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In vitro interactions between medicinal drugs and herbs on Cytochrome P-450 metabolism and P-glycoprotein transport

Thesis for the degree Philosophiae Doctor

Trondheim, October 2007

Norwegian University of Science and Technology
Faculty of Medicine
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In vitro interaksjoner mellom legemidler og urter på Cytokrom P450 metabolisme og P-glykoprotein transport.

Omsetningen av naturlegemidler og kosttilskudd har økt dramatisk de siste årene, og en økende aksept for urtebruk tilsier at forbruket fortsatt vil øke. Samtidig bruk av naturlegemidler/urter og legemidler forekommer ofte. Naturlegemidler og urter kan påvirke omsetningen av legemidler i kroppen og økt kunnskap rundt dette temaet er viktig for å fremme pasientsikkerheten.

Det er i hovedsak to systemer involvert i legemiddelomsetning og - transport som kan påvirkes av naturlegemidler: Cytokrom P-450 enzymer (CYP), som bryter ned legemidler, og P-glykoprotein (P-gp), som er involvert i transport av legemidler. Dette proteinet sitter bl.a. i tarmveggen og har som oppgave å transportere legemiddel tilbake i tarm igjen, og dermed begrense mengde legemiddel som blir tatt opp fra tarmen.

Hovedmålet med avhandlingen har vært, gjennom ulike teknikker i laboratoriet (*in vitro*), å få økt kunnskap om hvordan disse to systemene påvirkes av seks kommersielle produkter, johannesurt, salvie, valerianerot, Ginkgo Biloba, solhatt og hestekastanje, alle registrert som naturlegemidler i Norge.

I det første arbeidet undersøkte vi om urtene kunne påvirke dannelsen av CYP slik at det blir dannet mer av bestemte enzymer (induksjon) og dermed en økning i enzymaktivitet. Dette fører til at legemiddelnedbrytningen går fortere, og man får for lave konsentrasjoner av legemiddel i blod. Johannesurt, valerianerot og Ginkgo Biloba viste seg å øke aktiviteten til noen av enzymene.

Det andre arbeidet omhandlet hvordan aktiviteten til en spesiell CYP, CYP2D6, ble redusert (inhibert) under påvirkning av urtene. Effekten av etanol på dette enzymet ble også undersøkt. Alle urtene inhiberte CYP2D6, men den kraftigste virkningen hadde johannesurt, salvie og valerianerot. Etanol økte enzymaktiviteten ved lave konsentrasjoner, og reduserte aktiviteten ved høye konsentrasjoner.

CYP enzymet som omsetter flest legemidler heter CYP3A4. I det tredje arbeidet ble urtenes påvirkning på dette enzymet undersøkt. Vi undersøkte også om urtene kunne redusere P-gp aktivitet. Alle urtene påvirket både CYP3A4 og P-gp, med henholdsvis johannesurt og salvie, og Ginkgo Biloba og johannesurt, som de kraftigste inhibitorene av henholdsvis CYP3A4 og P-gp.

Samlet har vi vist at både CYPer og P-gp ble påvirket av urtene. I tillegg til johannesurt, foreslås Ginkgo Biloba og salvie som potensielle urter til å forårsake betydelige endringer i opptak og omsetning av legemidler i kroppen.

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Marthe and Mathilde, my two lovely daughters, must be mentioned. They are my pride and joy and it is wonderful to see them grow in all meanings of the word every day. This work is for you guys.

Trondheim, October 2007

Bent Håvard Hellum

Abbreviations

ATCC	American type culture collection
c-DNA	complementary deoxyribonucleic acid
CO	carbon monoxide
CPM	counts per minute
CYP	Cytochrome P450
DMEM	Dulbecco's modified eagles medium
DPM	disintegrations per minute
GC	gas chromatography
GLP	good laboratory practice
GMP	good manufacturing practice
HCl	hydrochloric acid
HMP	herbal medicinal product
HPLC	high pressure liquid chromatography
IC_{50}	inhibitor concentration decreasing enzyme activity by 50% compared to control activity
K_i	inhibitor constant
K_M	Michaelis-Menten constant for a substrate
µ	micro (10^{-6})
NaOH	sodium hydroxide
NADPH	nicotine amide diphosphate
nm	nanometer (10^{-9} meter)
NMA	Norwegian Medicinal Agency
nmol	nanomol (10^{-9} mol)
NOK	Norwegian kroner
P450	Cytochrome P450
P_{app}	apparent permeability
P-gp	P-glycoprotein (MDR1)
pmol	picomol (10^{-12} mol)
QC	quality control
RILD	Research Institute of Liver Diseases
SD	standard deviation
SJW	St. John's Wort

TEER	transepithelial electric resistance
UV	ultraviolet
V_i	velocity of metabolite formation in a enzymatic reaction with inhibitor present
V_{max}	maximum velocity of metabolite formation in an enzymatic reaction
wt	wild-type, the natural occurring genotype

Table of contents

1. List of papers

This thesis, which is based on the following papers (referred to by their Roman numerals in the text), is presented to the Faculty of Medicine, the Norwegian University of Science and Technology, for the Doctoral Degree Ph.D. in Molecular Medicine.

Paper I

Bent H. Hellum, Zhuohan Hu and Odd Georg Nilsen.

The Induction of CYP1A2, CYP2D6 and CYP3A4 by Six Trade Herbal Products in Cultured Human Hepatocytes. *Basic and Clinical Pharmacology and Toxicology* 2007; 100: 23-30.

Paper II

Bent H. Hellum and Odd Georg Nilsen.

The *in vitro* Inhibitory Potential of Trade Herbal Products on Human CYP2D6-mediated Metabolism and the Influence of Ethanol. *Basic and Clinical Pharmacology and Toxicology* 2007; 101: 350-358.

Paper III

Bent H. Hellum and Odd Georg Nilsen.

In vitro Inhibition of CYP3A4 metabolism and P-glycoprotein mediated transport by trade herbal products. *Basic and Clinical Pharmacology and Toxicology* 2007; submitted

2. Summary

2.1. Paper I

The Induction of CYP1A2, CYP2D6 and CYP3A4 by Six Trade Herbal Products in Cultured Human Hepatocytes.

Herbs have the ability to induce CYP enzymes, and six trade herbal products, containing SJW, common valerian, common sage, *Ginkgo Biloba, Echinacea Purpurea* and horse chestnut (all classified as HMPs in Norway), were investigated for their inductive potential on CYP1A2, 2D6 and 3A4. To ensure identity with the original products, the solvent applied for the extraction of herbal constituents, were the same as that of the producer. The *in vitro* testing system used was primary human hepatocytes, which were added specific CYP substrates and *in vivo* relevant concentrations of herbal extracts. Control hepatocytes were added classic inducers instead of herbal extracts. CYP mediated metabolite formation was determined by validated HPLC methodologies. SJW and common valerian were the strongest inducing herbs, while horse chestnut, common sage and *Echinacea Purpurea* in general inhibited the activities of the CYPs investigated. SJW increased the activity of CYP3A4, common valerian increased the activities of CYP3A4 and 2D6, while *Ginkgo Biloba* increased the activities of CYP1A2 and 2D6. An allosteric activation is suggested for the increased activity for CYP2D6. Results suggest that SJW, common valerian and *Ginkgo Biloba* might be candidates for *in vivo* significant interactions.

2.2. Paper II

The in vitro Inhibitory Potential of Trade Herbal Products on Human CYP2D6-mediated Metabolism and the Influence of Ethanol.

In paper II, the six herbal products containing commonly used herbs, in addition to ethanol, were investigated for their inhibitory potential on CYP2D6. The same extraction procedure as previously described was applied. CYP2D6 was expressed from baculovirus infected insect cells and dextromethorphan was used as substrate. Quinidine, a known CYP2D6 inhibitor was used as positive inhibitory control in the experiments, while formation of the metabolite dextrorphan was measured using HPLC. Ethanol demonstrated a biphasic effect on CYP2D6 activity, increasing it at low concentrations and decreasing activity at higher concentrations. All herbs inhibited CYP2D6 activity, and

SJW, common sage and common valerian were the most potent ones. CYP2D6 mediated metabolism was inhibited in an uncompetitive manner by SJW, while common valerian and common sage were non-competitive inhibitors. Common valerian might also be a mechanistic inhibitor of CYP2D6.

2.3. Paper III

In vitro inhibition of CYP3A4 metabolism and P-glycoprotein mediated transport by trade herbal products.

SJW, common valerian, common sage, *Echinacea Purpurea, Ginkgo Biloba* and horse chestnut were in paper III tested for their inhibitory effects on CYP3A4 mediated metabolism and P-gp efflux transport activity. Baculovirus expressed CYP3A4 was utilized and the herbs' influence on P-gp transport was investigated using Caco-2 cells. Testosterone and digoxin were used as substrates for CYP3A4 and P-gp, respectively, and the inhibitors ketoconazole and verapamil were included as controls. For CYP3A4, formation of the metabolite 6-OH-testosterone was quantified with a validated HPLC method, while P-gp transport was measured by liquid scintillation counting of radioactive ^{3}H-digoxin. All herbs inhibited both CYP3A4 activity and P-gp efflux transport, but to different extents. SJW and *Ginkgo Biloba* were the most potent inhibitory herbs of CYP3A4 and P-gp, respectively. There was no correlation between the inhibitory potential of the different herbs on the two measured activities. From the results of paper III, *Ginkgo Biloba*, horse chestnut, common sage, in addition to SJW, might have potential to cause significant *in vivo* herb- drug interactions.

