP3A based on *in vitro* experiments with cDNA expressed enzymes and microsomes, but when tested clinically SJW showed induction of CYP3A and P-gp systems [18]. Another example is the *in vitro* studies showing that milk thistle alters CYP3A metabolism, but later clinical studies showed that milk thistle had no clinical activity [64]. A study performed on *Rhodiola rosea* products revealed an *in vitro* inhibitory effect on cDNA expressed CYP1A2, 2D6 and 3A4, but when examined *in vivo* only CYP2C9 was significantly inhibited [32, 65].

All limitations must be carefully considered before making an interpretation whether the data could be extrapolated to humans and potentially poses a risk of interaction and, consequently, if clinical studies should be performed. The increased biotransformation *in vitro* compared to *in vivo,* together with unrealistic high concentrations of HMPs that most often will not reach the CYP enzymes results in unrealistic experimental $IC_{50}$-values. Even though extrapolation might not be possible, *in vitro* studies remain important as an early investigation before starting comprehensive and expensive *in vivo* experiments.

The inhibition experiments performed in this study are mainly to show that the developed method is functional to assess a herb's inhibition potential. Since the actual composition of hops is not known, the bioavailability of the possible inhibiting compounds is not known. Even though hops have shown inhibitory properties in this study, it cannot be concluded that hops

will inhibit CYP enzymes in humans. Since several studies have found that CYP2C9 was most affected by hops, some care should be taken when consummation of hops as HMP together with CYP2C9 metabolized drugs, such as mephenytoin and warfarin.

## 5.6 $IC_{50}$-values based on met/sub or met/IS

In section 4.4 results have been reported based on area ratio of met/sub, while in section 4.1-4.3, the results are reported based on area ratio of met/IS. The use of met/sub, also called metabolic area ratios, are common in clinical studies because it might enhance the calculated inhibitory effect and thus increase the sensitivity of the method, especially in studies where the detected amount of metabolite might be low [66]. Substrate uptake varies between persons, and using a metabolic ratio will give results that are more comparable from person to person, compared to data that only considers metabolite production, which is dependent on substrate availability.

Different studies report different use of metabolic ratios. For example, Ryu et al [28] in the development of Inje's cocktail used PXH/CAF, LOS/EXP, OME/OHO, DXM/DEX and no ratio for the quantification of MID and argues that these AUC ratios are correlated to systemic clearance. Another study by Thu et al [32], where Inje's cocktail was used to assess inhibition of a herbal product containing *Rhodiola rosea*, used EXP/LOS and OHM/MID based on what most other studies used, and argues the importance of using the same metabolic ratios in the same kind of studies to simplify comparisons.

To the authors knowledge, there are no specific guidelines regarding what kind of metabolic ratios should be used. $IC_{50}$-values calculated for hops investigated in this study can be compared (table 30) for metabolic area ratio (met/sub) and the metabolite alone (met/IS). No specific pattern could be detected, all of the $IC_{50}$-values are less than 50% different from each other, except the ethanol extracted $IC_{50}$ for CYP3A4 that shows a 66% difference. The difference between the use of met/IS and metabolic ratios can also be the reason the discrepancies in reported $IC_{50}$-values in this study with other studies of hops.

Since these studies are *in vitro,* the use of metabolic ratio might be unnecessary. The amount of substrate added to the cocktail are in excess and being kept identical for all experiment, and the pooled HLM are more homogenous than a group of persons. Because of the increased sensitivity and quality, it was decided to use a metabolic area of met/sub for all enzymes to mimic what is done in *in vivo* experiments.

# 6. Conclusion

Concluding remarks regarding the aims for this thesis outlined in section 2.

I. Inje's *in vivo* cocktail was introduced to our laboratory in an *in vitro* setting to have means for comparing *in vivo* and *in vitro results.* Inje's cocktail uses the probe substrates caffeine, losartan, omeprazole, dextromethorphan and midazolam for the CYP enzymes 1A2, 2C9, 2C19, 2D6 and 3A4, respectively. Since the metabolite of caffeine was not produced in the pilot study, caffeine was switched to phenacetin.

II. The *in vitro* cocktail method was optimized with regard to substrate concentration, HLM concentration and incubation time. The final conditions were: 20 µM phenacetin, 2 µM losartan, 2 µM omeprazole, 10 µM dextromethorphan and 10 µM midazolam incubated with 0.4 mg proteins/mL for 20 minutes. When adding a herbal extract the ethanol concentration in the cocktail should be less than 1%.

III. A standard operation procedure (SOP) was completed for the developed cocktail method, and can be seen in appendix D.

IV. A validated LC-MSMS method was adapted and re-validated with pre-run intra-and inter accuracy and precision (% CV) for the detection of the microsomal CYP substrates and metabolites. The original method used acetonitrile as organic HPLC phase, but acetonitrile was changed to methanol because of better detection of the metabolites.

V. The developed *in vitro* cocktail and the adapted LC-MSMS method was used to identify $IC_{50}$-values for hops. Two different types of hops were investigated, dried hops and the dietary supplement Hops Flowers. All investigated CYP enzymes were inhibited by hops, but to a different extent. The ethanol extracted dietary supplement gave the lowest $IC_{50}$-value at 0.022 mg/mL for CYP2C9, while water-extracted dietary supplement gave the highest $IC_{50}$-value at 2.4 mg/mL for CYP2D6. The order of inhibition, for all hops extracts investigated, was as following: CYP2C9 was the most affected, then 3A4, 2C19 or 1A2 and 2D6 being the least affected.

VI. Ethanol extracted hops gave $IC_{50}$-values in the range 0.022-0.76 mg/mL while water extracted hops gave $IC_{50}$-values in the range 0.025-2.4 mg/mL, indicating that ethanol is the more preferable extraction solvent and should be used for extraction of hops.

