

# An LC-MS/MS Based High Throughput Screening Method for Cytochrome P450 Inhibition Assay in a 384-well Plate Format

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## 1. Abstract

Screening for potential inhibition of cytochrome P450 (CYP) is critical to pharmaceutical drug discovery and development. High throughput screening (HTS) methods for the evaluation of CYP enzyme inhibition via fluorescence detection of a substrate's metabolite have been well established. However, when the inhibitor or its metabolite shows fluorescence and interferes with the fluorescence detection, a more specific technique will be needed to eliminate such interference. We have developed a 384-well plate HTS LC-MS/MS method using a sample volume of 27  $\mu$ L. The method specifically quantifies each CYP substrate's metabolite. This procedure has been validated for each of the 5 major CYPs enzymes (3A4, 2D6, 2C9, 2C19 or 1A2). The method has been validated using spiked standards and known inhibitors against the fluorometric method. Both methods agree very well. The method demonstrates high specificity to the target metabolites, high speed for screening, reduction of cost due to miniaturization, and effectiveness in direct use of assay plates from failed fluorometric detection. The method exhibits ease of use in sample cleanup by protein precipitation, fast chromatography, and data analysis within the Activity Base. In one 384-well assay plate, 32 compounds can be tested at 10 concentrations. It requires about 18 hours to complete the analysis of this plate on a single channel LC-MS/MS instrument. As a result, 32 compounds can be screened for CYP IC50s in this plate format in triplicate per day on Waters Micromass MUX system.

## 2. Introduction

In Vitro evaluation of Cytochrome P450 inhibition of five major isozymes (3A4, 2D6, 2C9, 2C19 and 1A2) has received a substantially increased attention in drug discovery phase. The fastest detection methodology is using fluorometric technique. However, the fluorometric technique will fail if the compounds or their metabolites fluoresce. To solve this issue, we have developed an LC-MS/MS based high throughput screening (HTS) method. The method is fast, highly specific and effective in utilizing the identical plate from the fluometric assay plate in a 384-well format.

## 3. Methods

Each CYP450 assay consists of an single isozyme/substrate mixture. Consequently only one metabolite from each substrate has been measured. There are four individual analytical methods for four isozymes, phenacetin (for ES+) and 4-nitrophenol (for ES-) have been used for internal standards. Quantitation is based on peak area ratios of analyte versus internal standard. Percent controls of each measured concentration versus the mean of high QCs are used for IC50 calculations.

### •Sample clean-up and preparation

Automatic liquid handler (Beckman Biomek FX) with a 384-head has been used for preparation of assay plates and sample clean-up. Protein precipitation was performed in a 384-well plate before samples injected. Each well contains a 27  $\mu$ L of sample aliquot.

### •Data handling in HTS format using ActivityBase.

•Liquid chromatograph using C8 column with a fast gradient and run time of 2 min

•4-Way MUX parallel analysis to increase mass spectrometer throughput

### •MS MRM Transitions for Analytes

| Analyte | MRM Transition | Ionization |
|---------|----------------|------------|
| HQ      | 146 > 91       | ES+        |
| AMHC    | 290 > 203      | ES+        |
| HFC     | 229 > 153      | ES-        |
| CHC     | 186 > 158      | ES-        |

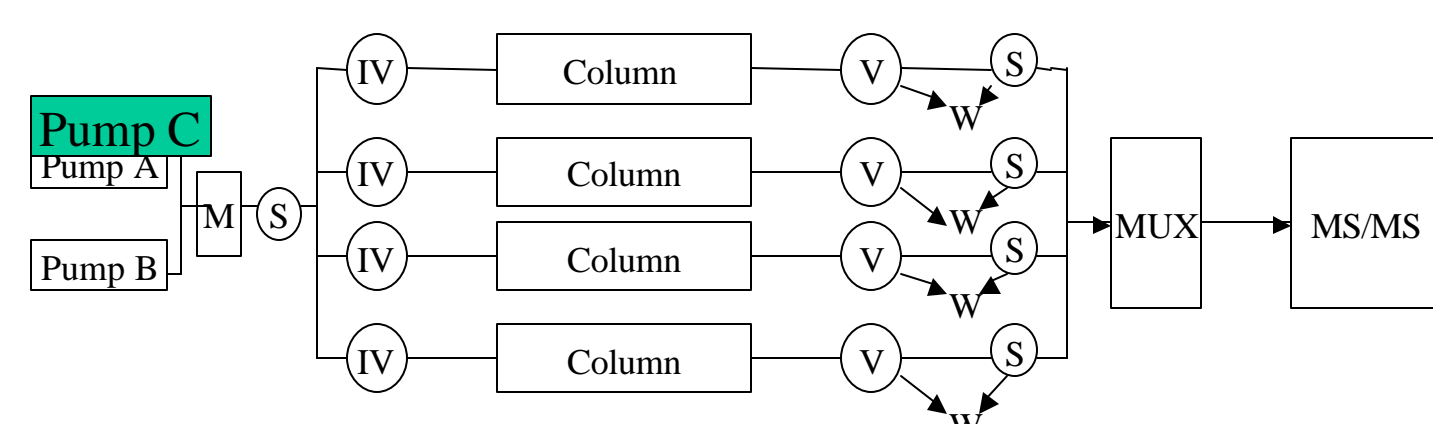
HQ: 7-Hydroxyquinoline; AMHC: (3-[2-(N,N-Diethyl-N-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin);