3. Introduction

3.1. Background

The use of herbs and other natural products in food, to alleviate pain, treat diseases, as intoxicants and so on emanates from ancient Chinese, Greek and Egyptian civilisations. Mandrake (*Mandragora officinarum*), an alkaloid-containing herb was used as a narcotic by the ancients, and exploited as a painkiller in the Middle Ages. Ancient civilisations were also aware of the more detrimental effects of some herbs on animals and humans, and used herbs for hunting and executions. The most famous poisonous herb is perhaps Poison Hemlock (*Conium maculatum*), which was used to kill the philosopher Socrates in ancient Greek. Many of today's most efficacious medicines are derived from the plant kingdom, for example the anticancer drug tamoxifen (from Pacific yew, *Taxus brevifolia*), vinblastine (from Madagascar Periwinkle, *Catharanthus roseus*) used in the treatment of leukaemia and the immunosuppressant drug cyclosporine (from the fungus *Tolypocladium inflatum Gams*, first isolated from a Norwegian soil sample taken from Hardangervidda).

Herbs as complementary and alternative medicine is gaining increasing popularity in today's world, as more and more people are taking different herbal medications, believing that herbs are safe and at least as efficacious as chemical drugs without the potential side effects (1-3). There is no rigorous control over either products or sales worldwide, as many people buy herbal products on the internet and outlets not under governmental control, or get them from herbal practitioners. Estimating total use or sales, is therefore very difficult. Top selling herbal remedies worldwide (approximate numbers) are given in table 1. In Norway, total sales of HMPs were in 2003 about NOK 55.6 million; however this constitutes only about 3% of the total sales of natural remedies, which was about NOK 1.8 billion (4).

The major problem is that many people taking herbs are already medicated with conventional drugs, in order to treat a disease. Herbs can interact with these drugs, changing drug absorption, disposition and/or elimination. The majority of these interactions can happen at one or more of three distinct sites: the Cytochrome P450 (CYP) system (section 3.2.1); the P-glycoprotein (P-gp) mediated efflux pump (section 3.2.2); and the plasma protein binding (section 3.2.3).

Table 1: Top selling herbal remedies worldwide

Herbal remedy	Sales in million dollars
Ginkgo Biloba	238.6
Garlic	160.0
SJW	147.7
Ginseng	120.9
Echinacea Purpurea	108.8
Saw palmetto	91.1

Source: The Hartman Group; F.D.C. reports

Indications of interactions with dietary phytochemicals and medicinal drugs were first described in the 1970s. Low plasma levels of the analgesic drug phenacetin were associated to the consumption of cruciferous vegetables. Constituents that are present in high amounts in such vegetables, called indoles, were presumed to be responsible for the interaction (5;6). Subsequent studies revealed that broccoli, when added to the diet of rats, increased the amount of P450s in the rats' liver and colon (7), and human studies showed that intake of broccoli selectively induced CYP1A2 (8;9), which is responsible for the metabolism of among others caffeine and phenacetin.

In recent years, quite many papers and investigations about interactions between phytochemicals and medicinal drugs have been published, and many of these are concerning drug-herb interactions. However, there is still a long way to go, considering the number of different herbal preparations available for intake.

The most well known herb to interact with drug metabolizing enzymes is St. John's Wort. SJW has been shown to modulate the enzyme activity of CYP3A4 both *in vitro* (10) and *in vivo* (11;12), and also to modulate the activity of the efflux protein P-gp (13). The herb causes an induction of CYP3A4 *in vivo*; the clinical consequence is a faster metabolization of drugs that are substrates of this enzyme. These drugs will then loose their effect as they appear systemically in sub therapeutic concentrations. This is especially critical when combined with drugs that have a narrow therapeutic range. Clinical examples include SJW and the immunosuppressant cyclosporine (14;15) and also oral contraceptives (16). SJW has a similar effect on the activity of P-gp, which actively

transports xenobiotics (e.g. drugs) out of cells. An induction of this protein will lead to increased efflux of drugs, and hence decrease the oral availability of these drugs. SJW is not the only herbal remedy modulating drug metabolism and transport, and the list includes ginseng, black peppers, *Ginkgo Biloba*, garlic, hawthorn, liquorice and milk thistle (17;18), among others.

3.2. Herbal remedies

Although many modern world medicines are derived from different phytochemicals, many people suffer from the misconception that herbs are totally safe to ingest, and without any side-effects, because they are "natural". However, dramatic effects can be seen when the herb and a medicinal drug is taken concomitantly (19;20), and to facilitate patient safety this issue is important to explore.

Recent investigations has reported that up to 49% of all consumers in the US had taken some kind of dietary supplement the last year, and 24% used herbal products regularly. Also, 16%, or 15 million people, took herbal products and medicinal drugs at the same time (21;22), and this without informing their primary physicians.

HMPs are usually botanical ethanolic extracts from root, stem, flower or leaves of herbal plants. After extraction, the ethanol is evaporated and the residue is put in capsules. The residue is then a very complex mixture of different herbal constituents, and thus one can have pharmacological and/or interacting effects from more than one chemical entity at a time. This is, among herbal medicine followers, regarded as positive, as the different chemical entities can act synergistically to create an effect. It is rare that only one constituent of an herb is responsible for the herb's total effect (23).

The six herbal remedies used in this thesis are all "single" components in six different commercially available herbal products. They are classified as HMPs by the Norwegian Medicinal Agency (NMA; Statens Legemiddeverk): St. John's Wort (Hypericum Stada®; *Hypericum Perforatum*), *Ginkgo Biloba* (Seredrin®), common sage (Nosweat®; *Salvia Officinalis*), common valerian (Valerina® Forte; *Valeriana Officinalis*), horse chestnut (Venastat®; *Aesculus Hippocastanum*) and *Echinacea Purpurea* (Echinagard®) (24). In

Norwegian pharmacies, these six HMPs sold for NOK 12.7 millions* in 2006 (25), however, most of the products can also be sold outside of pharmacies, so the number is underestimated.

In order to get a product classified as an "herbal medicinal product", an application has to be submitted to the NMA. The producer has to document the quality of the product (GMP), to the same level as set for ordinary medicinal drugs. However, documentation demands on safety and efficacy are less stringent than what is required for other medicinal drugs and are based on use in a historical and traditional perspective (26).

3.3. Sites of drug-herb interactions

3.3.1. Cytochrome P450 enzymes

The CYP system is a superfamily of heme-containing enzymes involved in phase I drug metabolism, responsible for the metabolism of many different xenobiotics, including chemical drugs, herbal constituents, toxic substances, and also some important endogenous compounds, for example steroids (27). Cytochrome P450 derives its name from the complex between Cytochrome P450 and CO, which absorbs light maximally at 450 nm. CYP enzymes are distributed in various tissues in the body, predominately expressed in liver, intracellularly in the endoplasmic reticulum, but may also be found in the respiratory tract, lungs, brain and the small intestine (28-30).

The CYP superfamily is divided into 18 families and 42 subfamilies in humans on the basis of their nucleotide sequence homology, covering 57 different human CYP genes (31;32), and inter-individual genetic variability is also high. Genetic polymorphisms have been detected for almost all CYPs, amongst others, CYP2D6, 2C19 1A2, 2C9 and 3A4 (33). This also means that there is a large variability in inter-individual metabolic rates and pathways of different drugs. Other factors, like environmental and patophysiological conditions, as well as dietary compounds, cigarette smoke and alcohol, can also contribute to variation in the expression and function of different P450s. While some CYPs play a role in the formation and elimination of endogenous compounds, others, especially those belonging to the families 1-3, seem to be there principally for the metabolism of

* sales from health food and food stores not included

xenobiotics (34). They represent around 70% of CYP enzymes in the human adult liver (35;36).

CYPs are capable of biotransforming xenobiotics by a number of oxidation reactions, to make lipophilic molecules more hydrophilic and easier for the body to excrete. The basic reaction of the enzyme family is mono-oxygenation, using NADPH (figure 1), but they are capable of catalyzing several types of oxidations, including: hydroxylation, epoxidation (of a double bond), dealkylation, dehydrogenation and cleavage of esters (37). They are also capable of catalyzing reducing reactions and isomerizations, and are thus very versatile (38). This versatility gives P450s broad substrate specificity, and explains their involvement in the many biotransformation reactions. However, P450s, also play an important role in bio-activating certain xenobiotics, for example "prodrugs", in order to make them pharmacologically active, or benzo[a]pyrene (in cigarette smoke) (39) in order to make the molecule carcinogenic. This is called metabolic activation.