# 7. Literature


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# Appendix A: MRM specifications for the MS

*Table A1: MSMS parameters for the validated method analyzing substrates and metabolites used in the experiments*

| ID | Q1 mass | Q3 mass | DP (volts) | CE (volts) | EP (volts) | CXP (volts) |
|---|---|---|---|---|---|---|
| Caffeine | 195.0 | 138.3 | 40.0 | 27.0 | 10.0 | 12.0 |
| Paraxanthine | 181.0 | 124.4 | 46.0 | 21.0 | 10.0 | 8.0 |
| Losartan | 423.2 | 207.2 | 101.0 | 31.0 | 10.0 | 12.0 |
| Exp-3174 | 437.2 | 235.0 | 111.0 | 25.0 | 10.0 | 14.0 |
| Omeprazole | 346.2 | 198.1 | 126.0 | 33.0 | 10.0 | 16.0 |
| 5-OH-Omeprazole | 362.2 | 214.1 | 46.0 | 17.0 | 10.0 | 12.0 |
| Dextromethorphan | 272.3 | 147.0 | 81.0 | 41.0 | 10.0 | 14.0 |
| Dextrorphan | 258.3 | 199.0 | 81.0 | 37.0 | 10.0 | 14.0 |
| Midazolam | 326.2 | 291.1 | 140.0 | 40.0 | 10.0 | 8.0 |
| 1-OH-midazolam | 342.2 | 203.2 | 131.0 | 37.0 | 10.0 | 10.0 |
| IS | 260.3 | 116.2 | 111.0 | 25.0 | 10.0 | 12.0 |
| Phenacetin | 180.0 | 110.0 | 56.0 | 29.0 | 10.0 | 6.0 |
| Acetaminophen | 152.0 | 110.0 | 16.0 | 25.0 | 10.0 | 8.0 |

DP: Declustering potential. CE: Collision Energy. EP: Entrance Potential. CXP: Collision Cell Exit Potential

# Appendix B: Extracted peaks for the analytes in the developed method

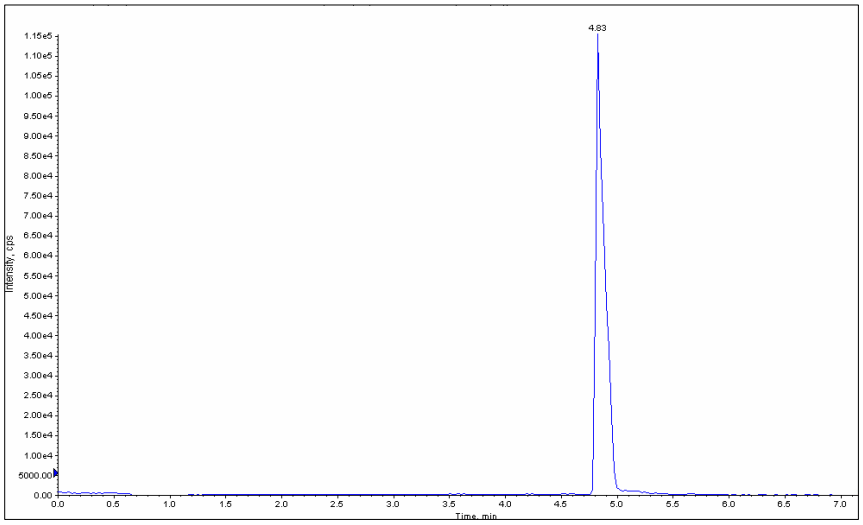


*Figure B1: LC-MSMS extracted peak of phenacetin with retention time 4.83 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

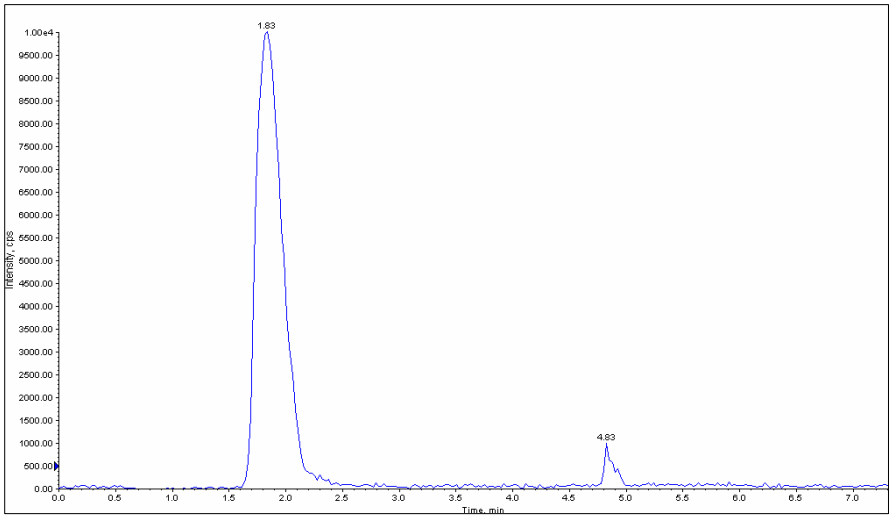


*Figure B2: LC-MSMS extracted peak of acetaminophen with retention time 1.83 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

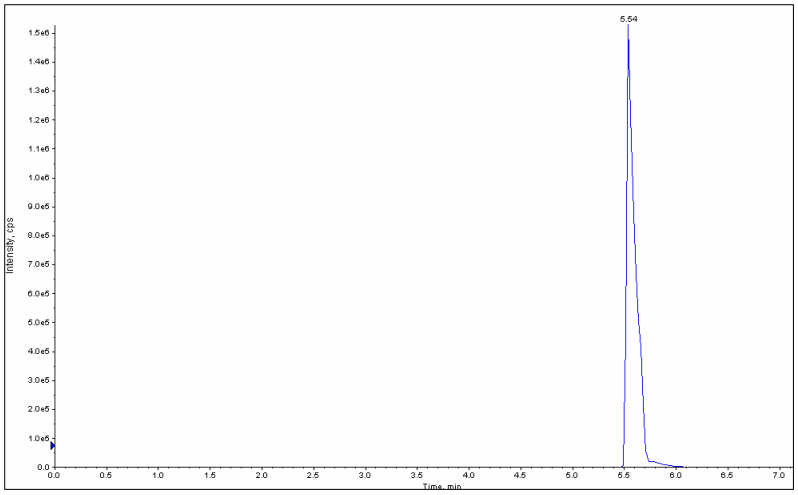


*Figure B3: LC-MSMS extracted peak of losartan with retention time 5.54 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