HFC: 7-Hydroxy-4-(trifluomethyl)-coumarin;

CHC: 3-Cyano-hydroxycoumarin.

### Parallel LC-MS/MS MUX System

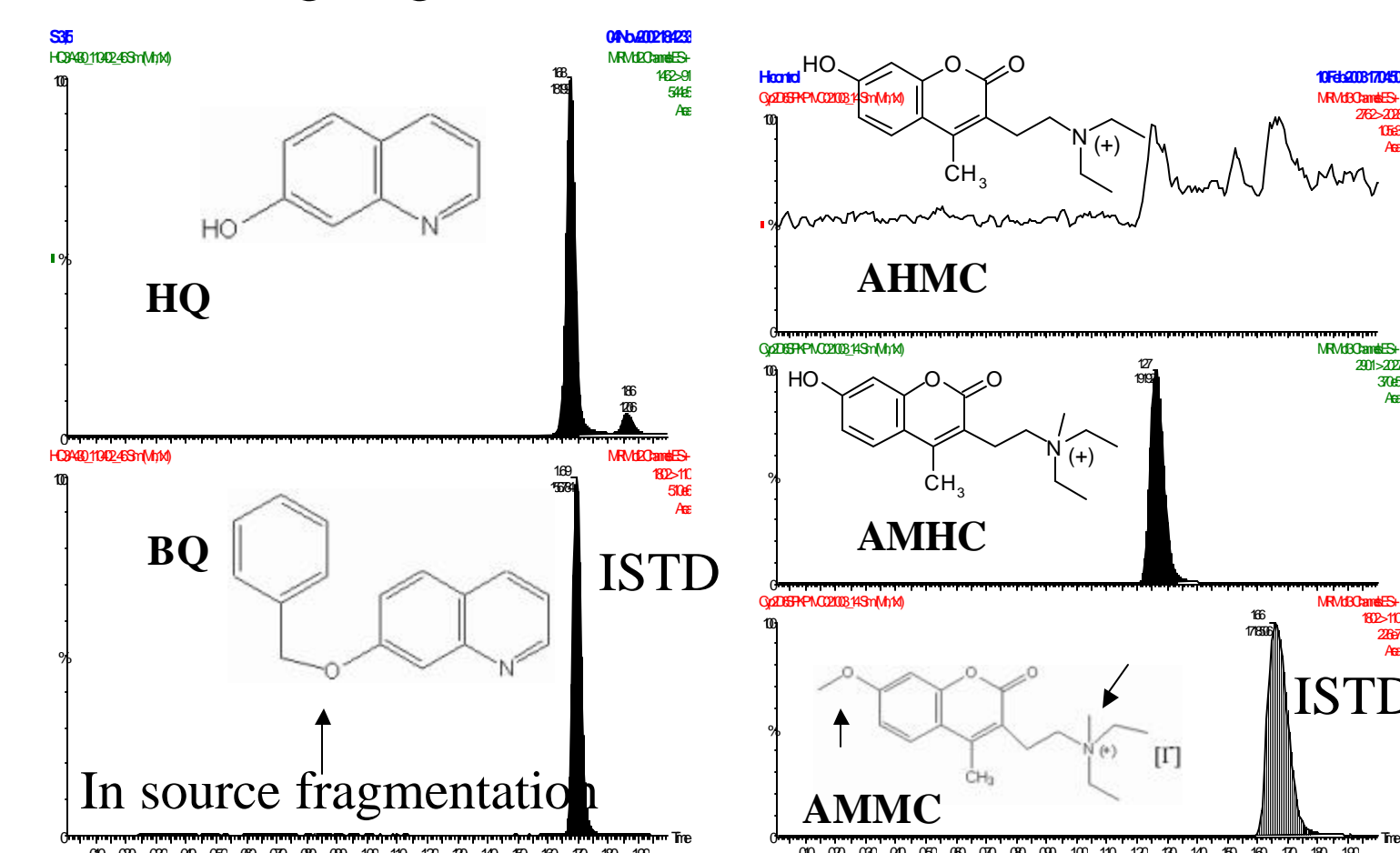
LC      Injector      Mass Spectrometer