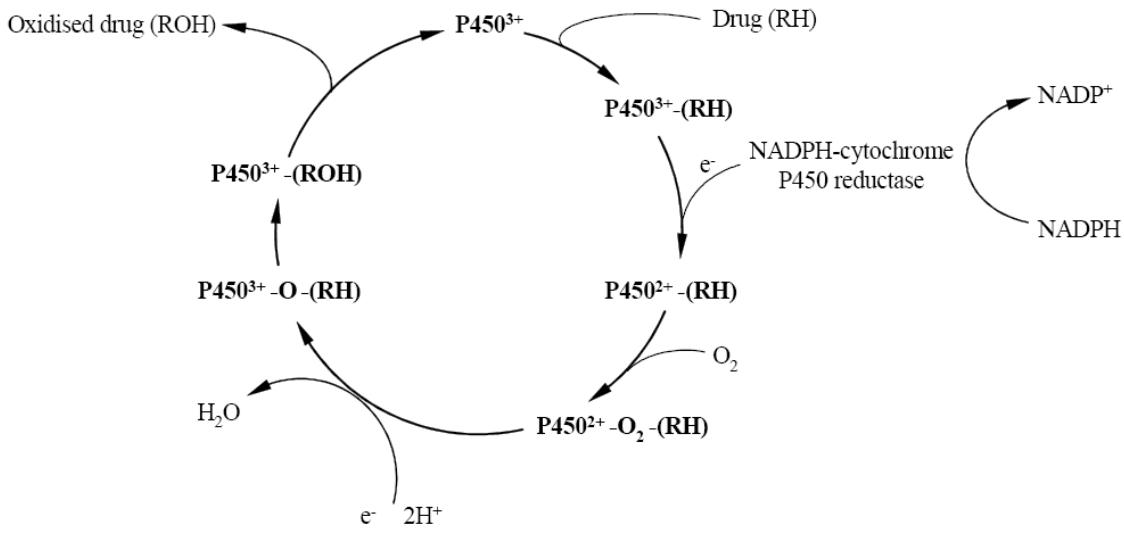

Figure 1: Catalytic cycle of Cytochrome P450 (adapted from Parkinson (37))

As previously mentioned variances in levels and activity of individual CYPs from one person to the next, are common, and can be attributed to environmental and/or genetic factors (40). Thus, increased CYP enzyme activity can be explained by: 1: stimulation of existing CYP enzymes, allosterism and conformal changes by a xenobiotic; 2: induction of the synthesis of CYP enzyme(s) by exposure to a xenobiotic; or 3: gene duplication,

leading to CYP over expression. On the other hand, decreased CYP enzyme activity can be attributed to: 1: inhibition and inactivation of existing CYP enzymes by a xenobiotic; 2: environmental factors that suppress CYP enzyme expression; or 3: genetic mutations that blocks synthesis, or generate inactive enzymes or enzymes with low activity. We are interested in what an herb can do (as a xenobiotic), and for increased and decreased enzyme activity, cases 1 and 2 are the most relevant for this thesis.

The type of inhibition can be either reversible or irreversible (mechanism-based inhibition). The most common type of enzyme inhibition is reversible inhibition and it can be divided further into competitive, uncompetitive, non-competitive and mixed-type inhibition. Reversible inhibitors act fast, is bound to the enzyme with weak bonds and do not destroy the enzyme. Competitive inhibition is characterized by competition between the enzyme substrate and inhibitor to bind to the same place in the active site of the enzyme; uncompetitive inhibition is characterized by binding of the inhibitor not to the free enzyme, but to the enzyme-substrate complex; and non-competitive inhibition is characterized by binding of the substrate and inhibitor at distinct different sites on the enzyme. However, elements of both competitive and non-competitive inhibition, mixed-type, are also seen (41). Mechanism-based inhibition (also called suicide inhibition) requires biotransformation of the inhibitor, and occurs via a metabolite intermediate complex or via strong covalent binding of reactive intermediates to some part of the enzyme. Mechanism-based inhibition is characterized by a concentration-, time- and NADPH- dependent enzyme inhibition, and the effect is usually long lasting. Herbs and isolated herbal constituents are shown to be both reversible and mechanistic-based inhibitors of CYPs (42-44).

Induction of CYPs is a slow regulatory process, involving nuclear receptors, and requires multiple dosing of the inducing compound. Many CYPs are inducible, e.g. 3A4, 2E1 and 1A2 (45). Induction will lead to increased amount of one or more CYPs, and, in a biological perspective, serves as a protective response to increase the detoxification activity. However, from a clinical perspective, it can lead to subtherapeutic levels of drug in plasma and therapeutic failure, or, in the case of prodrugs, it will increase the amount of active metabolite. As already mentioned, SJW is a CYP inducing herbal remedy and other *in vitro* examples include ginger and Wu wei zi (46).

3.3.2. P-glycoprotein

P-glycoprotein is a membrane bound protein which was first identified in drug-resistant Chinese hamster ovary cells (47) and later linked to the multidrug resistance of cancer cells. It is therefore also called multidrug resistance protein 1 (MDR1). It belongs to the ATP-binding cassette (ABC) family of transporters (48;49), and has an important role in the absorption, distribution and excretion of xenobiotics in mammals (50). P-gp is found in high levels on the apical surface of epithelial cells in kidneys, adrenal glands, liver, pancreas, small intestine and colon and also in the blood-brain barrier (51-53).

P-gp in the small intestine plays an important role in the absorption of orally administered drugs. Situated in the cell membranes, P-gp effectively limit the amount of drug that is gaining systemic access by working against the diffusion or absorptive process of the drugs, and actively transporting its substrates out of the cell and back into the intestinal lumen (figure 2) (54-56). Changes in P-gp activity will therefore affect the systemic concentration of xenobiotics. Inhibiting P-gp mediated efflux, will lead to an enhanced concentration of a drug systemically, with possible adverse or even toxic reactions. Opposite, an induction or activation of P-gp by a xenobiotic will lead to an enhanced efflux activity, and hence a low systemic concentration of a given drug, with a concomitant loss of therapeutic effect. This is especially critical when dealing with drugs that have a narrow therapeutic window. Increasing evidence indicate that herbs also have an ability to modulate P-gp mediated efflux activity, *in vitro* as well as *in vivo*, by both inhibiting P-gp activity and inducing protein expression (57). CYP3A4 and P-gp are also reported to have significant substrate overlap (58).

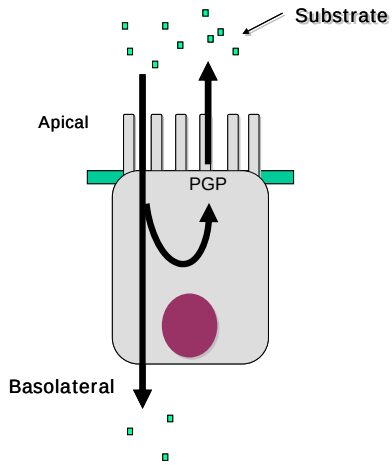

Figure 2: Schematic illustration of P-gp efflux

3.3.3. Plasma protein binding displacement

Although it is beyond the scope of this thesis, I will also mention briefly the third site for a possible interaction between medicinal drugs and herbs; the level of plasma protein binding. There are three main proteins that medicinal drugs will bind to in plasma: albumin, α-1-acid glycoprotein and lipoproteins, where the two first are the most important ones.

Data on modulation of plasma protein binding by herbs are very limited, compared to what exist on CYP and P-gp and the clinical implications of protein binding displacement of a drug in, is debated (59). An interaction at this level might lead to unwanted large fluctuations of free active drug in plasma for longer periods of time. This might have implications especially for drugs with a narrow therapeutic window.

4. Aims of the thesis

The main objectives of this thesis were to gain further knowledge about the drug pharmacokinetic inhibition potential and inhibition mechanisms of six trade herbal products registered in Norway as HMPs. This by using *in vitro* techniques for CYP metabolism and P-gp efflux transport.

Addressed questions were:

- Will ethanol, as an essential herbal extraction medium, influence the selected *in vitro* assays?
- Do the six selected herbal products inhibit or induce CYP mediated metabolism, if so, in what order?
- What kind of inhibition is exerted on the CYP mediated metabolism by the selected herbs?
- Are any of the herbal products mechanistic inhibitors of CYP mediated metabolism?
- Do the six selected herbal products inhibit P-gp mediated efflux transport, if so, in what order?
- Are the herbal effects on CYP3A4 mediated metabolism related to P-gp transport activity, if so, how?
- Do any of the investigated herbs possess a probable *in vivo* pharmacokinetic drug inhibition potential?

5. Methods

In the present thesis, several different methods and procedures were used. These are described in detail in each paper. Some considerations on methods and procedures are given below.

5.1. Herbal extractions and concentrations

The trade herbal products were grounded and added ethanol at appropriate concentrations (same as in the original extraction). Herbal constituents were then extracted at 30°C with constant stirring for 1 hour. The solution was centrifuged, and the ethanol containing herbal constituents was transferred to a preweighed beaker. The residue was reconstituted in ethanol, and the extraction procedure was repeated. The two ethanolic extracts were pooled, solvent was evaporated and residue was weighed and resuspended in an exact amount of ethanol at the given strength, to give herbal stock solutions with known concentrations.

Echinacea Purpurea, which was in liquid form, was evaporated, weighed and reconstituted in a smaller, known amount of 20% ethanol.

5.2. Isolation of human hepatocytes

The human liver was donated following the Shanghai Donation Regulation (China) and informed consent. All serology data were normal; no information was given on smoking habits. Cause of death was trauma.

Primary human hepatocytes were prepared by a modified collagenase perfusion technique described by Li et al. (60). In short: Liver is cut into approximately 40 gram pieces and they are perfused through a single blood vessel with a digestion medium containing collagenase for 10-12 minutes. Liver specimen is then transferred to a beaker and is cut open to release hepatocytes. The cell suspension is filtered into 50 ml centrifuge tubes, and centrifuged/washed in digestion medium. Pellets are resuspended in digestion medium, counted in a haemocytometer and viability is determined by the trypan blue dye exclusion method. After another centrifugation, cell pellets are resuspended in supplemented cell medium to the right cell density and plated onto appropriate culture plates. The hepatocytes are evaluated with microscopy immediately and 2-4 hours after

plating to ensure correct morphology. The method is well established in the RILD laboratory, and gives a good yield of viable hepatocytes.

5.3. CYP incubations

5.3.1. Incubations with human hepatocytes

To establish basal levels of activity, hepatocytes were exposed to specific CYP substrates (100 µM; 1A2: phenacetin; 2D6: dextromethorphan; 3A4: testosterone). Incubation medium containing substrates were added and cells were incubated for 1 hour. Reaction was stopped with ice cold methanol, suspension was centrifuged and the supernatant was analysed on an HPLC for CYP-specific metabolites.

Hepatocytes exposed to herbs or selective inducers (added in the incubation medium) were added specific substrates after 120 hours, and incubated as described above.

