Nutritional conditioning
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General Introduction
Nutritional Conditioning:
The Effect of Short-Term Fasting on Drug Metabolism

This thesis focuses on the effects of fasting on drug metabolism. This introduction summarizes the major concepts of drug metabolism and the rationale for the studies described in this thesis.

Determinants of drug disposition

Patients respond differently to drug therapy. This is often due to differences in drug disposition and may result in treatment failure, increased side effects or even toxicity. The disposition of drugs in patients can be described by four basic processes: absorption, distribution, metabolism and excretion (ADME). In addition to the administered dose, the net effect of these four processes determines plasma drug concentrations. In general, it is important that the plasma concentrations are within a therapeutic range to achieve safe and effective drug treatment. If drug concentrations are below this range, the drug may be ineffective whereas if drug concentrations exceed this range, toxicity may occur.

There are many factors that contribute to variability in drug disposition and, more specifically, in drug metabolism. These factors include genetic, physiologic, pharmacologic, environmental factors and nutritional status, such as fasting (Figure 1).

Figure 1: Factors that contribute to variability in drug metabolism
Drug metabolism consists of different phases (Figure 2). In phase I, reactive and polar groups are introduced into their drug substrates by oxidation, reduction or hydrolysis. Cytochrome P450 (CYP) enzymes play an important role in phase I drug metabolism because of their ability to catalyze the oxidative biotransformation of most drugs.\textsuperscript{2} CYP enzymes are microsomal enzymes which are abundantly present in the liver, gastrointestinal tract, lung and kidney.\textsuperscript{3} This superfamily of enzymes consists of families and subfamilies of enzymes classified based on their amino acid sequence. In humans, five CYP isoforms predominantly present in the liver are frequently involved in drug metabolism: CYP1A2, CYP2D6, CYP2C9, CYP2C19 and CYP3A4.\textsuperscript{1} Together, these isoforms are responsible for more than 70% of all phase I dependent metabolism of drugs.\textsuperscript{1}

\textbf{Figure 2:} Phases of drug metabolism

In phase II, the drug metabolites formed in phase I are conjugated with endogenous molecules such as glucuronic acid or glutathione. These metabolites become more polar which enhances further renal or biliary excretion. The phase II metabolizing or conjugating enzymes consist of many superfamilies of enzymes including uridine diphosphate-glucuronosyltransferases (UGTs) involved in glucuronidation of drugs, sulfotransferases (SULTs) involved in sulfation of drugs and glutathione-S-transferases (GSTs) involved in the conjugation of the reduced form of glutathione (GSH) to the drug.\textsuperscript{3} UGTs represent the majority of phase II metabolizing enzymes catalyzing the transfer of glucuronic acid from UDP-glucuronic acid to a number of acceptor substrates including steroid hormones, bile acids, bilirubin and drugs.\textsuperscript{4} Approximately 10% of the top 200 prescribed drugs are glucuronidated by UGTs.\textsuperscript{5} These enzymes represent two families (UGT1 and UGT2) including 9 human family UGT1 enzymes, 3 subfamily UGT2A and 8 UGT2B enzymes.\textsuperscript{6} UGT1A1 is the most familiar UGT isoform because of its important role in bilirubin con-
jugation. Genetic variants have shown to decrease UGT1A1 enzyme activity which can lead to jaundice as for the syndroms of Gilbert and Crigler-Najjar. Furthermore, impaired UGT1A1 enzyme activity is associated with toxicity of the oncolytic agent irinotecan. UGT1A1 is also involved in acetaminophen (paracetamol) metabolism. Besides UGT1A1, other UGT isoforms are important in drug metabolism. For example UGT1A4, UGT2B4 and UGT2B7 which are involved in the metabolism of midazolam (Figure 3).

Sulfotransferases (SULTs) catalyze the transfer of a sulfonate group (-SO_3^-) from the co-factor 3'-phosphoadenosine-5'-phosphosulfate to an acceptor substrate. These enzymes can conjugate with a wide variety of xenobiotic substrates and drugs (e.g. acetaminophen) but also with endogenous substrates such as steroids, catecholamines, and thyroid hormones.

