THE INFLUENCE OF SEVERE HEPATIC DYSFUNCTION ON THE METABOLIC CAPACITY OF THE LIVER IN CHILDREN: PROJECT PLAN.

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1. Introduction

The cytochrome P450 (CYP) family plays an important role in the biotransformation of many drugs and endogenous compounds. Mainly located in the liver, their expression and activity may alter in case of liver disease. The nature of these changes in enzyme activity caused by liver disease have been well described in adults, but these data cannot be extrapolated to children for two reasons. First, liver pathologies most common in adults (alcoholic cirrhosis and chronic hepatitis B/C) differ from those in children (cystic fibrosis, biliary atresia, acute liver failure...). Secondly, the activity of the CYPs also varies with age in healthy individuals.

2. Aim

This project aims to identify the changes in hepatic metabolism by the main CYP isoforms in children with severe hepatic dysfunction. These data could help identifying those drugs for which extra caution is necessary when used in children with liver disease. Besides this direct clinical relevance, the collected data are essential in the development of a Physiologically Based PharmacoKinetic-model for the development of new drugs for this specific patient population.

3. Sample collection and processing

• Samples are taken from the explanted livers of children undergoing liver transplantation (e.g. Figure 1.) within 15 minutes after explantation of the liver.

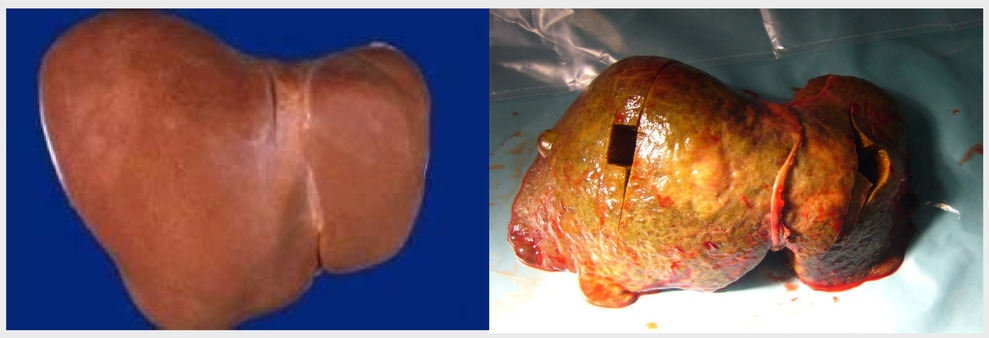

Figure 1: left: healthy human liver; right: liver from 6-month-old girl with biliary atresia.

• Liver samples are processed to microsomes during a process of differential centrifugation (Figure 2). The microsomal pellet is resuspended in a glycerol containing buffer and is snap frozen in liquid nitrogen. Microsomes can be stored at -80°C for 5 years without any substantial loss of CYP activity[1].

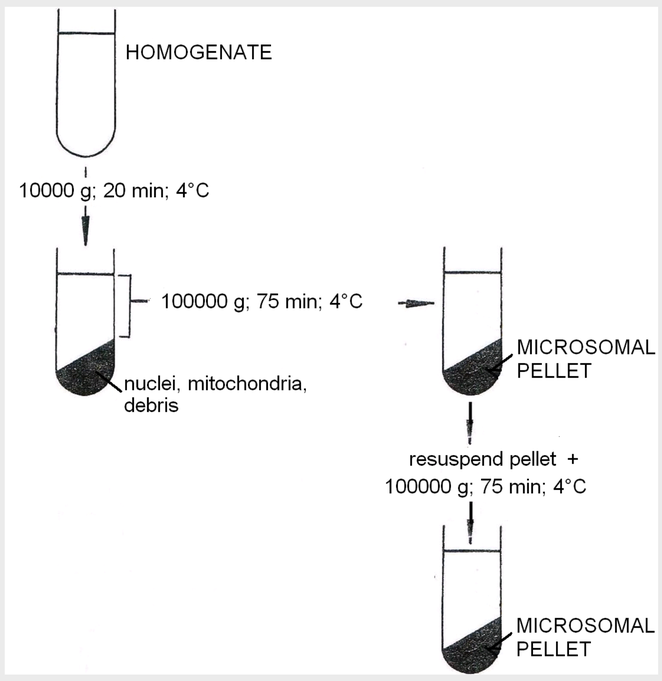

Figure 2: preparation of microsomes through differential centrifugation.

• Protein and CYP content are determined using the method of Bradford[2] and Matsubara[3], respectively.

4. Determination of enzyme activity

• To determine the activity of the six most important isoforms, the microsomes are incubated with probe substrates that are selectively metabolized by a specific isoform. The probes used are listed in Table 1.

CYP	Substrate	Concentration (µM)	Metabolite	Transition (m/z)	Polarity
1A2	Phenacetin	50	Acetaminophen	152>110	ESI+
2C9	Tolbutamide	100	4-OH-tolbutamide	285>186	ESI-
2C19	S-mephenytoin	100	4'-OH-mephenytoin	235>150	ESI+
2D6	Dextromethorphan	5	Dextrorphan	258>157	ESI+
2E1	Chlorzoxazone	50	6-OH-chlorzoxazone	184>120	ESI-
3A4	Midazolam	5	1-OH-midazolam	342>203	ESI+

Table 1: the 6 probe substrates used in the incubation assays.

• The microsomal incubation mixtures contain 0.25mg/mL microsomal protein, 0.2M phosphate buffer (pH 7.4), 1mM NADPH and the substrates in a concentration approximately equal to their K_m values (see Table 1). Incubations can be performed with each substrate individually, but to increase throughput, co-incubation with multiple probes is possible. The incubations are performed in a shaking heating block at 37°C. The reactions are terminated by adding cold acetonitrile, containing the internal standard, and by putting the tubes on ice. After mixing, samples are centrifuged at 20000g for 10 min at 4°C and the supernatant can be readily used for analysis.

• The method for simultaneous quantification of the metabolites is being developed. Supernatant can be directly injected onto a reversed-phase column. Gradient elution, using water containing 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B), will be used to separate the metabolites. Solvent B will be increased linearly from 5% to about 80%.

• Metabolites will be detected with a triple quadrupole mass spectrometer using both positive and negative electrospray ionization (ESI). In order to have a selective method, the mass spectrometer will be operated in the MRM mode. For each metabolite, two productions will be followed. The productions used for quantification are listed in Table 1.

• Enzyme activity is expressed in pmol metabolite formed/ (mg protein x minute).

4. Future plans

• The method for metabolite quantification requires further development and validation.

• This study was already approved by the Ethical Committee of Ghent University Hospital, but in order to enlarge the amount of samples, a multi center study will be set up.

• Genotyping of all patients will be necessary.

5. References

1. Yamazaki H, Inoue K, Turvy CG, Guengerich FP, Shimada T. Effects of freezing, thawing, and storage of human liver samples on the microsomal contents and activities of cytochrome p450 enzymes. *Drug Metabolism and Disposition* 1997;25(2):168-174.

2. Bradford MM. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Anal Biochem* 1976;72(1-2):248-254.

3. Matsubara T, Koike M, Touchi A, Tochino Y, Sugeno K. Quantitative determination of cytochrome P-450 in rat liver homogenate. *Anal Biochem* 1976 Oct;75(2):596-603.

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