5.3.2. Incubations with c-DNA expressed CYP3A4

The CYP3A4 Supersomes® (BD Gentest) were stored at -80 C until use and reported to contain 132 pmol CYP3A4/mg protein. Incubation mixtures contained 20 nM CYP3A4, 0.1 mM testosterone (substrate) and a NADPH-regenerating system in 400 µl 0.1 mM potassium-phosphate buffer at physiological pH. Incubations could also be added ketoconazole (control inhibitor) or herbal extracts in different concentrations. The incubation mixture was added ice cold methanol after 10 minutes to stop the reaction, and, after centrifugation, it could be applied directly on the HPLC without any interference from herbal constituents.

5.3.3. Incubations with c-DNA expressed CYP2D6

The CYP2D6 Supersomes® were wt (CYP2D6*1, BD Gentest), stored at -80 C until use and were reported to contain 137 pmol CYP2D6/mg protein. Incubation mixtures contained 10 nM CYP2D6, 6 µM dextromethorphan (substrate) and a NADPH-regenerating system in 400 µl 0.1 mM potassium-phosphate buffer at physiological pH. Incubations could also be added quinidine (control inhibitor) or herbal extracts in different concentrations. The incubation mixture was added ice cold acetonitrile after 25 minutes to stop the reaction.

Dextrorphan was extracted with sodium carbonate (pH 12) and toluene, and back-extracted with 0.1 M hydrochloric acid. The acid was evaporated, and the residue (dextrorphan) was reconstituted in 400 µl mobile phase and transferred to HPLC vials.

5.4. Caco-2 cells

Caco-2 cells, obtained from ATCC, were maintained in supplemented DMEM. Cells of passage 35-45 were seeded in permeable inserts and incubated for 3 weeks (figure 3). Cell medium was changed every second day.

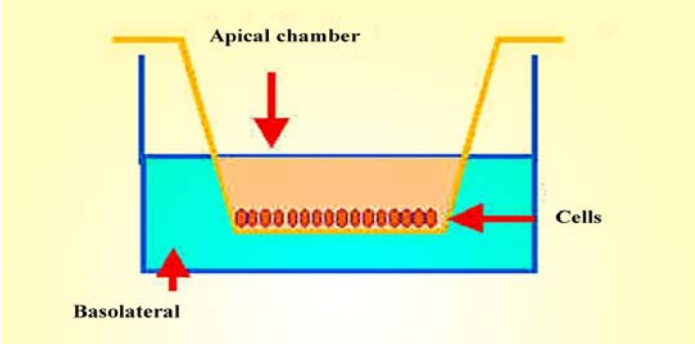

Figure 3: Schematic model of the Caco-2 system used in paper III

5.4.1. P-gp transport and accumulation

Labelled ^{3}H-digoxin (substrate) was added to the donor compartment, apical or basolateral, depending on the direction measured. Verapamil or herbs were added to both donor and acceptor compartments in equal concentrations. Cells were then incubated while mixed carefully for 90 minutes. 100 µl was taken from each of the compartments and transferred to scintillation vials.

After termination of the transport experiments, the cells were solubilised with NaOH for 15 minutes and neutralized with HCl. 100 µl of the cell lyseate was transferred to scintillation vials to measure the amount of intracellular accumulated ^{3}H-digoxin.

5.4.2. Cell monolayer integrity

To obtain good data from P-gp experiments, it is imperative that the integrity of the cell monolayer is not compromised. The monolayer integrity was monitored using TEER measurements and transport of ^{14}C-mannitol.

TEER is found by measuring the electric physical resistance on the surface of the cells and is a way to assess the permeability of the cellular epithelial monolayer. TEER was measured in each well both prior to and after the experiments. Cells showing TEER values below the cut-off value (500 Ω cm^2) were disregarded.

Cell monolayer integrity during transport experiments was also monitored using ^{14}C-mannitol. Permeability of ^{14}C-mannitol through the epithelial cell monolayer is supposed to be low, and the cut-off value was set to $1.0x10^{-6}$ cm/s, a limit suggested by others for adequate cell integrity (61).

5.5. HPLC

5.5.1. Basic HPLC theory

An HPLC consists in short of a pump, sample injector, column and a detector. A solvent (mobile phase) is continuously pumped through the machinery (figure 4).

HPLC separations involve both the mobile phase(s) and the stationary phase in the column. The mobile phase is usually composed of water and an organic solvent (plus other additives), while the stationary phase usually consists of hydrophobic ("water hating") materials. The analytes are then separated on basis of their hydrophobicity. The less polar (more hydrophobic) compounds are more attracted to the hydrophobic stationary phase and the more hydrophobic a compound is, the stronger it will attach. Hence, the less polar compounds will be eluted last from the column. Of course, the chemical composition of the mobile phase will also govern how strongly the compounds are retained (more polar = more retained).

A general liquid chromatographic process will be like this (figure 4): A mobile phase is pumped at a fixed rate through the system (and mixed, if required) by the pump. A "plug" of sample is introduced into the mobile phase by the injector, without having to stop the flow or getting air into the closed system. The sample, containing a mixture of components, is carried to the top of and into the column. Here, some components will have greater affinity for the stationary phase in the column than for the mobile phase and they will be retained in the column longer. The components that are not retained so strongly are carried down and out of the column by the mobile phase. Eventually, all

components in the sample are carried down and out. The detector is set to respond to a physiochemical property of the target component, and the response is digitally amplified and sent to a computer system, where it is recorded as a chromatogram (see figure 6).

The retained compounds will elute as peaks in the chromatogram. The retention time (time until a given peak show up) of a compound provide the qualitative aspect of the chromatogram (visualizes the degree of separation), and should be equal under identical system conditions. The peak height or area is decided by the quantity of the compound. When this height/area is compared with standards of known concentration, the actual amount of compound can be determined.

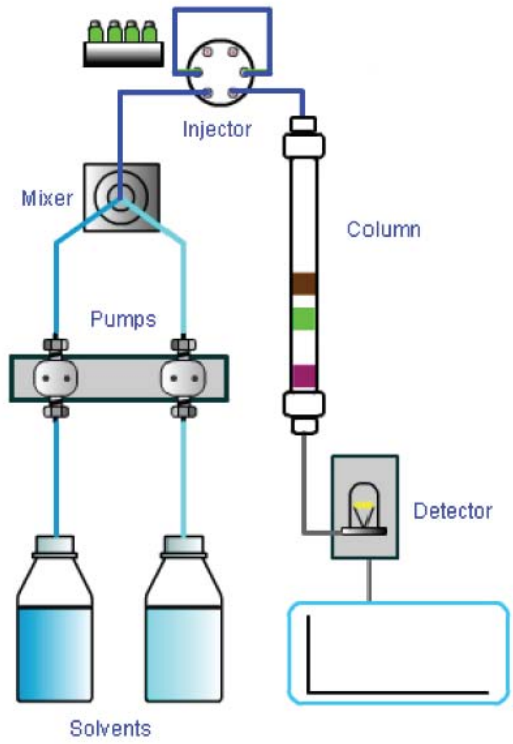

Figure 3: Schematic overview of the LC process (adapted from Crawford Scientific: "Theory of HPLC")

5.5.2. Detection of CYP metabolites by HPLC

HPLC was used to quantify the metabolites from the different CYP incubations (CYP1A2: acetaminophen; CYP2D6: dextrorphan; CYP3A4: 6β-hydroxy-testosterone). As the experiment described in paper I was conducted and the results analyzed at the

RILD laboratory in Shanghai, the details in the described HPLC methods will differ from what was used in papers II and III.

CYP1A2: Acetaminophen was separated on a Luna 5µ phenyl-hexyl (150 x 4.6 mm) column from Phenomex using a mobile phase of 10% acetonitrile, 15% methanol, 75% water (solvent A) and a solution of 60% methanol, 10% acetonitrile and 30% water. A programmed gradient of the two eluents was used to obtain a faster and more efficient separation of the incubation mixture. Acetaminophen was detected with UV light and quantified using a standard curve of pure acetaminophen in different concentrations.

CYP2D6 (paper I): Dextrorphan was separated on a Nucleosil 5µC18 100A (250 x 4.6 mm) from Phenomex using a mobile phase with 35.3% acetonitrile, 1.2% acetic acid, 0.1% trifluoroacetic acid and 63.4% water. Dextrorphan was elueted isocratically and detected with fluorescence. Quantitation was done by comparing peaks with a standard curve of pure dextrorphan.

CYP3A4 (paper I): 6β-hydroxy-testosterone was separated on a Luna 5µ phenyl-hexyl (150 x 4.6 mm) column from Phenomex using the same two eluents as for CYP1A2, however, a different programmed gradient was used. 6β-hydroxy-testosterone was also detected with UV light and quantified using a standard curve of pure 6β-hydroxy-testosterone in different concentrations.

CYP2D6 (paper II): Dextrorphan in paper II was separated on a Zorbax Eclipse XDB C18 (150 x 4.6 mm) from Agilent using 0.01 M potassium phosphate with 18% acetonitrile. Dextrorphan was detected with UV light and quantified using a standard curve of pure dextrorphan in different concentrations.

CYP3A4 (paper III): 6β-hydroxy-testosterone was separated on a Supelco LC18 5µ (150 mm x ¼") from Supelco using 50% methanol and 50% water as mobile phase. 6β-hydroxy-testosterone was elueted isocratically and detected with UV light. Quantitation was done by comparing peaks with a standard curve of pure 6β-hydroxy-testosterone.