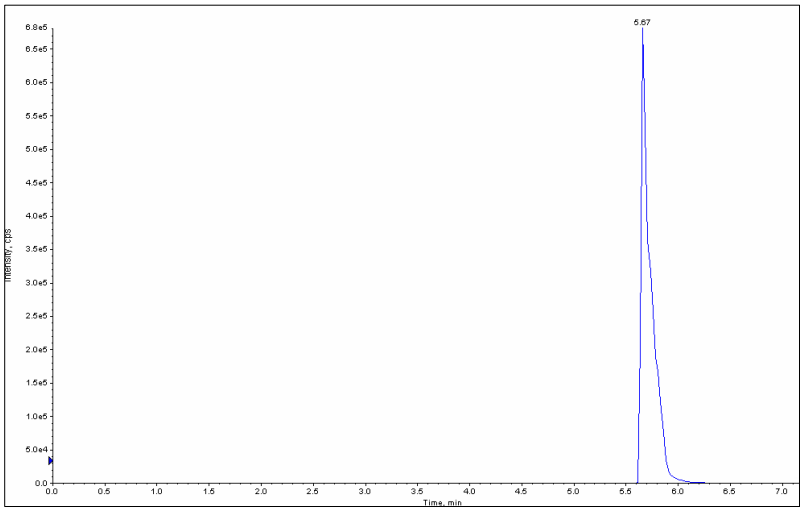


*Figure B4: LC-MSMS extracted peak of EXP-3174 with retention time 5.67 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

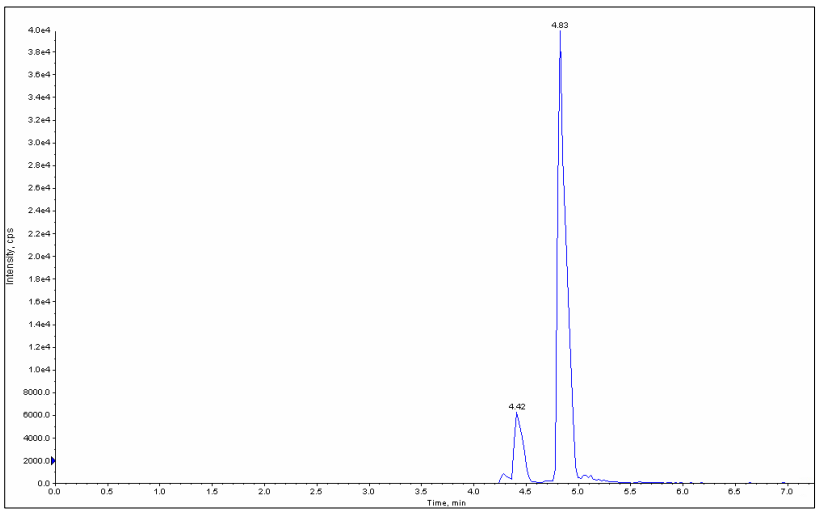


*Figure B5: LC-MSMS extracted peak of omeprazole with retention time 4.83 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

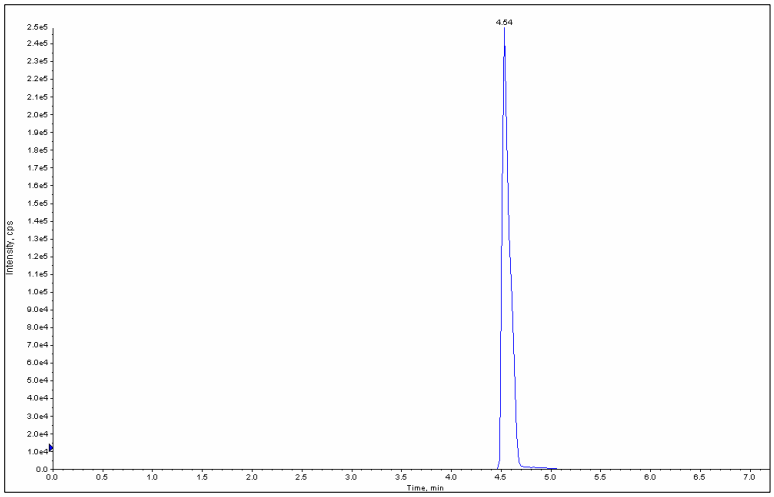


*Figure B6: LC-MSMS extracted peak of 5-OH-omeprazole with retention time 4.54 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

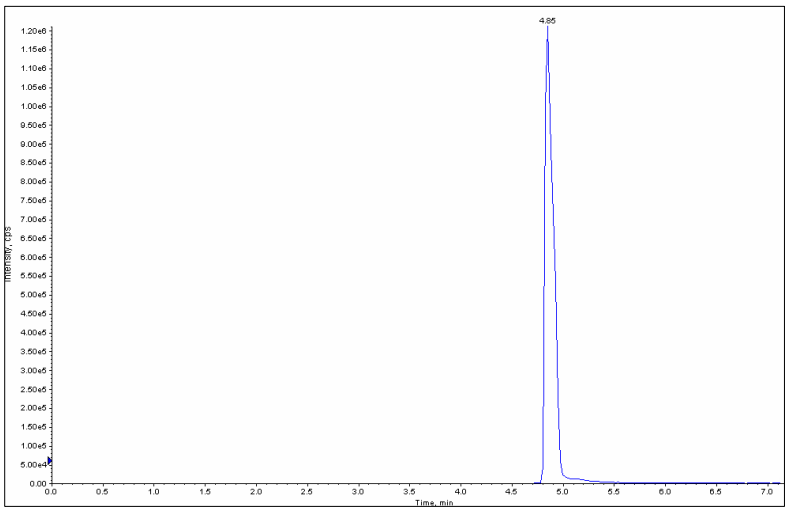


*Figure B7: LC-MSMS extracted peak of dextromethorphan with retention time 4.85 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

