# High sensitive and high throughput analysis of diltiazem metabolites using LC/TOF-MS

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## Introduction

LC/MS is significantly important in qualitative and the quantitative analysis in the drug metabolism and the drug kinetics today. The separation with LC is a very big factor because it needs to measure the metabolites from among a complex matrix, and typically a separation can be obtained by using slow gradient at long measurement time (i.e. about 60 min per one measurement ). Recently a lot of the columns with a small particle diameter are used in many applications and the high resolution and the speed-up measurement in LC analysis become in common. This time, we examined high sensitivity, high throughput metabolite analysis by using LC/TOF-MS with a small particle packed column.

Fig.1 micrOTOF II

## Device & Method

### Sample preparation

***In vitro* metabolism of Diltiazem (DTZ) in human liver S9:** DTZ, NADPH regeneration system solution A (NADP+, Glucose-6-phosphate, $MgCl_2$ in $H_2O$), NADPH regeneration system solution B (Glucose-6-phosphate dehydrogenase in sodium citrate buffer), human liver S9 were mixed in 100 mM phosphate buffer (pH 7.4). The mixture was incubated at 37 deg C overnight (approx. 17 hrs). The control sample was prepared without DTZ added. [Final concentration in incubation mixture; 100 μM DTZ, 1.6 mM NADP, 3.3 mM Glucose-6-phosphate, 0.4 U/mL Glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$, 2 mg/mL S9 protein]

**Solid phase extraction (SPE) of incubation mixture:** The incubation mixture was extracted on YMC Dispo SPE C18 column (100 mg/mL) as follows and injected onto LC/TOF-MS.

1. Condition with 2 mL MeOH
2. Equilibrate with 2 mL 0.1% $CH_3COOH$
3. Load 250 μL sample
4. Elute with 1 mL MeOH
5. Dry extract and resolve in 500 μL $H_2O$

### LC/TOF-MS conditions

**HPLC: Agilent 1200SL system**
Column: Hydrosphere C18 (YMC Co., Ltd.), 5 μm, 150x2.0 mmI.D.
Eluent: A) 20 mM $HCOONH_4$ B) Methanol
Gradient: 25%B (0-3.0min), 25-80%B (3-21min), 80%B (21-24min)
Flow Rate: 0.2 mL/min
Column Oven Temperature: 37 deg C
Injection volume: 3 μL
**Mass instrument: micrOTOF II** (Bruker Daltonics)
ESI, positive mode, m/z 100 – 1000

### High throughput LC/TOF-MS conditions

**HPLC: Agilent 1200SL system**
Column: **YMC-UltraHT Hydrosphere C18** (YMC Co., Ltd.), **2 μm, 50x2.0 mmI.D.**
Eluent: A) 20 mM $HCOONH_4$ B) Methanol
Gradient: 25%B (0-0.5min), 25-80%B (0.5-3.5min), 80%B (3.5-4min)
Flow Rate: 0.4 mL/min
Column Oven Temperature: 37 deg C
Injection volume: **1 μL (reduced to 1/3)**
**Mass instrument: micrOTOF II** (Bruker Daltonics)
ESI, positive mode, m/z 100 – 1000

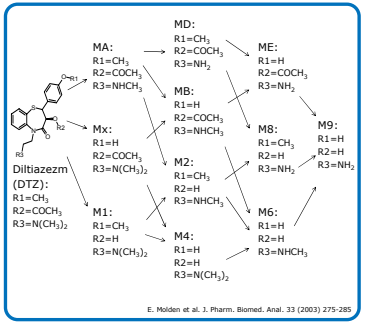


Fig.2 Structure of Diltiazem and its metabolites

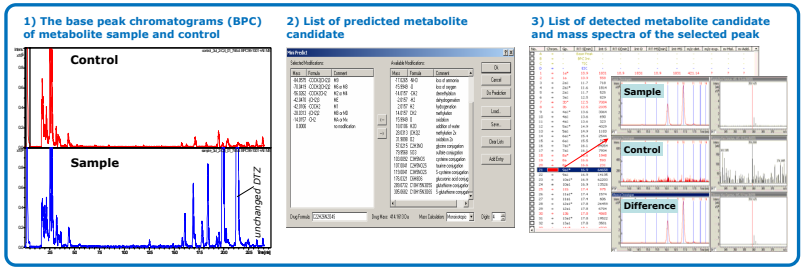


Fig.3 Analysis of DTZ metabolites by LC/TOF-MS and software for metabolite identification (Metabolite Tool)