5.6. Liquid scintillation counting

5.6.1. Basic scintillation theory

Liquid scintillation counting is a standard laboratory method for measuring radiation from beta-emitting nuclides. Samples are dissolved in a "scintillation cocktail", which contains organic solvents and solutes (or fluors). This mixture is designed to capture the energy from the beta emission transforming this energy into light: energy from the emitted beta particle excites the solvent molecule, which transfers its energy to the fluors. The now excited fluor molecules dissipate the energy by emitting (blue) light, which is detected and converted to an electrical signal by a photomultiplier tube inside the counter (figure 5). The total number of photons from the fluor molecules constitutes the scintillation. Ideally, every emitted beta particle would result in an emitted photon; however this is usually not the case. There is a discrepancy between the counted photons per minute (CPM) and the actual number of nuclear decays per minute (DPM). The ratio CPM/DPM is therefore called counting efficiency. Counting efficiency usually range from about 30% for low-energy emitters (e.g. ^{3}H) to nearly 100% for high-energy beta emitters (e.g. ^{32}P). Materials in the scintillation solution can interfere with the process leading to the emitting of light, e.g. absorbance of light by chemical entities. This interference is called quenching, and will lead to a reduced counting efficiency. Quenching can usually be corrected for through careful sample preparation or the use of internal standards.

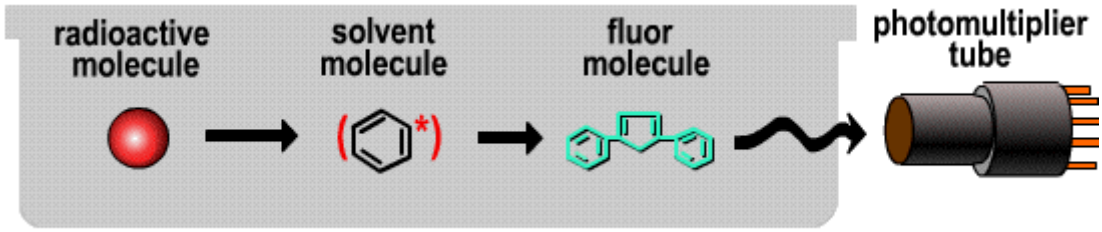

Figure 5: Overview of the liquid scintillation process (adapted from J. Thompson: "Sample Preparation and Liquid Scintillation Counting", Perkin Elmer)

5.6.2. Liquid scintillation counting of ^{3}H-digoxin

To quantify the amount of ^{3}H-digoxin that was accumulated intracellular and on the acceptor side in the P-gp experiments, the 100 µl removed were mixed with an Optiphase

scintillation cocktail, and then counted for 10 minutes in a pre-programmed Beckman scintillation counter. No quenching was observed.

Vials with known concentrations of ^{3}H-digoxin were included, and used to construct standard curves and calculate counting efficiency (28.5%).

5.7. Pharmacokinetic calculations and plots

The enzyme activities were expressed as "pmol (metabolite formed) x 10^{6} $cells^{-1}$ x min^{-1}" for hepatocytes incubations and "pmol (metabolite formed) x pmol CYP^{-1} x min^{-1}" for incubations with c-DNA expressed enzymes. Transport of ^{3}H-digoxin was expressed as P_{app} values (cm/s) or net flux (nmol x cm^{-2} x h^{-1}).

The apparent Michaelis-Menten constant K_m and the maximal reaction velocity, V_{max}, were determined from Lineweaver-Burke plots and plots of the formation of metabolite in relation to the substrate concentration used. IC_{50} values were calculated from plots of metabolite formation (CYP) or net flux (P-gp) in relation to inhibitor and herbal concentrations. The lines were fitted with linear (Lineweaver-Burke) and non-linear (IC_{50}) regressions using Sigmaplot (Sigmaplot for Windows, ver. 10.0. 2006, Systat Software, Inc., San Jose, CA, USA).

5.8. Statistics

Results were expressed as mean values $\pm$ SD, where appropriate, in the text, tables and figures. In all studies, the minimum statistical significance level was set *a priori* at $p<0.05$.

Two-sample Student's t-tests were used to test the effects of herbs and modulators on enzyme activity and P-gp transport, and ANOVA was applied to test the statistical significance of the effects on each herbal concentration. Statistical analysis were performed on SPSS (SPSS for Windows, Rel. 13.0. 2004, SPSS Inc., Chicago, IL, USA).

6. Results and Discussion

6.1. Herbal issues

Working with "impure" products containing multiple, often unknown, constituents, raises some challenges you will not meet when working with pure single chemical entities, such as a medicinal drug.

Interference from herbal constituents in the HPLC chromatogram was initially a problem, and was discovered when working with the method for CYP2D6 in paper II. At the low UV wavelength (220 nm), constituents in the herbs clouded the chromatogram (figure 6), and the metabolite was impossible to distinguish. This was overcome by an acid-base extraction, exploiting the ionic properties of dextrorphan, thus removing many of the unwanted peaks. Fortunately, this was limited to the UV detection in the assay for CYP2D6 only. At higher wavelengths, the absorbance from herbal constituents was negligible, and no extraction was necessary for adequate resolution of metabolite peaks.

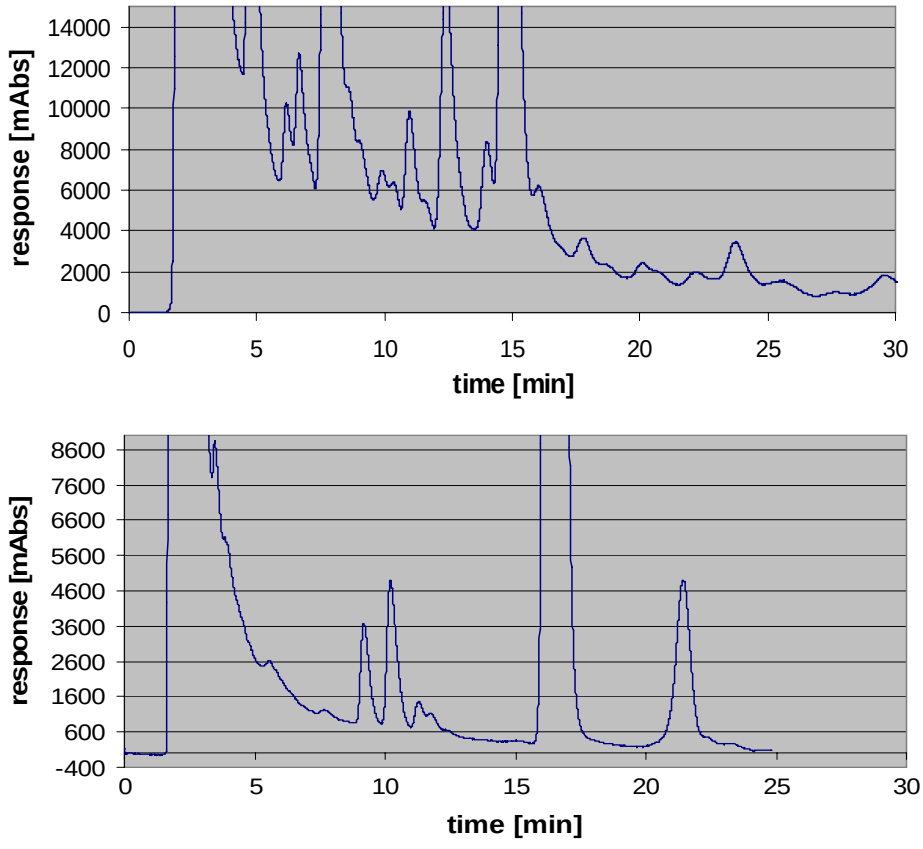

Figure 6: top: HPLC chromatogram (UV-220 nm) of supernatant after incubation with Common Sage; bottom: same chromatogram after acid-base extraction, dextrorphan can be identified as the peak with retention time of 10.2 minutes.

Testing presumed *in vivo* relevant herbal concentration was important, in order to make the *in vitro- in vivo* extrapolation (however dependent on other factors as well) more credible. Bioavailability of herbal constituents will obviously be an important factor here (figure 7). As the bioavailability of total herbs and herbal constituents usually is unknown, some estimates had to be done. However, there are a few published reports measuring the plasma steady-state concentration of isolated herbal constituents (62-65), and these were used as a basis for extrapolations and calculations of adequate and relevant herbal concentrations to use in *in vitro* experiments. This was thoroughly discussed in paper I.

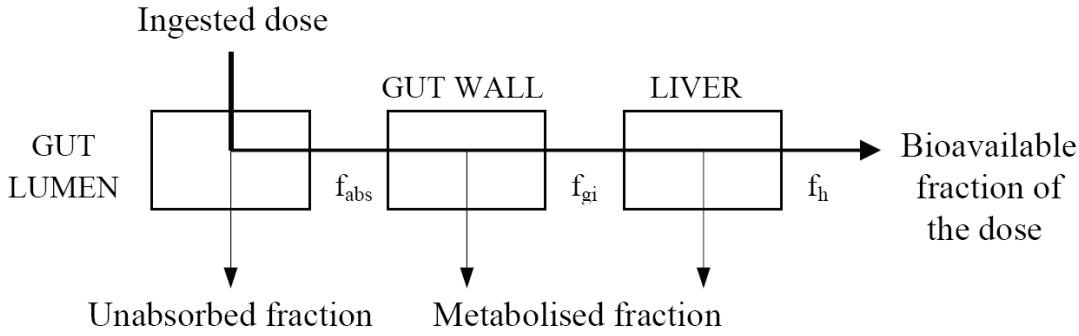

Figure 7: Illustration of the mechanisms involved in bioavailability of ingested xenobiotics.

6.2. Induction of CYP enzymes

Primary human hepatocytes as an *in vitro* system is excellent for investigating P450 drug metabolism, drug-dug interactions and drug-herb interactions. All co-factors and other cell components are present in physiological relevant concentration, and the resemblance to an *in vivo* situation is high.

In paper I, the six selected herbs were tested for their inductive potential on the major and important CYP1A2, 2D6 and 3A4. Together, these enzymes metabolize a large majority of currently used drugs.