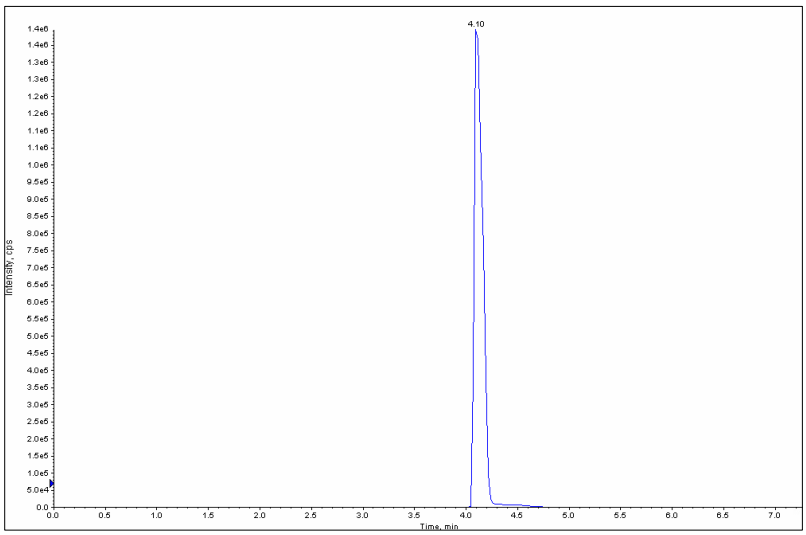


*Figure B8: LC-MSMS extracted peak of dextrorphan with retention time 4.10 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

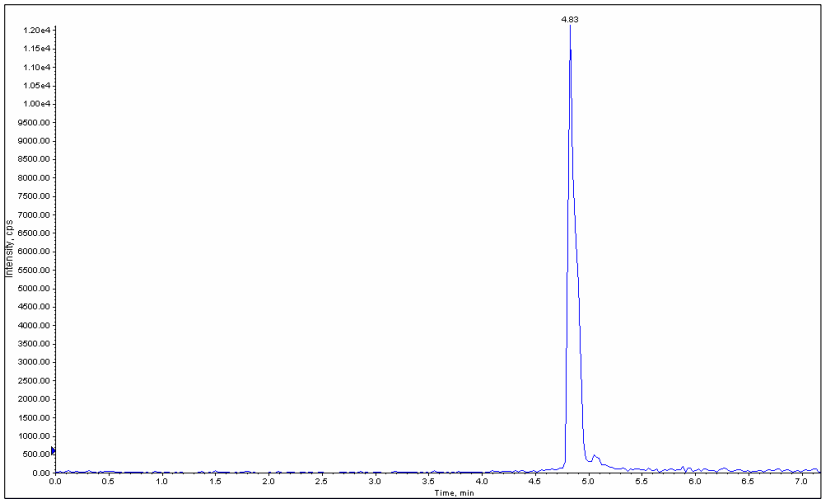


*Figure B9: LC-MSMS extracted peak of midazolam with retention time 4.10 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

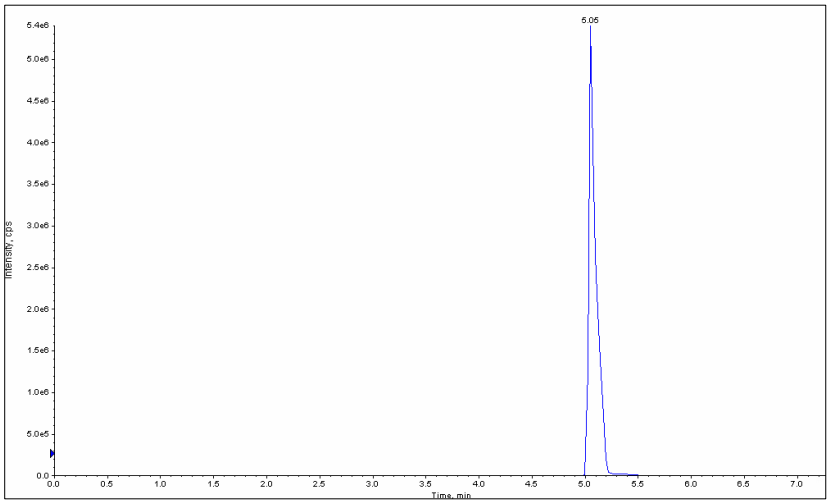


*Figure B10: LC-MSMS extracted peak of 1-OH-midazolam with retention time 5.05 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

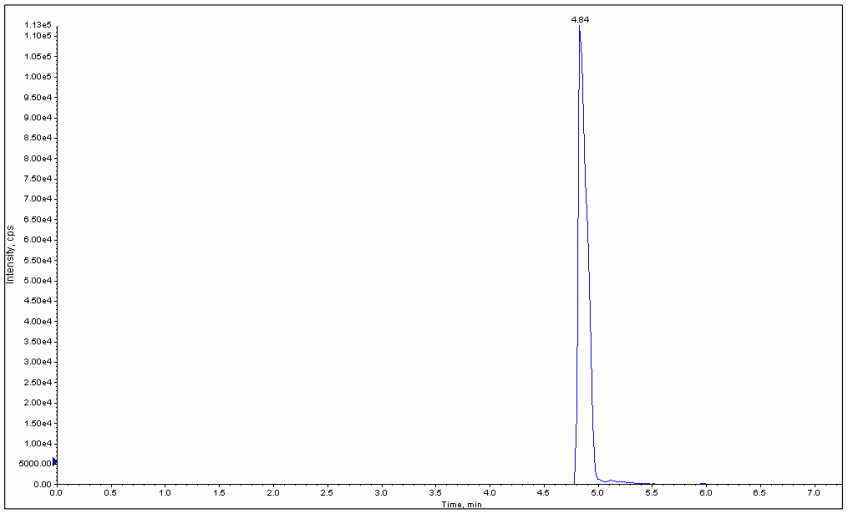


*Figure B11: LC-MSMS extracted peak of the internal standard propranolol with retention time 4.84 min from a chromatogram of a standard containing 0.2 µM of each analyte.*

