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# OPTIMIZATION OF THE DERIVATIZATION OF SIX PROBE METABOLITES FOR THE IN VITRO DETERMINATION OF CYTOCHROME P450 ACTIVITY IN CHILDREN WITH HEPATIC IMPAIRMENT.

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## 1. Introduction

In previously published studies, reduced cytochrome P450 enzyme activity was detected in adult patients with hepatic impairment. The activity of this essential enzyme system in children with severe hepatic dysfunction, has not yet been assessed. For an in vitro determination of the activity of the six most important isoforms, 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4, hepatic microsomes can be incubated with specific probe substrates that are selectively metabolized by one of the isoforms.

## 2. Aim

Enzyme activity is expressed as amount of metabolite formed per milligram of protein and per minute. To quantify the amount of metabolite formed, a quantification method was developed using HPLC-MS/MS (Micromass, Quattro II). This method had two major limitations: insufficient sensitivity was seen for the detection of some of the metabolites, and some metabolites could only be detected in the negative electrospray ionization mode. To overcome these limitations, derivatization of the molecules was considered.

## 3. Method optimization: HPLC-MS/MS

**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*

**Sample matrix :**

- 0.25 mg/ml microsomal protein
- 0.2 M phosphate buffer (pH 7.4)
- 1 mM NADPH
- 1.15% KCl
- spiked metabolites

**MS/MS Method (Micromass Quattro II)**

- Capillary voltage: 3.6 kV
- Gas flow: drying gas 450 L/h and nebulising gas 6 L/h
- Source temperature : 120°C

**HPLC Method (Kontron)**

- Luna C18 Column 3 µm 50 x 2.0 mm, 100Å (Phenomenex) and Alltima C18 Pre-column 5 µm 7.5 x 2.1 mm (Grace)
- Eluent A: Water + 0.1% formic acid
- Eluent B: Acetonitrile + 0.1% formic acid
- Flow: 200 µl/min
- Partial loop injection (10 µl)