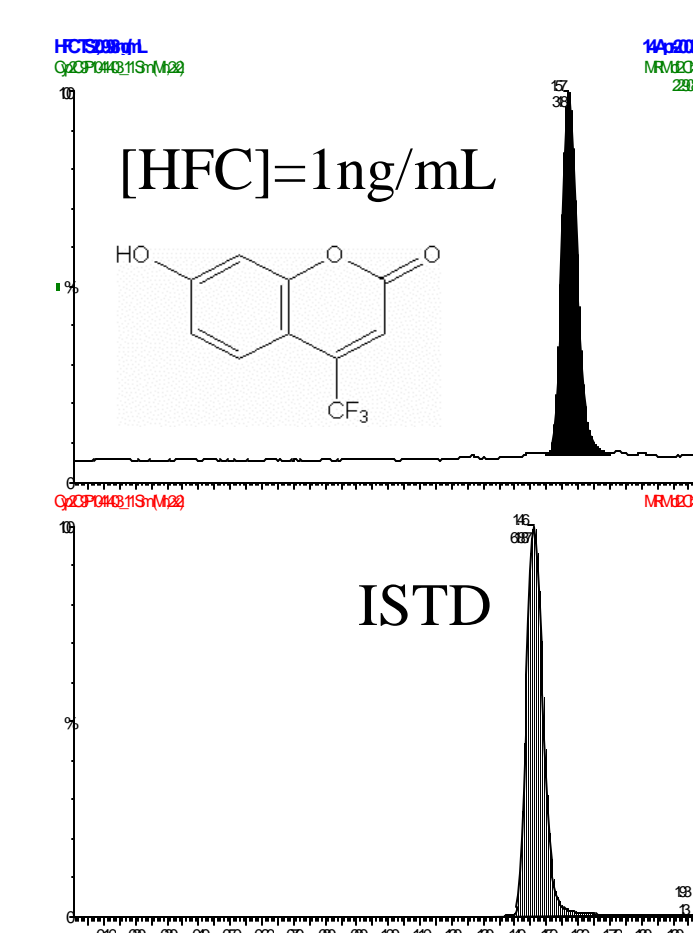
M: Mixer; S: Splitter; IV: Injection valve; V: Devaulting valve; W: Waste

## 4. Results

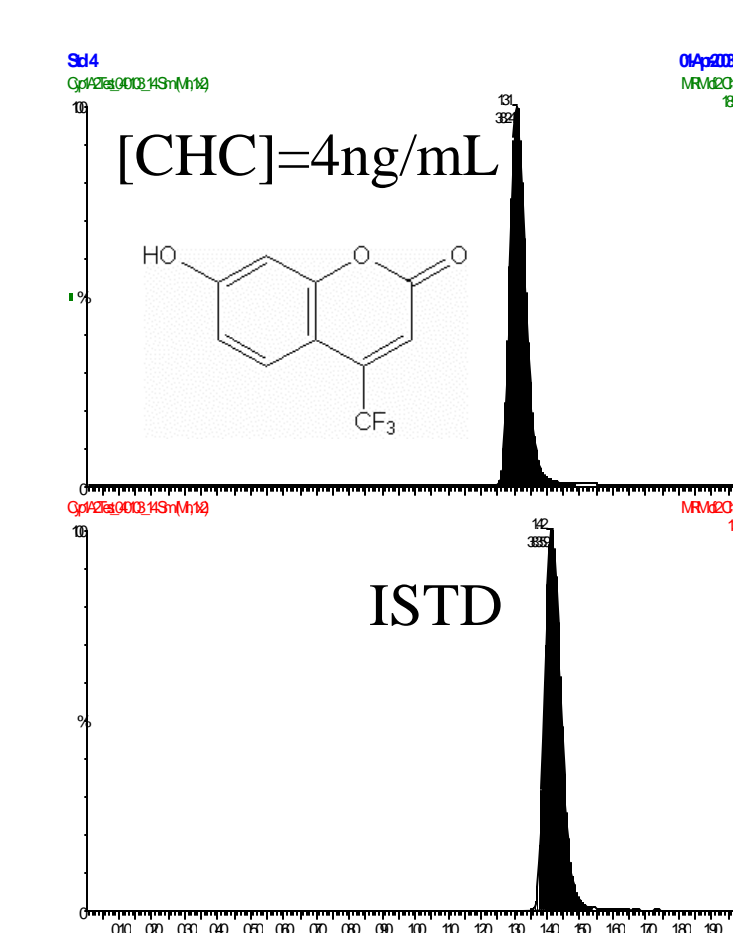
### 3A4\_HQ/BQ Method    2D6\_AMHC/AMMC Method



