# IN VITRO CYTOCHROME P450 ACTIVITY IN CHILDREN WITH SEVERE HEPATIC DYSFUNCTION: A UPLC-MS/MS METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF SIX PROBE METABOLITES

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## 1. Introduction

A vast majority of drugs depend on the cytochrome P450 enzyme (CYP) system for their metabolism. As this system is mainly located in the liver, the CYP activity may be affected by liver disease, consequently causing alterations in the pharmacokinetics of drugs. The nature of these changes has not yet been investigated in children with severe hepatic dysfunction. Therefore, a study was set up to determine the in vitro activity of the six most important isoforms (1A2, 2C9, 2C19, 2D6, 2E1 and 3A4) in hepatic microsomes from this specific population.

## 2. Objectives

Development and validation of a UPLC-MS/MS method for the simultaneous quantification of six probe metabolites following the FDA Guidance for Industry on Bio-Analytical Method Validation (2001).

## 3. Methods

### Probe substrates and metabolites

| CYP | Substrate | | Concentration (µM) | Metabolite | |
|---|---|---|---|---|---|
| 1A2 | Phenacetin | (PH) | 50 | Acetaminophen | (AP) |
| 2C9 | Tolbutamide | (TB) | 100 | 4-OH-tolbutamide | (HTB) |
| 2C19 | S-mephenytoin | (ME) | 100 | 4'-OH-mephenytoin | (HME) |
| 2D6 | Dextromethorphan | (DM) | 5 | Dextrorphan | (DX) |
| 2E1 | Chlorzoxazone | (CZ) | 50 | 6-OH-chlorzoxazone | (HCZ) |
| 3A4 | Midazolam | (MDZ) | 5 | 1-OH-midazolam | (HMDZ) |

*Table 1: Probe substrates and metabolites*

### Microsomal incubations:

As a proof-of-concept, microsomes prepared from the tissue from a partial liver resection of an adult patient with hepatic metastasis were analysed.

Incubation medium contained:

- 0.25 mg/ml microsomal protein
- 0.2 M phosphate buffer (pH 7.4)
- 1 mM NADPH
- Substrates in a concentration near their apparent Km
- 1.15% KCl

After exactly 15 minutes incubation in a shaking heating block at 37°C, reactions were terminated by adding a cold reagent, containing formic acid, acetonitrile and the internal standard (chlorpropamide; CP), and by putting the tubes on ice. After mixing, samples were centrifuged at 20000xg for 10 min at 4°C and the supernatant was injected onto the UPLC column.

### UPLC Method (Waters Acquity)

- BEH C18 Column 1.7 µm 2.1x50 mm and VanGuard Pre-column 1.7 µm 2.1x5 mm (Waters)
- Column temperature: 35 °C
- Eluent A: Water + 0.1% formic acid
- Eluent B: Acetonitrile + 0.1% formic acid
- Flow: 400 µl/min
- Full loop injection (20 µl)
- Retention times (min): AP 0.99; HTB 1.68; HME 1.65; DX 1.45; HCZ 1.46; HMDZ 2.09; CP 2.82

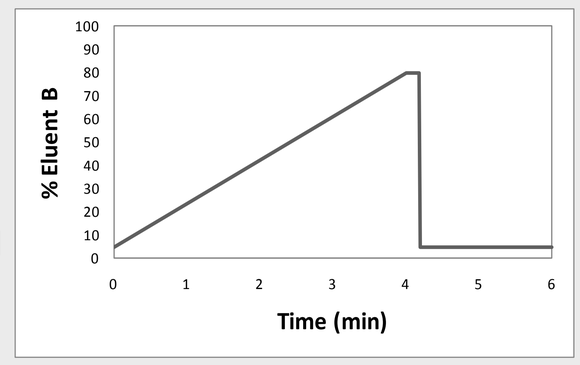


*Figure 1: UPLC gradient profile*

### MS/MS Method (Micromass Quattro Ultima)

- Capillary Voltage (kV): +3.25 and -2.80
- Gas flow (L/h): cone 175 and desolvation 575
- Temperature (°C): source 150 and desolvation 400

| | Ionization mode | Precursor (m/z) | Quantifier (m/z) | Qualifier (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|---|---|
| AP | ESI + | 152,10 | 110,00 | 93,00 | 12 | 28 |
| HTB | ESI - | 285,09 | 185,60 | - | 24 | 12 |
| HME | ESI + | 235,41 | 150,00 | 133,00 | 24 | 14 |
| DX | ESI + | 258,00 | 156,70 | 132,80 | 22 | 28 |
| HCZ | ESI - | 183,83 | 119,80 | 147,70 | 25 | 14 |
| HMDZ | ESI + | 342,04 | 323,70 | 202,80 | 22 | 19 |

*Table 2 : Individual MS parameters for the six probe metabolites*

## 4. Results

### Validation data

- Calibration curves: see Table 3
- LOQ: lowest calibrator
- Precision: 1.10 – 9.6 % (RSD)
- Accuracy: 84.59 – 109.83%
- Good selectivity
- Matrix Effect: calibration curves with and without microsomes differ significantly (see Figure 2)

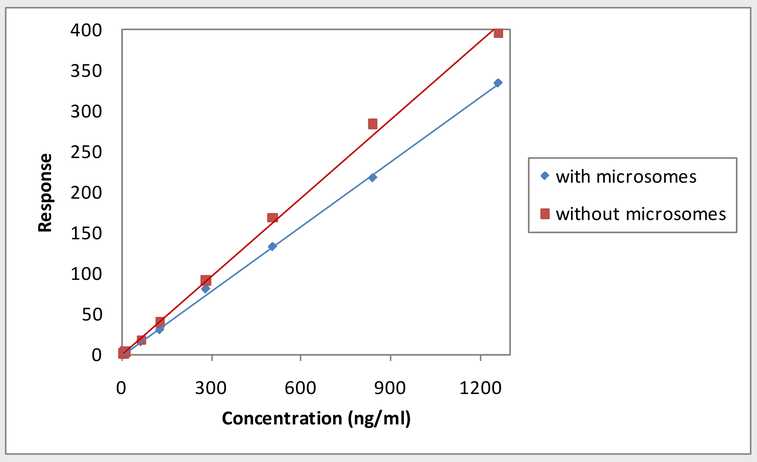


*Figure 2: AP calibration curves with and without microsomes*

| | Fitting | Weight | Range (ng/ml) | |
|---|---|---|---|---|
| AP | linear | $1/x^2$ | 2,80 | 1260 |
| HTB | quadratic | 1/x | 0,80 | 1500 |
| HME | quadratic | 1/x | 4,32 | 1943 |
| DX | linear | $1/x^2$ | 0,55 | 246,9 |
| HCZ | quadratic | 1/x | 9,11 | 1025 |
| HMDZ | linear | $1/x^2$ | 1,47 | 659,5 |

*Table 3: Calibration curves*

### Patient data

| CYP | Metabolite | Activity in pmol/(mg x min) |
|---|---|---|
| 1A2 | AP | 3510,45 |
| 2C9 | HTB | 467,16 |
| 2C19 | HME | 77,94 |
| 2D6 | DX | 465,51 |
| 2E1 | HCZ | 1718,08 |
| 3A4 | HMDZ | 595,16 |

*Table 4: Enzyme activity in microsomes prepared from human adult liver from a patient with hepatic metastasis*

The calculated activities are in the range of previously published results.

## 5. Conclusion

This UPLC-MS/MS method enables the simultaneous quantification of six probe metabolites. After the validation, the method was applied to a human sample, and proved to be useful. Currently, the first samples from the clinical study are being analyzed by this protocol.

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