SJW induced CYP3A4, as expected, increasing CYP3A4 activity to nearly 700% of control at the lowest concentration tested (8 µg/ml) and 1100% at 80µg/ml, confirming the inducing potential on CYP3A4 for this herb. Inhibition of all three enzymes occurred when hepatocytes were exposed to 800µg/ml SJW, indicating that there also is a concentration factor in this herb's modulation of CYP3A4 activity.

While *Ginkgo Biloba* did not increase CYP3A4 activity, it showed interesting opposite effects on CYP2D6 and 1A2. CYP1A2 mediated metabolism was increased at low *Ginkgo Biloba* concentrations and decreased at high concentrations, while the opposite occurred for 2D6 mediated metabolism, with increased activity at high concentrations and decreased activity at low concentrations. This is a good example of observed effects when investigating herbs as a whole, and not isolated compounds. It is suggested that effects like these are observed when different herbal constituents dominates at different herbal concentration. It also reflects how one herb can act on differently on the different P450s.

Common sage and *Echinacea Purpurea* did not show any inducing effects on the three CYP enzymes investigated, but rather a generally inhibitory potential. Worth noticing, is the apparent potent inhibition of CYP3A4 activity by all concentrations of Common sage.

Common valerian increased both CYP2D6 and 3A4 activity in a dose dependent manner, up to 140 and 200%, respectively, at the highest herbal concentration tested. The *in vivo* relevance is questionable, as the herbal concentrations are too high. However, effects on intestinal CYP3A4 might happen, as concentrations of herbal constituents here can be quite high after intake. A couple of reports investigating this herb in humans showed no apparent effect on CYP3A4 mediated metabolism of alprazolam (66) or midazolam (67). The same two reports also included CYP2D6 mediated metabolism of dextromethorphan and chlorzoxazone, respectively, and reported no effect.

6.3. The influence of ethanol on CYP2D6

Ethanol influence and herbal modulation on CYP2D6 mediated metabolism of dextromethorphan was investigated in paper II. During the initial stages of experimental design it occurred to us that ethanol, in which the herbal constituents were dissolved, could have a more substantial impact on CYP2D6 activity than anticipated. Results

showed that ethanol increased CYP2D6 mediated metabolism of dextromethorphan, max up to 175% of control, in concentrations up to 1.1% and inhibited the enzyme when ethanol concentration was further increased. At 15% ethanol, CYP2D6 activity was nearly abolished. The large increase in activity was surprising, conformal changes and/or allosteric effects are suggested as a probable mechanism of action. This also shows the importance of controlling the final concentration of ethanol and other organic solvents in incubations, as the effects at rather low concentrations can be dramatic.

6.4. Inhibition of CYP2D6

In paper II, herbal inhibition on CYP2D6 mediated metabolism was investigated, IC_{50} values for the three strongest herbal inhibitors (from a screening study) was calculated and inhibitory patterns and mechanisms of these herbs were evaluated.

SJW, Common Sage and Common Valerian were the strongest herbal inhibitors, with IC_{50} values of 67$\pm$ 6.9, 796$\pm$ 45 and 1660$\pm$ 155 μg/ml, respectively. SJW showed an uncompetitive inhibition pattern, while the two others inhibited CYP2D6 in a non-competitive pattern. The time- and NADPH-dependent inhibition by common valerian indicated that this herb might be a mechanism-based inhibitor of CYP2D6.

The CYP2D6 inhibition by SJW was stronger than for both common sage and common valerian, confirming the *in vitro* inhibition potential of this herb. Other *in vitro* studies, investigating both isolated herbal constituents and total herbal extracts, have shown both inhibition and no inhibition (68-71). The results are difficult to compare, and the discrepancies might be caused by different substrates, different solvents and different testing systems used. The *in vivo* evidence of CYP2D6 inhibition by SJW is lacking and the published reports indicate that the herb has no effect on CYP2D6 activity in the human body (72-75). However, the possibilities of an interaction with narrow width therapeutics can not be ruled out. The uncompetitive inhibition pattern shown by SJW towards CYP2D6 activity in paper II is in contrast to the non-competitive pattern found by Obach et al. (76) by hyperforin, a major constituent in SJW. This illustrates the benefit of investigating total herb extract, as the uncompetitive inhibition pattern probably is a result of a net effect caused by multiple constituents in SJW.

Common valerian and common sage were the second and third strongest herbal inhibitor towards CYP2D6 activity; however, they are not likely to produce any significant interactions with this enzyme *in vivo*. This is also in line with two studies (77;78) in humans showing no effect on CYP2D6 by common valerian. In paper I, common valerian increased CYP2D6 activity in human hepatocytes. The apparent difference in effect might be caused by different testing systems or dosing regime. In paper I, a general inhibition potential towards CYP2D6 was demonstrated by common sage. No other *in vitro* or *in vivo* reports are published on this herb towards P450 interaction. Common sage and common valerian both inhibited CYP2D6 in a non-competitive fashion, but only common valerian had the characteristics of a mechanism-based inhibitor. Although the IC_{50} value of this herb was quite high, this type of inhibition can totally inactivate enzymes, and one should be aware of the potential, especially in people genotyped as slow CYP2D6 metabolizers.

Horse Chestnut, *Ginkgo Biloba* and *Echinacea Purpurea* were more modest inhibitors of CYP2D6, inhibiting 42, 25 and 28% of enzyme activity, respectively, at the maximum herbal concentration tested. These results are confirmed by other investigations, both *in vitro* and *in vivo*, regarding CYP2D6 inhibition by *Echinacea Purpurea* and *Ginkgo Biloba* (72;79-81). One *in vitro* study has shown potent inhibition of CYP2D6 by alcoholic extracts of *Echinacea Purpurea* and also extracts of (common) valerian (82). An assay based on fluorescence was used. In the study referred to in paper I, both *Echinacea Purpurea* and Horse Chestnut seemed to inhibit CYP2D6 activity. The apparent total inhibition by Horse chestnut was attributed cytotoxicity, while the discrepancy with *Echinacea Purpurea* remains unresolved.

6.5. Inhibition of CYP3A4

As a part of the study referred to in paper III, the inhibitory potential of the six herbs towards CYP3A4 was investigated. SJW was clearly the most potent herbal inhibitor, with an IC_{50} as low as 15.4 ± 2.5 µg/ml, followed by Common Sage, *Ginkgo Biloba*, Common Valerian, Horse Chestnut and *Echinacea Purpurea*, the latter with an IC_{50} value as high as 5 mg/ml.

CYP3A4 is the most studied P450 when it comes to drug-herb interactions. Despite of this, *in vitro* investigations rarely report an IC_{50} value of the investigated herbs, and it seems that the results are very depended on what substrate and solvent used, and also what kind of testing system has been applied. There are reports of an *in vitro* inhibitory potential of SJW towards CYP3A4 mediated metabolism (83-85) when SJW has been given as a single dose, also in inducible human hepatocytes. In paper I, SJW also inhibited CYP3A4 in human hepatocytes after long term exposure at the highest concentration tested, indicating that the induction/inhibition of CYP3A4 not only is dependent on long-term/short-term exposure, but maybe concentration. In one human *in vivo* investigation, where a single dose of SJW was administered, inhibition of CYP3A4 was demonstrated (86), so the inhibition is probably more than only an *in vitro* effect. Another human study, however, using midazolam as a substrate, showed no effect on the CYP3A4 mediated metabolism of this substrate (87). Regardless, people usually take SJW in a long term perspective, and, thus, the main focus will be the CYP3A4 induction (88).

Significant presystemic inhibition of CYP3A4 might happen with Common Sage according to the results in paper III, as the reported IC_{50} value of Common Sage inhibition might well be present in the small intestine after intake. However, the concentration needed for 50% inhibition, is probably too high for systemic effects to happen. In paper I, Common Sage indicated a non-concentration dependent potent inhibition of CYP3A4 mediated metabolism, another indication that this herb might have a potential for an *in vivo* significant interaction with CYP3A4.

Common Valerian, *Ginkgo Biloba* and Horse Chestnut are not likely to cause any problems when ingested together with CYP3A4 substrates, as the results from the experiments with c-DNA expressed CYP3A4 indicate a rather low inhibitory potential. This is also in line with previous *in vitro* investigations of Common Valerian and *Ginkgo Biloba,* showing a modest inhibition (89-91), however, isolated compounds from *Ginkgo Biloba* seem to be quite potent inhibitors of CYP3A4 (92). Human studies, on the contrary, show little or no effect of these two herbs on CYP3A4 activity (72;93-95), although Uchida et al. (96) reported significant inhibitory effects of *Ginkgo Biloba* in a recent study. No other pharmacokinetic data has been found on the herb Horse Chestnut.

Echinacea Purpurea, previously shown to be a moderate to potent inhibitor of CYP3A4 *in vitro* (97-99), was the weakest herbal inhibitor in the study referred to in paper III. The *in vivo* interaction potential of this herb also seems weak in another study (100), however, the authors of the study conclude that further research into this herb is warranted.

6.6. Inhibition of P-gp

The other part of paper III was to evaluate the inhibitory potential for the six herbs on P-gp efflux transport activity in Caco-2 cells. The use of Caco-2 cells in P-gp transport experiments is well established, and considered to be a good *in vitro* model. As CYP3A4 and P-gp share many substrates (101), it was also interesting to see if the inhibitory potential of the herbs correlated in some way.

The most potent herbal P-gp inhibitor was *Ginkgo Biloba*, with an IC_{50} value of 24 µg/ml. This is a concentration that most likely will be present in the small intestine after intake, and thus indicates an *in vivo* interaction potential between P-gp and *Ginkgo Biloba*. In a recent *in vitro* study (102), investigators noted a modest inhibition of P-gp by this herb, but in rats, the effect was opposite, indicating an *in vivo* induction of P-gp in these animals after long term administration (103). Another study has shown that constituents in *Ginkgo Biloba* are substrates for P-gp (104), which might have implications for the bioavailability of other P-gp substrates.