# Appendix C: Lineweaver-Burke plots to calculate $K_m$

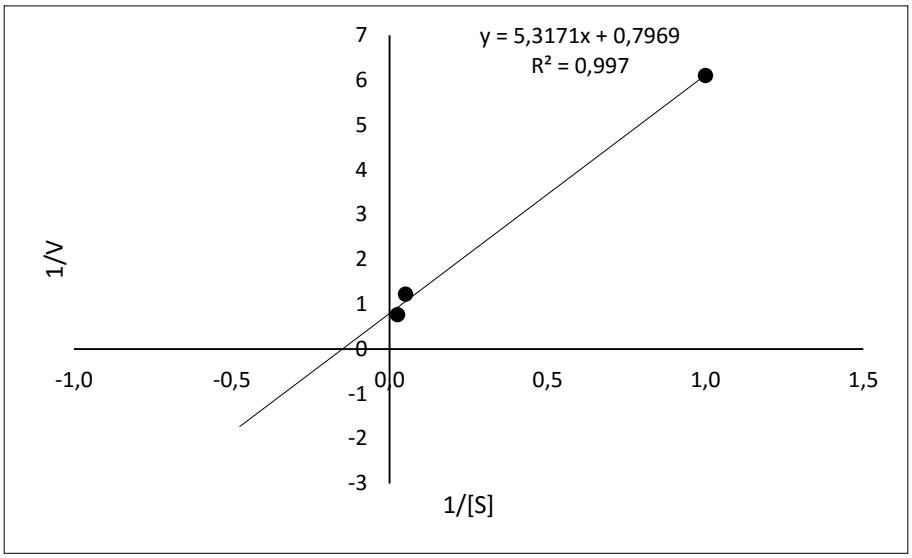


*Figure C1: Lineweaver-Burke plot for CYP1A2.*

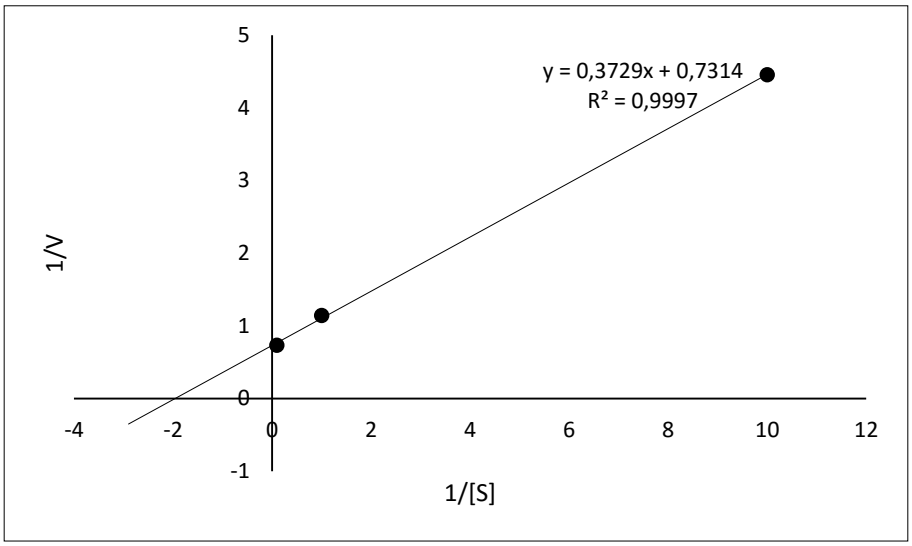


*Figure C2: Lineweaver-Burke plot for CYP2C9.*

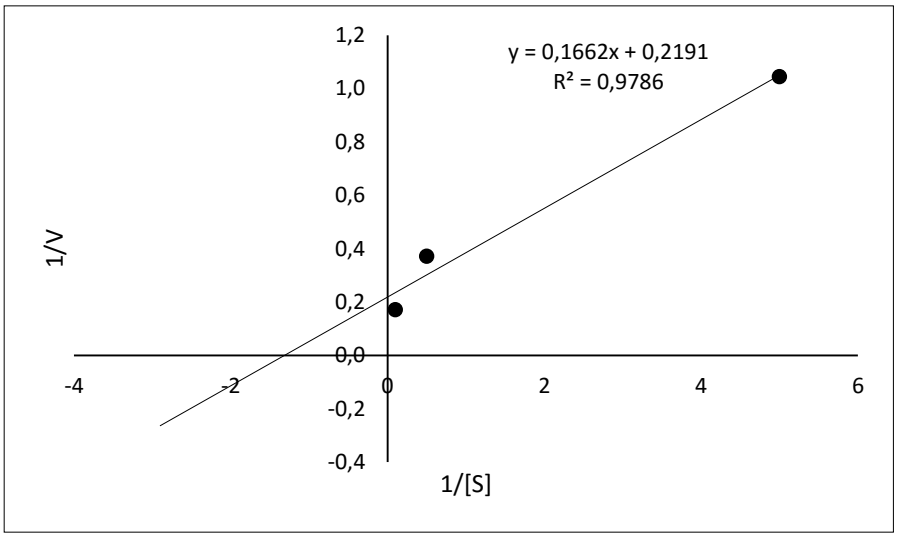


*Figure C3: Lineweaver-Burke plot for CYP2C19.*

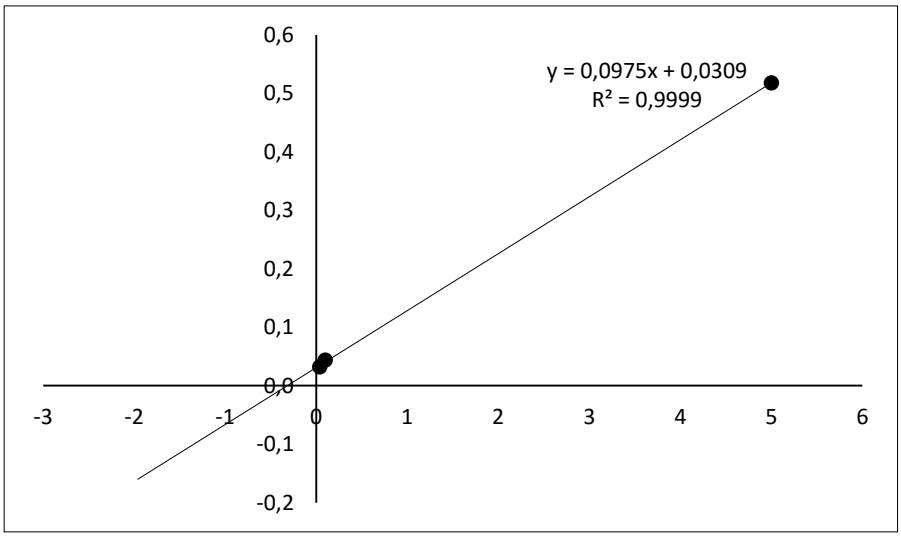


*Figure C4: Lineweaver-Burke plot for CYP2D.*

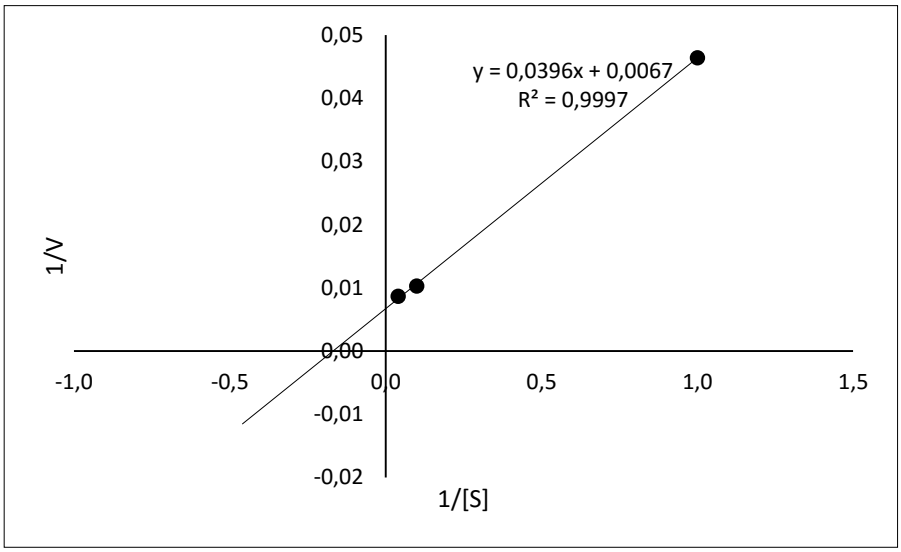


*Figure C5: Lineweaver-Burke plot for CYP3A4.*

**Appendix D: Standard Operating Procedure (SOP) for simultaneous determination of CYP activities in human microsomes**

# SOP

Department of Cancer Research and Molecular Medicine

The Medical Faculty

NTNU

# *In vitro* CYP cocktail to study inhibition in human liver microsomes

| | |
|---|---|
| SOP-Number: | E60 |
| Revision Number: | 1 |
| Copy no.: | |
| Revision date: | 26.04.2016 |
| Last revision date: | |
| Classification: | Intern |

| | | |
|---|---|---|
| **Edited by:** | (title/sign) | Student Marita Gustavsen |
| **Approved by:** | (title/sign) | Associate Professor Bent Hellum |
| **Reviewed by:** | (title/sign) | Student Marita Gustavsen |

# 1. PURPOSE

The purpose of this experiment is to determine the inhibition potential of a xenobiotic towards the enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 using pooled human liver microsomes. The inhibition potential is determined by measuring the conversion of specific CYP substrates to their respective metabolites; phenacetin to acetaminophen, losartan to EXP-3174, omeprazole to 5-OH-omeprazole, dextromethorphan to dextrorphan and midazolam to 1-OH-midazolam, during 20 minutes incubation. The conversion is compared to a control with no inhibitor and measured by a validated HPLC-MSMS procedure.

# 2. Equipment and supplies

## 2.1 Laboratory apparatus

- Waterbath 37ºC
- Timer
- Vortexer
- Kubota centrifuge
- An LC-MSMS system consisting of
  - Shimadzu LC20AD LC system
  - Waters XBridge $C_{18}$-column (3.5 µm, 2.1×100 mm)
  - AB SCIEX Triple Quad$^{TM}$ 5500

## 2.2 Equipment

- Coniform glass test tubes
- Pipettes of 5-50, 20-200 and 200-1000 µl and tips
- Multipipette w/tips, 1 ml and 2.5 ml
- Insulating box and ice
- HPLC vials and caps

## 2.3 Solutions for the incubation assay

All solutions should be prepared the day before the experiment.