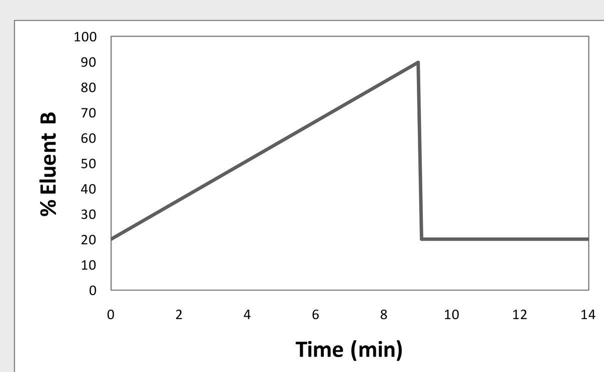


*Figure 1: HPLC gradient profile*

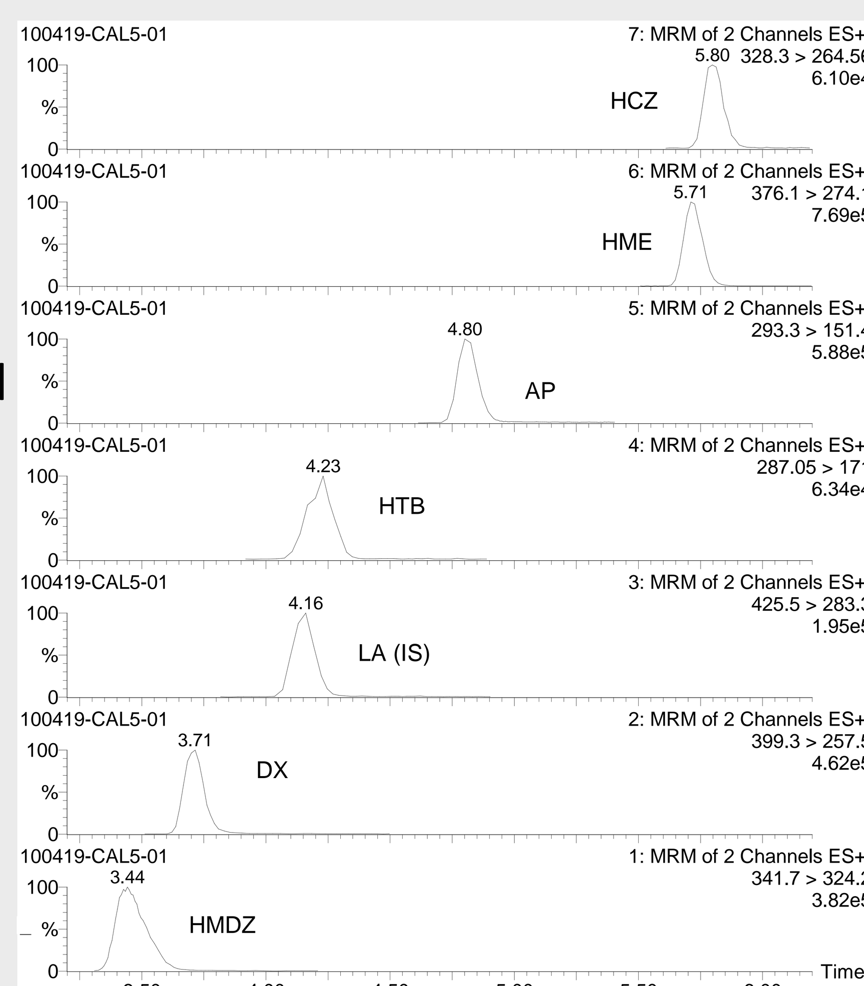


*Figure 2: Chromatogram of a calibrator obtained with the final method*

## 4. Method optimization: derivatization

- Derivatization reagent: Pyridine-3-sulfonyl chloride hydrochloride (PS) versus 4-(1H-pyrazol-1yl)benzenesulfonyl chloride (PBS): derivatization of the aromatic OH-function (in semi-aqueous reaction environment)
  PS was chosen for the following reasons:
  - good fragmentation pattern
  - shorter retention times

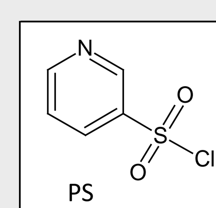


*Figure 4: Structure of derivatization reagent*

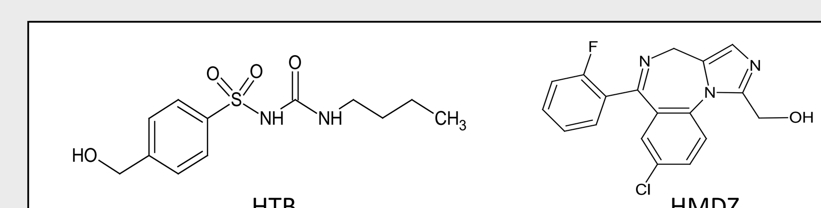


*Figure 3: Structure of the two underivatized metabolites*

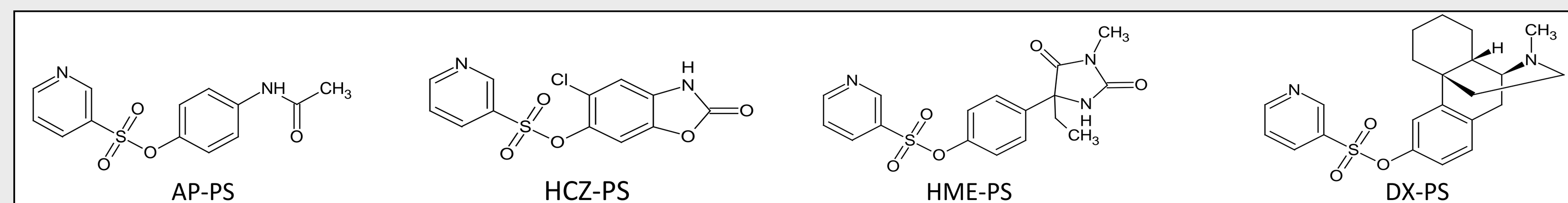


*Figure 5: Structures of the four derivatizated metabolites*

- Other parameters optimized and interpreted based on sensitivity:

| Parameter | Values |
|---|---|
| Temp (°C), heat block | 40 - 55 - 60 - 70 - 80 - 90 |
| Time (min) | 5 - 10 - 15 - 20 - 30 |
| pH | 9 - 9,5 |
| Cool down time (min) | room temp 10 - ice 5 - ice 10 |
| Amount PS (µl) | 50 - 60 - 70 |
| Microwave 850 Watt (sec) | 10 - 20 - 30 - 40 |

**Final derivatization protocol:**

- 200 µl sample
- + 10 µl 1.75 M NaOH
- + 10 µl internal standard (Levallorphan 1.25 µg/ml)
- + 70 µl 1mg/ml PS in acetonitrile
- vortex mixing
- derivatization reaction for 20 seconds in microwave (850 Watt)
- vortex and cool on ice for 10 min
- transfer to 250 µl insert for injection (note: no concentration step !)

| | Ionization mode | Precursor (m/z) | Quantifier (m/z) | Qualifier (m/z) | Cone voltage (V) | Collision energy (eV) | Retention time (min) | LOQ (ng/ml) |
|---|---|---|---|---|---|---|---|---|
| AP-PS | ESI + | 293,30 | 151,40 | 109,10 | 40 | 18 | 4,80 | 5,6 |
| HTB | ESI + | 287,05 | 171,00 | 188,00 | 35 | 18 | 4,23 | 25,0 |
| HME-PS | ESI + | 376,10 | 274,10 | 132,10 | 45 | 23 | 5,71 | 6,9 |
| DX-PS | ESI + | 399,30 | 257,50 | 199,02 | 50 | 30 | 3,71 | 1,1 |
| HCZ-PS | ESI + | 328,30 | 364,56 | 144,25 | 35 | 17 | 5,80 | 128,1 |
| HMDZ | ESI + | 341,70 | 324,20 | 203,20 | 40 | 20 | 3,44 | 2,3 |

*Table 2: Individual parameters for the six (derivatizated) probe metabolites*

## 5. Conclusion

This quantification method shows clear improvement compared to the previously developed method: only positive electrospray ionization is necessary, and the limits of quantification of all metabolites are in the range needed for the particular preclinical pharmacokinetics application. The procedure is quick and easy to perform without various extraction/re-concentration steps. This method is now in the process of full validation.

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