Inhibition of P-gp by SJW, after short term administration, has been shown previously, both *in vitro* (105) and *in vivo* (106), and this thesis confirms these findings. Although acute exposures of SJW result in inhibition, the net effect during chronic exposure is, as with CYP3A4, an induction and increased efflux transport activity by P-gp (107-109). Both SJW and *Ginkgo Biloba* contain phytochemicals called flavonoids, which are thought to cause these interactions with P-gp (110;111).

Horse Chestnut demonstrated an IC_{25} value of 70 µg/ml. If this herb has an interaction potential with P-gp *in vivo*, is uncertain, though the reported concentration that inhibits 25% is susceptible to appear in the small intestine. No other publication exists on the herb and P-gp activity.

Common Valerian, Common Sage and *Echinacea Purpurea* did not reach 50% inhibition event at the highest concentration tested, however, the reported IC_{25} value of Common Sage might cause significant *in vivo* inhibition. An effect from the other two is more dubious.

Although the kinetic values derived in the study, net flux and apparent permeability, correlated well, the correlation between herbal inhibition of CYP3A4 and P-gp was lacking in this study, showing that there are constituents in the herbs that has effect on one of the proteins and not the other.

6.7. Considerations on model systems

Several models have been and are being used for evaluation of drug-drug interactions, and the same systems are usually applicable for studying drug-herb interactions. Common *in vitro* models include c-DNA expressed CYP enzymes, liver microsomes, liver slices, hepatocytes and immortalised cell lines, while *in vivo* models include animals and humans. One can use perfused organs (*in situ*), and also a non biological method, by simulating interactions on computers, *in silico*. A brief discussion on available methodology is included in this thesis:

6.7.1. c-DNA expressed CYP enzymes

Isolated heterogeneous human CYP enzymes have been commercially available for some years. Single enzymes can for example be expressed in bacteria (112), yeast (113) and baculovirus systems (114). With these *in vitro* systems, one can investigate the effect on one single CYP without the influence of other enzymes. They are also suitable for studying mechanistic aspects of CYP inhibition, easy to use and cost-effective. However, the *in vivo* predictive value of the data obtained has been debated, due to the fact that enzymes are studied in isolation and lack the complement of other hepatic enzymes, as is the case with hepatocytes or microsomes (115). It is important to remember when interpreting the data that there are different amounts of enzymes *in vivo* and the co-factor supply may also be of importance.

c-DNA expressed CYP enzymes were used in papers II and III.

6.7.2. Liver microsomes

Liver microsomes are derived from the endoplasmic reticulum after homogenisation of whole liver samples and differential ultracentrifugation of liver homogenates. Microsomes are probably the most widely used *in vitro* system to study drug metabolism, they contain the whole spectre of P450 enzymes in the liver and can be used to study CYP inhibition (116;117).

Liver microsomes are easy to prepare, use and store, however, some enzyme activity might be lost during the preparation. This can be corrected by the addition of different co-factors (e.g. NADPH), and, when added appropriate co-factors, enzyme activities are comparable of those in intact tissue (118). Enzymatic activities are also shown to be stable during prolonged storage, given that the original fresh liver tissue is handled correctly (119;120).

6.7.3. Liver slices

Precision-cut liver slices closely resemble the liver as an organ, retaining cell morphology, and also contain a wide range of drug metabolizing enzymes. Inhibition and induction can be investigated, and since transport systems are intact, they can also be used to study modulation of cell membrane transport (121;122).

The maintenance of liver slices is very demanding. Special slicers are needed, in order to get liver slices as thin as possible, to facilitate oxygen and nutrient transportation. Although effective cryopreservation methods have been developed (123), the time of use is limited. Compared to hepatocytes, studies have shown lower metabolic capacity and cellular uptake in liver slices (124), and this might have influenced their popularity.

6.7.4. Hepatocytes

Hepatocytes (figure 8) contain the full spectre of drug metabolizing enzymes, both phase I and II, and are therefore a valuable tool for studying herbal influence on P450s. Hepatocytes also contain relevant co-factors in physiologically correct concentrations. Cultured human hepatocytes are currently the most recommended tools for studying inhibition and induction of CYP enzymes (125-127). However, use of primary hepatocytes is limited due to the restricted availability of human liver tissue, which can be

obtained from whole livers (that are e.g. unfit for transplants) or from surgical biopsies. To maintain normal cellular physiology and intercellular contacts in hepatocytes, special matrix configurations are needed and technical skills must be learned. All experiments with hepatocytes must be conducted in a sterile environment using sterile techniques. It is imperative for the experiments to avoid contamination, as it is in all research using live cells.

Recent years, several cryopreservation methods have been developed, however, efficient use of a single batch of hepatocytes can only occur for a short period of time (128;129).

Cultured human hepatocytes were used in paper I.

6.7.5. Immortalised cell lines

Immortalised cell lines expressing one or more drug metabolizing enzymes has been used for many year, and the most common ones are called HepG2 and BC2 cell lines (from liver) and A549 (from lung) (130-132). However, the expression of drug metabolizing enzymes in these cells is not always adequate, and their uses in CYP interaction studies are limited. Genetically engineered cell lines have been developed, in an effort to increase the expression of CYPs, however, most approaches has failed (133).

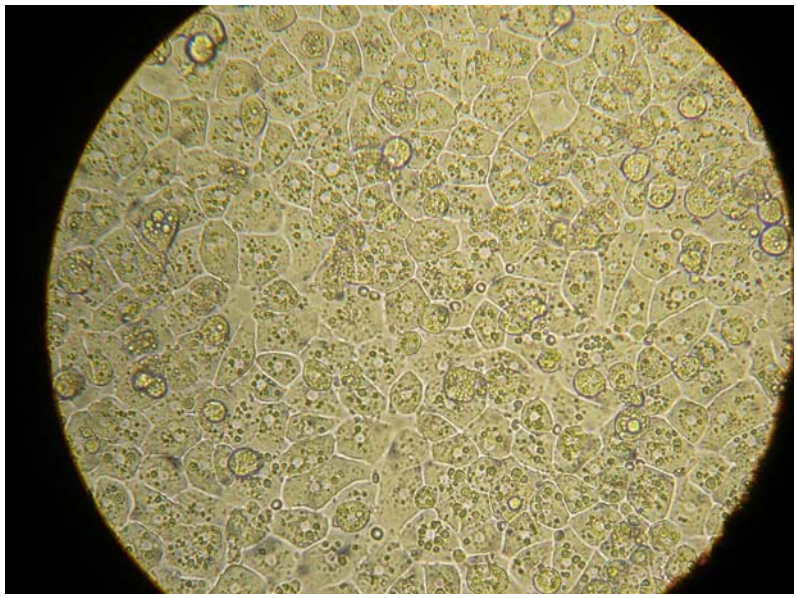

Figure 8: Cultured human hepatocytes (RILD laboratory)

Recently a new cell line was introduced, HepaRG, derived from a human liver tumour (134). Several liver-specific functions are intact and the correct morphology is present (135;136). The expression and amount of different CYPs are also satisfactory, thus, this can be a tool for the future (137).

There are a variety of cell lines expressing drug transporters; however, common ones used in P-gp research are Caco-2, originating from human colon cancer cells, MDCK-MDR1 (from dog kidney) and CHO (Chinese Hamster Ovary) cells. Another cell line, called LS-180, also derived form a human colon carcinoma, express many P450s and also P-gp, and many sees this cell line as a good *in vitro* tool to study induction on both P-gp and CYP enzymes (138;139). Caco-2 cells, used in paper III, are considered to be a good *in vitro* tool to study P-gp interactions; however, the preparations of these cells are quite time consuming. The cells need about 3 weeks of developing before they can be used. In this period of time, the cells will have differentiated into a polarized monolayer membrane, and experiments can be carried on. As with the hepatocytes, correct sterile techniques are important, as cells are susceptible to contaminations, and especially mycoplasma infections.

6.7.6. Perfused livers

Perfused isolated liver, usually from rats, is a valuable tool for CYP metabolism studies. Lobular architecture and microcirculation is intact, there is a close resemblance to physiological conditions and data are easy to collect. However, the metabolic profiles differ from humans and also CYP enzymes are different in rats compared to man, although similarities are high, with up to 70% homology in sequences (140). Basic surgical skills are required for correct excision of the livers.

6.7.7. Animals

Animals like rats, dogs, monkeys and mice are used in CYP interaction studies. Using animals, one can investigate a drug-herb interaction in an *in vivo* environment, but the correlation to humans is not always evident, as the metabolic profile of a xenobiotic compound usually differs from species to species (141), and so the data can be difficult to interpret. Animal testing is also quite expensive.

Chimeric mice with humanized liver (142-144) and genetically modified mice expressing human CYPs (145) have been developed in recent years, in order to try to correct for the interspecies difference. This work is at the starting line and the use is very limited. The models need further validation and characterisation.

6.7.8. Humans

Using humans would of course provide the ultimate answer to our inquires about drug-herb interactions, but human investigations are very time-consuming and expensive. Also, the obtained results will be holistic, and will not always elucidate for example mechanisms or whether the drug-herbal interactions take place in the gut or liver.

6.7.9. Computational *in silico* models

The use of computer-based methods in drug-drug interaction research has expanded rapidly the last years. Several systems using rule- and knowledge based databanks and different algorithms have been introduced. Computational methods, however, is probably not very good for studying drug-herb interaction yet, as there might be too many uncertainties tied to how herbal constituents act *in vivo*, and correct parameters are difficult to obtain.