NB! Some of the solutions may be stored. Check if there is anything left and the production date. If necessary, make new ones.

| Solution | pH | Shelf life | Stored in | SOP | Comments |
|---|---|---|---|---|---|
| 0.5 M $KH_2PO_4$ (kaliumdihydrogenfosfat) | Ca 4.3 | 1 year | R1 | S01 | |
| 0.5 M $K_2HPO_4$ (kaliumhydrogenfosfat) | Ca 9.4 | 1 year | R1 | S02 | |
| 0.1 M KPO-buffer | 7.4 | 3 weeks | R1 | S03 | Check pH |
| 5 mM NADPH | | 4 weeks | F1 (-20 ºC) | S60 | |
| Distilled water | | | Bench | | |
| 400 µM phenacetin | | 3 months | R1 | S60 | |
| 40 µM losartan | | 3 months | R1 | S60 | |
| 40 µM omeprazole | | 3 months | R1 | S60 | |
| 200 µM dextromethorphan | | 3 months | R1 | S60 | |
| 200 µM midazolam | | 3 months | R1 | S60 | |
| 20 mg microsomal proteins/mg | | | F2 (- 80ºC) | | |
| Protein precipitation solution | | | R1 | S60 | |
| Herbal extracts | | 2 weeks | R1 | | w/ ≤1 % ethanol |

## 2.4 Solutions for the LC-MSMS measurements

| Solution | pH | Shelf life | Stored in | SOP | Comments |
|---|---|---|---|---|---|
| Methanol HPLC grade | | | Chem. room | | |
| Formic acid | | | R1 | | |
| Distilled water | | | Lab | | |

# 3. Procedure

The experiment is performed in 3 parallels.

1. Mark the test tubes and place them in an appropriate rack.
2. Prepare the working area by starting the **water bath** and placing the **timer** and the **vortexer** appropriately.
3. Fill an insulating box with ice from the washroom.
4. Place the protein precipitation solution on ice.
5. Put **human liver microsomes (HLM)** (found in F2) on bench for rapid thawing. Register which vials are taken out in the Balance sheet for HLM (check Lot.no).
6. If aliquots with 50 µL HLM is already in freezer, use them first.
7. **NB! This step is not necessary to do before each experimental session.**
   Divide the HLM from one vial to 10 Eppendorf tubes with 50 µL in each, keep the number of tubes necessary for the experiment on ice, and freeze the rest in F2.
8. Add 450 µL **0.1 mM KPO-buffer** to the Eppendorf tube with HLM to make 500 µL 0.4 mg proteins/mL HLM solutions for the experiment.
9. Find all the **substrates**, **the herbal extracts/inhibitor**, **the NADPH-solution** and the 0.1 M KPO-buffer.
10. Dilute the herbal extract to 6-10 decreasing concentrations.

11. Mix the reagents in the glass tubes as described in the table below, with three paralells of the reference sample, and three parallels for each concentration of the diluted herbal extract.

| | **Blank** | **Reference** | **With Herb** |
|---|---|---|---|
| **400 µM Phenacetin** | 10 µL | 10 µL | 10 µL |
| **40 µM Losartan** | 10 µL | 10 µL | 10 µL |
| **40 µM Omeprazole** | 10 µL | 10 µL | 10 µL |
| **200 µM Dextromethorphan** | 10 µL | 10 µL | 10 µL |
| **200 µM Midazolam** | 10 µL | 10 µL | 10 µL |
| **5 mM NADPH** | - | 40 µL | 40 µL |
| **Herb** | - | - | 40 µL |
| **0.1 mM KPO Buffer** | 110 µL | 70 µL | 30 µL |
| **Total** | 160 µL | 160 µL | 160 µL |

12. Put the test tubes in water bath with agitation (100-110 rpm) at 37.8 ºC and let them temper for 5 minutes.
13. Prepare multipipette with 1 ml tip.
14. Remove the first test tube from the water bath.
15. Add 40 µl **0.4 mg/ml HLMs** and start the timer simultaneously.
16. Vortex, and put the test tube back in the water bath, preferably on a new place to make track of the additions.
17. Remove the next test tube and at t = 15 sec, add 40 µL HLMs.
18. Vortex, and replace in the water bath.
19. Repeat every 15 seconds to all the tubes have received HLMs.
20. At **t = 20.00 min**, add 100 µL ice-cold **protein precipitation solution** to tube 1, vortex and place on ice.
21. Prepare the next tube and add ice-cold protein precipitation solution at **t = 20 min and 15 sec**. Vortex and place on ice.

22. Repeat every 15 sec to all the tubes have received ice cold protein precipitation solution and been put on ice.
23. Centrifuge samples at 3500 rpm for 12 minutes.
24. Transfer the supernatant to HPLC vials.
25. Analyse production of substrates and metabolites with the following LC-MSMS conditions:

| LC-MSMS parameters | Set conditions |
|---|---|
| **Cooling rack temperature** | 15 ºC |
| **Injection volume** | 5 µL |
| **Flow rate** | 0.3 ml/min |
| **Column oven temperature** | 30 ºC |
| **Mobile phase A** | Water w/ 0.1 % formic acid |
| **Mobile phase B** | Methanol w/ 0.1 % formic acid |
| **ESI mode** | Positive |
| **IonSpray voltage** | 4500 volt |
| **Temperature** | 575 ºC |
| **Curtain gas flow, nitrogen** | 16.0 bar |
| **Collision gas flow, nitrogen** | 8.0 bar |
| **Ion source gas 1 flow, nitrogen** | 60 bar |
| **Ion source gas 2 flow, nitrogen** | 50 bar |
| **Run time** | 7.5 min |

| ID | Q1 mass | Q3 mass | DP (volts) | CE (volts) | EP (volts) | CXP (volts) |
|---|---|---|---|---|---|---|
| **Phenacetin** | 180.0 | 110.0 | 56.0 | 29.0 | 10.0 | 6.0 |
| **Acetaminophen** | 152.0 | 110.0 | 16.0 | 25.0 | 10.0 | 8.0 |
| **Losartan** | 423.2 | 207.2 | 101.0 | 31.0 | 10.0 | 12.0 |
| **Exp-3174** | 437.2 | 235.0 | 111.0 | 25.0 | 10.0 | 14.0 |
| **Omeprazole** | 346.2 | 198.1 | 126.0 | 33.0 | 10.0 | 16.0 |
| **5-OH-Omeprazole** | 362.2 | 214.1 | 46.0 | 17.0 | 10.0 | 12.0 |
| **Dextromethorphan** | 272.3 | 147.0 | 81.0 | 41.0 | 10.0 | 14.0 |
| **Dextrorphan** | 258.3 | 199.0 | 81.0 | 37.0 | 10.0 | 14.0 |
| **Midazolam** | 326.2 | 291.1 | 140.0 | 40.0 | 10.0 | 8.0 |
| **1-OH-midazolam** | 342.2 | 203.2 | 131.0 | 37.0 | 10.0 | 10.0 |
| **IS** | 260.3 | 116.2 | 111.0 | 25.0 | 10.0 | 12.0 |