The major biological function of glutathione-S-transferases (GSTs) appears to be defense against reactive and toxic electrophiles such as reactive oxygen species (ROS) that arise through normal metabolic processes. Many of these ROS are formed by oxidative reactions catalyzed by CYP enzymes (phase I drug metabolism). For example, a small percentage (5%-10%) of acetaminophen is converted by CYP enzymes to the reactive and hepatotoxic metabolite N-acetyl-p-benzoquinone-imine (NAPQI). NAPQI can be detoxified by GST-mediated conjugation with endogenous glutathione to nontoxic metabolites (Figure 4).

Fasting and drug metabolism

Animal studies
Preclinical studies have shown that fasting can alter phase I and phase II drug metabolism by modulating the activity of the different enzymes involved. Already in 1965, Kato and Gillette established that fasting of female rats enhances the activity of ‘almost all’ drug metabolizing enzymes in liver microsomes. Later studies have shown that starvation of rats led to an increase in the hepatic CYP2e1-mediated demethylation of dimethylnitrosamine. Starvation of rats for 3 days also resulted in a doubling of total CYP levels in the kidney. In contrast with this upregulation of CYP enzymes, the male-specific isoform CYP2c11 was down-regulated by starvation in the liver of rats. Similarly, CYP2c13 and CYP2b2 levels were down-regulated by fasting. Fasting can also affect phase II drug metabolism. For example, Xu et al. have shown that fasting increased hepatic UGT isoforms Ugt1a1, -1a6, -1a7, -1a9, -2b1, -2b5, -2a3, -3a1, and -3a2 mRNA expression in mouse liver. Furthermore, Ding et al. have shown that fasting induces expression of genes encoding CYP enzymes (Cyp2b10), UGT (Ugt1a1) and SULT (Sult2a1) in mice liver.
**Figure 3:** Simplified metabolic pathway of midazolam

**Figure 4:** Simplified metabolic pathway of acetaminophen
Chapter 1

The effects of fasting on drug metabolizing enzymes in experimental models can be explained by the altered activity of nuclear transcription factors. The nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) serve as xenosensors, which regulate the activity of many of the drug metabolizing enzymes in animals.\(^{21,22}\) Interestingly, animal studies have shown that short-term fasting increases the activity of both CAR and PXR.\(^{20,22}\)

Fasting increases cyclic adenosine monophosphate (cAMP) and activates protein kinase A (PKA), which in turn induces peroxisome proliferator-activated receptor coactivator-1\(\alpha\) (PGC-1\(\alpha\)). PGC-1\(\alpha\) then interacts with hepatocyte nuclear factor-4\(\alpha\) and induces CAR after which CAR induces CAR target genes.\(^{22}\) This is a physiologically relevant response, since CAR deficient mice are defective in fasting adaptation and lose more weight during prolonged fasting.\(^{20}\) Compared to CAR, PXR is activated by a larger variety of endogenous and exogenous compounds including steroids (e.g. progesterone, corticosterone, dexamethasone), antibiotics (e.g. rifampicin), antimycotics, bile acids and many herbal compounds.\(^{22,23}\) Experimental studies have also demonstrated that fasting activates PXR in mice liver.\(^{24}\) In accordance, fatty acids, which increase readily during short-term fasting due to increased lipolysis of triglycerides in adipose tissue, activate CAR and to a lesser extend PXR.\(^{25}\)