## Experimental & Results

First of all, samples were acquired on the general LC/TOF-MS condition; column particle diameter 5 μm, column size 150 X 2 mm, flow rate 0.2 ml/min, run time 24 min, and injection volume 3 μl. As a result, an excellent separation was obtained, and a lot of metabolites such as de-methylation (-$CH_2$), de-ethylation (-$C_2H_4$) and de-acetylation (-$COCH_2$) were detected (Figure 3).

The metabolites were confirmed by high resolution extracted ion chromatogram (hrEIC) of their precise mass which is available in using the high resolution of TOF, and the formula and isotope pattern analysis to the mass spectrum of the detected peak (Figure 4, Table 1).

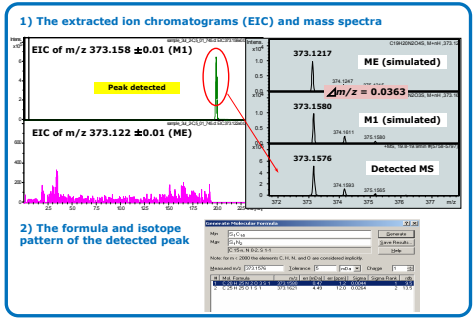


Fig.4 Confirmation of the de-acetylated metabolite by the high resolution of TOF and the formula/isotope pattern analysis

Table 1 List of the main metabolites detected

| Metabolite | Formula | *m/z* expect | *m/z* detect | Δ*m/z* (mDa) | RT (min) |
|---|---|---|---|---|---|
| Drug | C22H26N2O4S | 415.1686 | 415.1700 | 1.38 | 21.6 |
| -CH2 | C21H24N2O4S | 401.1530 | 401.1535 / 401.1533 | -0.53 / -0.35 | 17.8 / 18.4 |
| -COCH2 | C20H25N2O3S | 373.1580 | 373.1590 | -0.98 | 19.9 |
| -(CH2)2 | C20H22N2O4S1 | 387.1373 | 387.1366 / 387.1380 | 0.68 / -0.65 | 15.4 / 18.9 |
| -CO(CH2)2 | C19H22N2O3S | 359.1424 | 359.1416 / 359.1425 | 0.81 / -0.08 | 16.1 / 16.9 |

Next, samples were acquired on the high throughput LC/TOF-MS condition that the particle diameter of the column was reduced to 2 μm, the length of the column was shortened to 50 mm, and the flow rate was fastened to 0.4 ml/min in order to raise throughput. As the sensitivity improvement was expected, the injection volume was reduced to 1 μl. As a result, all metabolites that had been obtained on the previous condition were detected. It was found that sensitivity has improved because detection intensity was equal to the previous condition though the injection volume was reduced. In addition, it was found to be able to maintain high resolution even if the column length was shortened by reducing the particle size because the resolution of the peak in chromatogram was equally to the previous condition (Figure 5).

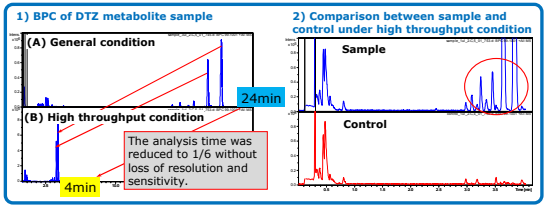


Fig. 5 High throughput LC/TOF-MS analysis of DTZ metabolites

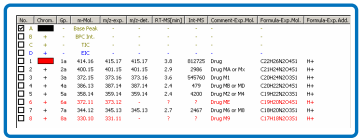

Fig. 6 Expected metabolite list of Metabolite Tool

Black: detected metabolites
Red: n. d.

## Conclusions

1. **Metabolite analysis using LC/TOF-MS with small particle size column achieved speed-up of analysis time and provided excellent separation.**
2. **The metabolites were confirmed without MS/MS analysis because of the excellent performance of the high resolution TOF, the formula and isotope pattern analysis.**