6.7.10. Future *in vitro* models

New technologies and future applications for drug metabolism and interaction studies include bioartificial liver systems (146;147) and stem cell-derived cultures (148). Preliminary published data indicate that technologies like these are an interesting possibility, but they need more work.

6.8. Methodological considerations

6.8.1. Chemicals

In enzymatic studies, it is important that compounds (substrates, inducers and inhibitors) interact with their targets (enzymes/proteins) in the form and concentration predefined for the experiment. The substrates, metabolites, inducers and inhibitors used in this study were carefully prepared, and stored according to manufacturer's recommendations. The sources of these compounds and other chemicals used have been described in papers I-III.

6.8.2. Ethanol and herbal preparations

In order to add the HMPs to the incubation mixtures, effective solubilisation of the herbal constituents are important and also that the concentrations of the herbal constituents are known. Also, it is a point in keeping the herbal preparations as identical to the original HMP as possible. Inquiries were made to the producers about what kind of solvent the herbs were originally extracted in. All herbs, except common sage, were originally extracted in ethanol (20 – 60%), and the same concentration of ethanol was applied in the laboratory during our extractions. The extractions took place at 30°C in order to facilitate mass transfer and at the same time not to destroy temperature sensitive constituents, if any. Solvent was evaporated from extracts, weighed, and resuspended in an exact amount of ethanol at the given strength, to give herbal stock solutions with known concentrations and presumably identical composition as the original product.

6.8.3. Measurements and analyzes

In this thesis, the experimental results from metabolism studies were analysed using validated HPLC methodologies, while results from P-gp transport experiments were analysed by liquid scintillation counting.

It is important that the results obtained in scientific studies can be trusted. Precise and validated methodologies for analysing results of experiments are needed, so that data can be collected in a thorough and rigorous manner (149;150). This applies, of course, not only to analytical methods, but should permeate all laboratory work, and is called GLP. In order to comply this, all aspects of experimental work was subject to extensive testing and validation during development. Stability of stock- and standard solutions were checked, inter- and intra-day differences of assays were monitored and linearities of incubations regarding substrate concentrations, CYP concentrations and time were decided. Several QCs were included in all analytic runs.

To obtain a good and reproducible separation on the HPLC, several instrument and chemical parameters need to be optimised: mobile phase composition and pH, injection volume, flow rate, detection parameters and column temperature. In addition, the size and chemistry of the bonded phase in the column and the dimension of the column itself must be adequate to separate the analytes in question.

Recent years, a combination of HPLC or gas chromatography (using a gaseous mobile phase), combined with mass spectrometry, is used to get an even better separation and resolution of analytes.

6.9. Clinical relevance of *in vitro* findings

One should be very careful when making *in vivo* extrapolations from *in vitro* collected data. There are numerous factors that come into play, such as intestinal and systemic concentrations of the compounds studied (both substrate and herbal constituents) including bioavailability, pharmacokinetic values (IC_{50} and K_i), binding of compounds to serum protein, the relative content of CYP enzymes participating in the metabolism in liver and small intestine, effect of active transport systems in and out of cells and other possible pathways of metabolism. When it comes to drug-drug interactions, several of these factors can be measured or simulated using *in silico* techniques, however, as for herbal constituents, and for total herb extracts in particular, most of these factors are unknown.

Although there are many unknown factors when extrapolating, the herbal concentration in the gut and small intestine can be approximated. CYP enzymes and P-gp present in the small intestine are thus exposed to these concentrations. CYP3A4 and P-gp are functionally co-localized in the small intestine (151). Herbal preparations showing potent *in vitro* inhibition on both of these systems are prone to give rise to, at least, presystemic significant effects *in vivo*, increasing or decreasing drug bioavailability through either inhibition or induction, as shown for SJW. As shown in paper III, SJW, *Ginkgo Biloba* and common sage show potent inhibition on both systems at reasonable low concentrations, and synergistic effects on CYP3A4/P-gp might well happen. Systemic effects of these herbal preparations are more difficult to assess as the plasma concentrations of herbal constituents are mostly unknown. However, it seems that the herbal concentrations giving potent inhibitory effects are too high to be reached systemically, except maybe for that of SJW (for both CYP2D6 and 3A4).

In paper 1, *Gingko Biloba* increased CYP1A2 activity at the lowest dose tested, which might indicate this herb to be a candidate for an *in vivo* effect, although a previous study showed no effect of this herb on CYP1A2 activity in humans (72). The use of different

substrates is another source of discrepancy when comparing *in vitro* results and extrapolating to *in vivo* situations. In the previously mentioned study, caffeine was used.

7. Conclusions

In general, one can conclude that the P450s investigated and P-gp are all susceptible to modulations in their activity by the six selected herbs. With the addressed questions in mind, the following conclusions are made:

- Will ethanol, as an essential herbal extraction medium, influence the selected *in vitro* assays?
    - Yes, ethanol showed a biphasic effect on CYP2D6 metabolism, with a significant increased activity at low concentrations and decreasing activity at higher concentrations. For CYP3A4, only a decreased activity was observed. These effects should be adjusted for during the *in vitro* assays and should also be noticed for the *in vivo* situation.
- Do the six selected herbal products inhibit or induce CYP mediated metabolism, if so, in what order?
    - CYP mediated metabolism was modulated in different degrees by the six herbal preparations, both induction and inhibition were demonstrated. SJW was the most potent inhibitor of CYP metabolism in general, but also the strongest herbal inducer (CYP3A4), followed by common sage, *Ginkgo Biloba*, common valerian, horse chestnut and *Echinacea Purpurea*. For increased activity in hepatocytes, SJW was followed by common valerian and *Ginkgo Biloba*.
- What kind of inhibition is exerted on the CYP mediated metabolism by the selected herbs?
    - SJW inhibited CYP2D6 mediated metabolism in an uncompetitive manner, while common valerian and common sage inhibited CYP2D6 in a non-competitive manner. The difference in inhibition pattern indicates that, while SJW bind to an enzyme-substrate complex, the two other herbs inhibit the enzyme by binding to a different site on the enzyme than does the substrate.
- Are any of the herbal products mechanistic inhibitors of CYP mediated metabolism?
    - Yes, common valerian showed characteristics of a mechanism based inhibitor towards CYP2D6 mediated metabolism. As this type of inhibition is

irreversible, care should be taken when combining CYP2D6 metabolized medicinal drugs and common valerian, especially in those genotyped as poor metabolizers. A toxic effect of common valerian is indicated.

- Do the six selected herbal products inhibit P-gp mediated efflux transport, if so, in what order?
    - Yes, P-gp was inhibited by all the six herbs, but to different degrees. *Ginkgo Biloba* and SJW were the most potent ones, followed by horse chestnut, common sage, common valerian and *Echinacea Purpurea*
- Are the herbal effects on CYP3A4 mediated metabolism related to their effects on P-gp transport activity, if so, how?
    - The inhibition by the six herbal preparations in our investigation on CYP3A4 mediated metabolism and P-gp transport activity did not seem to correlate. However, the sample size was considered small and a correlated effect on the two systems by other herbal preparations cannot be ruled out.
- Do any of the investigated herbs possess a probable *in vivo* pharmacokinetic drug inhibition potential?
    - Yes, besides SJW, *Ginkgo Biloba* and common sage are suggested to be potential candidates to cause *in vivo* significant herb-drug pharmacokinetic interactions when both CYP and P-gp effects are considered. As no herb-drug interaction data are earlier presented for common sage, and this herb shows a high inhibitory potential towards both CYP3A4 and 2D6, and P-gp transport, this herb should be considered for further *in vivo* pharmacokinetic investigations.

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129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

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132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.
139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

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141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.

142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
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168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.

174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
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183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
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186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS

203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
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227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING

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233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS

260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
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267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT
273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS
280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OG PATHOLOGICAL GAMBLING IN NORWAY

288. Svein Artur Jensen: THE PREVALENCE OF SYMPTOMATIC ARTERIAL DISEASE OF THE LOWER LIMB
289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
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291. Anne Engum: DEPRESSION AND ANXIETY – THEIR RELATIONS TO THYROID DYSFUNCTION AND DIABETES IN A LARGE EPIDEMIOLOGICAL STUDY
292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
293. Jon Olav Drogset: RESULTS AFTER SURGICAL TREATMENT OF ANTERIOR CRUCIATE LIGAMENT INJURIES – A CLINICAL STUDY
294. Lars Fosse: MECHANICAL BEHAVIOUR OF COMPACTED MORSELLISED BONE – AN EXPERIMENTAL IN VITRO STUDY
295. Gunilla Klensmeden Fosse: MENTAL HEALTH OF PSYCHIATRIC OUTPATIENTS BULLIED IN CHILDHOOD
296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN
297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

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299. Janniche Hammer: GLUTAMATE METABOLISM AND CYCLING IN MESIAL TEMPORAL LOBE EPILEPSY
300. May Britt Drugli: YOUNG CHILDREN TREATED BECAUSE OF ODD/CD: CONDUCT PROBLEMS AND SOCIAL COMPETENCIES IN DAY-CARE AND SCHOOL SETTINGS
301. Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
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304. Lasse Løvstakken: SIGNAL PROCESSING IN DIAGNOSTIC ULTRASOUND: ALGORITHMS FOR REAL-TIME ESTIMATION AND VISUALIZATION OF BLOOD FLOW VELOCITY
305. Elisabeth Olstad: GLUTAMATE AND GABA: MAJOR PLAYERS IN NEURONAL METABOLISM
306. Lilian Leistad: THE ROLE OF CYTOKINES AND PHOSPHOLIPASE A_2s IN ARTICULAR CARTILAGE CHONDROCYTES IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS
307. Arne Vaaler: EFFECTS OF PSYCHIATRIC INTENSIVE CARE UNIT IN AN ACUTE PSYCIATHRIC WARD
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