**Human studies**

Interestingly, the role of short-term fasting, defined as the abstinence of food and drinks except water for a period of 24 to 72 hours, on drug metabolism in general, and on individual enzyme activities in particular, has hardly been studied in humans. In 1994, O’Shea et al. studied the effect of short-term (36 hours) fasting on CYP2E1 probe chlorzoxazone.\(^{26}\) Fasting was associated with a reduction in the oral clearance of chlorzoxazone which, however, was in contrast with previous findings in rats demonstrating that CYP2E1 levels were induced by fasting. The authors discussed this may reflect an interspecies difference in CYP2E1 regulation but conclude that it more likely reflects destruction of the enzyme by lipid peroxidation resulting from the prolonged period of fasting. Additional research on the effect of short-term fasting on other phase I or phase II enzymes involved in human drug metabolism is lacking. Nonetheless, the activities of CAR and PXR are increased by fasting as shown by preclinical studies and since both transcription factors are involved in the regulation of phase I and phase II drug metabolizing enzymes in animals, short-term fasting may contribute to both intra- and inter-individual variations in human drug metabolism as well.\(^{13,27}\) Moreover, fasting related consequences such as weight loss or cachexia are common in patients. For example, the prevalence of cachexia ranges from about 10% in patients with chronic heart failure or COPD to approximately 70% in patients with advanced cancer.\(^{28}\) Furthermore,
it should be realized that a major part of the metabolic features of the fasting response can be readily stimulated, for example by only skipping breakfast and lunch in humans.\textsuperscript{21}

Considering the experimental data, changes in drug metabolism due to alterations in nutritional conditions such as short-term fasting (e.g. 36 hours) may potentially alter the concentration of drugs in humans, resulting in treatment failure, or, conversely, in unwanted side effects.

**The use of probe drugs to study drug metabolism**

A well-established method to study enzyme activity in humans is by using enzyme selective probe drugs.\textsuperscript{29} This is commonly used in drug development to study drug–drug interactions but can also be used for pharmacological research purposes or as an approach to individualize pharmacotherapy. In addition to the administration of a single enzyme-specific probe drug at a time, it is possible to simultaneously administer multiple types of enzyme-specific probe drugs, which is commonly referred to as the “cocktail approach”. Preferably, the probe drugs used in such a cocktail should be (1) selective for the individual metabolizing enzyme, (2) highly sensitive to changes in activity of the respective enzyme, (3) unaffected by other mechanisms of drug metabolism such as P-glycoprotein or other transporters (phase III), (4) (commercially) available and (5) should not interact with each other.\textsuperscript{30} In 2009, Turpault \textit{et al.} studied the pharmacokinetics (PK) of selective substrates of the five CYP isoforms frequently involved in phase I drug metabolism: caffeine (CYP1A2), S-warfarin (CYP2C9), omeprazole (CYP2C19), metoprolol (CYP2D6), and midazolam (CYP3A4).\textsuperscript{31} They observed no PK interaction between the probe drugs when administered as a cocktail, relative to the probes administered alone. The lack of interaction between the probe drugs indicates that this cocktail can well be used \textit{in vivo}.

For assessment of phase II drug metabolism of drugs that also undergo phase I metabolism, studying the PK of only the parent probe drug will not be sufficient. Alternatively, phase II drug metabolism may be studied by the analysis of a parent drug together with its metabolites. For example, midazolam which is predominantly hydroxylated by CYP3A4 (phase I) to 1-OH-midazolam and further metabolized by UGT2B4/2B7 (phase II) to 1-OH-midazolam-glucuronide (Figure 3). Another example is acetaminophen which is metabolized by glucuronidation to acetaminophen-glucuronide, sulphation to acetaminophen-sulphate and to acetaminophen-glutation by glutathione-S-transferases (Figure 4).\textsuperscript{32}
Aim of the thesis

The overall aim of this thesis was to assess the role of fasting as a nutritional modulator of drug metabolism. Proof of concept studies in animals and healthy subjects were performed to gain insight in the effects of short-term fasting on phase I and phase II mediated drug metabolism.

Part I: Preclinical Study

Part I, Chapter 2, describes a preclinical study in mice in which the role of the constitutive androstane receptor (CAR) in fasting-induced alterations in Cytochrome P450 (CYP)-mediated drug metabolism was investigated. Preclinical studies have shown that the expression of CYP enzymes is partly regulated by CAR. Furthermore, fasting affects the expression of both P450 enzymes and CAR in animals. This study therefore investigated whether fasting-induced alterations in CYP-mediated drug metabolism are mediated by CAR. A drug cocktail consisting of five probe drugs for human CYP enzymes (caffeine (CYP1A2), metoprolol (CYP2D6), omeprazole (CYP2C19), midazolam (CYP3A4) and S-warfarin (CYP2C9)) was administered to wild type and CAR deficient mice. The mice were either fed ad libitum or fasted for 24 hours. Blood was sampled at predefined intervals to determine exposure of the probe drugs and hepatic mRNA expression was measured of the mice orthologous for the five CYP enzymes.