### 2C9\_HFC/MFC Method

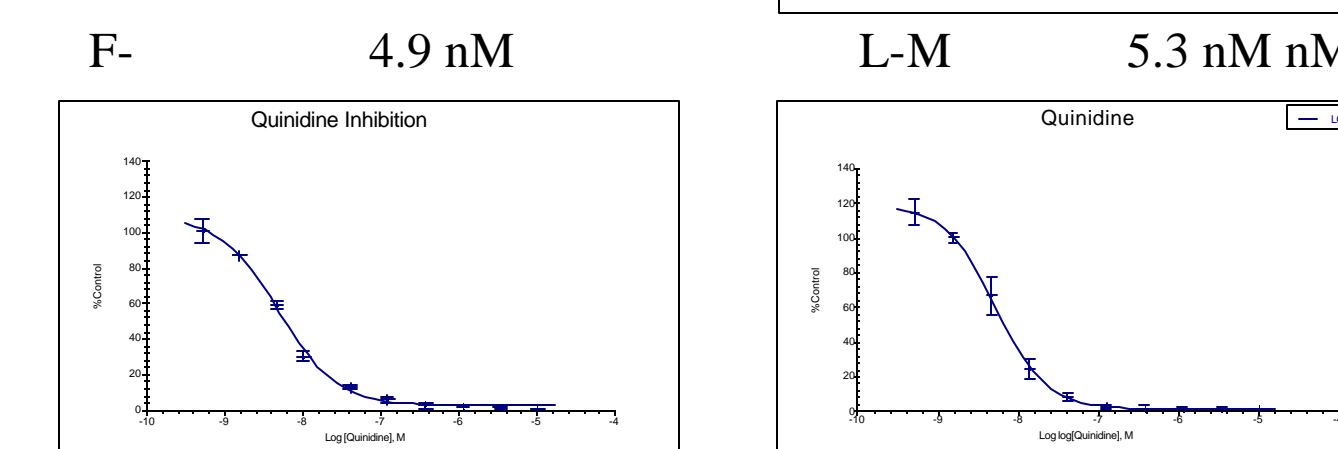


### 1A2/2C19\_CHC/CEC Method



### IC50 Calculations from ActivityBase

Enzyme: 2D6  
Inhibitor: Quinidine  
Substrate: AMMC  
Metabolite: AMHC  
Replicate n = 3



F: Fluorescence; L-S: Single LC-MS/MS; L-M: MUX LC-MS/MS

### Comparison of IC50 Values from Fluorometric and LCMS Detection

| Inhibitor              | IC50 Value ( $\mu$ M) |        |        |
|------------------------|-----------------------|--------|--------|
|                        | (F)*                  | (L-S)* | (L-M)* |
| Ketoconazole (3A4)     | 0.55                  | 0.47   |        |
| Quinidine (2D6)        | 0.0046                | 0.0049 | 0.0059 |
| Sulfaphenazole (2C9)   | 0.17                  | 0.18   |        |
| Furafylline (1A2&2C19) | 3.5                   | 4.6    |        |

F: Fluorescence; L-S: Single LC-MS/MS; L-M: MUX LC-MS/MS

## 5. Conclusions

- Four LC-MS/MS analytical methods for the 5 major CYP inhibition assays (3A4, 2D6, 2C9, 2C19 and 1A2) have been validated using spiked standards (Hi QCs and calibration standards). The IC50 values of known inhibitors measured from these methods agree very well with those generated from fluorometric detection.
- The LC-MS/MS methods are fast. In triplicate 384-well plates, 32 compounds can be screened per day (about 20 hours on MUX) at 10 concentration points.
- These methods result in a cost reduction due to assay miniaturization and in enhancing the effectiveness by integration of the LC-MS/MS method with the process flow of the fluometric CYP450 assays.

### Validation

- Accuracy      within  $\pm 20\%$  of relative errors
- Precision       $\leq 20\%$  (CVs)
- Analytical recovery  $\geq 80\%$
- Linearity       $R^2 \geq 0.98$
- Range          1 – 100 ng/mL
- Stability      Assay plate stable at  $-20^\circ\text{C}$  over one week  
Treated samples stable at room temperature over 24 hours

### Data Format