24. Analyse the incubation samples together with a standard curve and quality controls, using the method “Injestest2.rbd”

# 4. Deviations from the procedure

# Appendix E: Poster presented at the 2016 NSFT winter meeting at Beitostølen

## *In vitro* inhibition of human CYP enzymes by hops (*Humulus lupulus*). A "cocktail" study

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## Background

**An increasing** number of people use herbs and herbal preparations together with conventional medicines. This increases the need for knowledge about possible dangerous combinations that may lead to changed drug pharmacokinetics. In recent years, there have been an increased focus on interactions between phytochemicals and medicinal drugs, and quite many papers have been published exploring this subject. However, there is still a long way to go, considering the number of different herbal preparations available for human intake.

**Hops (*Humulus lupulus*,** figure 1) is a herb often added to calming herbal preparations in combination with other herbs proposed to have calming properties. In addition, tea made of hops is recommended for everything from sleep deprivations to menopausal problems in women.

Figure 1: Hops (*Humulus lupulus*)

**The aim of this study** was to use a "cocktail" method with human liver microsomes to simultaneously assess *in vitro* inhibition by hops on the cytochrome P450 enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4.

## Material and Methods

**Herbal constituents** from dried hops were extracted using either a 50% ethanol solution or water.

**Human liver** microsomes (0.4 mg/ml protein content, Gentest) from a pool of donors were incubated for 20 minutes in a potassium-phosphate buffer containing NADPH (1mM) and hops using phenacetin (20 µM), losartan (2 µM), omeprazole (2 µM), dextromethorphan (10 µM), and midazolam (10 µM) as substrates for CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively. Total incubation volume was 200 µL. The incubation reaction was terminated by adding 100 µL ice cold 50:50 acetonitrile-methanol solution containing the internal standard (IS) propranolol. Samples were centrifuged and transferred to LC-MSMS-vials.

**The CYP** substrates and the metabolites acetaminophen (CYP1A2), EXP-3174 (CYP2C9), 5-OH-omeprazole (CYP2C19), dextrorphan and 1-OH-midazolam were analyzed in the same run by a validated LC-MSMS method.

**The metabolite/substrate** area ratio were plotted against the concentration of hops in inhibition curves and non-linear regression was used to determine $IC_{50}$-values.

## Results

**Inhibition curves** of the different extracts of hops can be seen in figure 2, the corresponding $IC_{50}$ values are summarized in table 1.

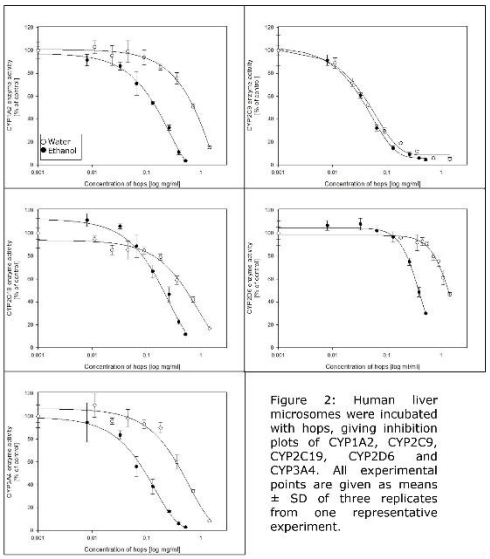


Figure 2: Human liver microsomes were incubated with hops, giving inhibition plots of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. All experimental points are given as means ± SD of three replicates from one representative experiment.

Table 1: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 $IC_{50}$-values [mg/ml] for water and ethanol extracted hops. All experimental values are given as means ± SD (n ≥ 6) from at least two independent experiments.

| | Extraction solute | water | 50% ethanol |
|---|---|---|---|
| **Hops $IC_{50}$ [mg/ml]** | **CYP1A2** | 0.75 ± 0.008 | 0.18 ± 0.03 |
| | **CYP2C9** | 0.060 ± 0.01 | 0.046 ± 0.005 |
| | **CYP2C19** | 0.66 ± 0.2 | 0.21 ± 0.06 |
| | **CYP2D6** | 1.3 ± 0.01 | 0.37 ± 0.09 |
| | **CYP3A4** | 0.51 ± 0.07 | 0.088 ± 0.008 |

**Ethanol extracted** hops was in general a stronger inhibitor, giving lower IC50-values compared to water extracted hops. The strongest inhibition was shown for CYP2C9, with an $IC_{50}$-value 0.060 mg/ml and 0.046 mg/ml for water and ethanol extract, respectively, while CYP2D6 was least affected with an $IC_{50}$-value of 1.3 mg/ml and 0.37 mg/ml for water and ethanol extract, respectively.

## Conclusion

**Extracts from** hops inhibited all CYP enzymes investigated, CYP2C9 was most affected.

***In vitro*** «cocktail» inhibition studies are cheap, effective and relatively easy to perform. *In vitro* «cocktail» inhibition studies are useful to get increased knowledge of possible CYP interactions between herbal and conventional medicines.

*Acknowledgements*: Financial support from the Faculty of Medicine, NTNU, Trondheim
The analysis were performed at Proteomics and Metabolomics Core Facility (PROMEC), NTNU, Trondheim