Part II: Effects of Fasting on Phase I Drug Metabolism

In Part II, the results of studies on the clinical effects of short-term fasting on phase I drug metabolism are presented. First, Chapter 3 describes the development and validation of an LC-MS/MS method for the simultaneous quantification of the five probe drugs used as substrates of five CYP isoforms that play an important role in human drug metabolism: caffeine, metoprolol, omeprazole, midazolam and S-warfarin. The validated method was used in the clinical study described in Chapter 4 in which the concentrations of the five orally administered probe drugs were determined in healthy subjects after an overnight fast (control) and after a period of short-term fasting (36 hours). The clinical study was combined with a preclinical study in rats to compare the effects of short-term fasting on hepatic mRNA expression of the orthologous CYP isoforms with the five studied CYP enzymes in humans.

In Chapter 5 the outcomes of a randomized, controlled, crossover study performed in healthy subjects are presented in which the effects of short-term fasting on systemic CYP-mediated drug metabolism were studied. In order to distinguish between the effects of short-term fasting on bioavailability and systemic clearance, the previously used drug cocktail was administered on four occasions: an oral (1) and intravenous (2) administration after an overnight fast (control) and an oral (3) and intravenous (4) administration after 36 hours of fasting. Differences in pharmacokinetic parameters of the
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Probe drugs between the control and the fasting interventions were analyzed using the nonlinear mixed-effects modeling software NONMEM.

Besides bioavailability and systemic clearance, plasma protein binding is an important factor in drug metabolism. For example, decreased plasma protein binding due to hypoalbuminemia leads to an increase in free plasma fraction of a drug which alters the pharmacokinetic parameters of the drug. To determine if the effects of fasting described in chapters 4 and 5 were not due to an effect of fasting on protein binding instead of CYP-mediated metabolism, Chapter 6 describes a study performed on the effects of short-term fasting on plasma protein binding of the five probe drugs.

Part III: Effects of Fasting on Phase II Drug Metabolism

In Part III, the clinical effects of fasting on several drug metabolizing enzymes involved in phase II drug metabolism are presented.

In Chapter 7 the effects of both short-term fasting and a short-term high fat diet (HFD) on CYP3A4 and UGT-mediated (UGT1A4, UGT2B4/2B7) midazolam metabolism are described. Nine healthy subjects received a single intravenous administration of 0.015 mgkg⁻¹ midazolam after (1) an overnight fast (control), (2) 36 hours of fasting and (3) an overnight fast after three days of a HFD consisting of 500ml of cream supplemented to their regular diet. Differences in pharmacokinetic parameters between the three interventions of midazolam and its main metabolites 1-OH-midazolam and 1-OH-midazolam-O-glucuronide were analyzed using NONMEM (Figure 3).

In order to study the effects of fasting on phase II drug metabolizing enzymes not only involved in UGT-mediated glucuronidation but also in SULT-mediated sulfation and GST-mediated conjugation with glutathione, acetaminophen (paracetamol) was used as a probe. First, Chapter 8 describes the development and validation of an LC-MS/MS method to simultaneously quantify acetaminophen and six of its main metabolites: acetaminophen-glucuronide, acetaminophen-sulphate, 3-methoxy-acetaminophen, acetaminophen-glutathione, 3-cysteinly-acetaminophen and acetaminophen-mercapturate (Figure 4).

Chapter 9 presents the results of a randomized, controlled, crossover study in healthy subjects in which the effects of short-term fasting on exposure of the parent compound acetaminophen and six of its main metabolites have been studied.

Summary and General Discussion

In Chapter 10, the main findings presented in this thesis are summarized and the current benefits and limitations are discussed together with how these insights on the effects of short-term fasting on drug metabolism may be used to further optimize pharmacotherapy.
References

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