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# High-throughput cytochrome P450 (CYP) inhibition screening via cassette probe-dosing strategy
## II. Validation of a direct injection/on-line guard cartridge extraction–tandem mass spectrometry method for CYP2D6 inhibition assessment

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**Abstract**

A highly efficient direct injection/on-line guard cartridge extraction–tandem mass spectrometry (DI/GCE–MS–MS) method has been validated for high-throughput evaluation of cytochrome P450 (CYP) 2D6 inhibition potential using human hepatic microsomes and 96-well microtiter plates. Microsomal incubations were terminated with formic acid, centrifuged, and the resulting supernatants were injected for DI/GCE–MS–MS analysis. Due to the novel use of an extremely short $C_{18}$ guard cartridge, this method exhibits several advantages, such as no sample preparation, excellent on-line extraction, short run time (2.5 min), and minimized source contamination and performance deterioration. The DI/GCE–MS–MS method demonstrates acceptable accuracy and precision for the quantification of dextrorphan, a marker metabolite of dextromethorphan mediated by CYP2D6, in microsomal incubations. The CYP2D6 inhibition assay has been validated using quinidine as a known selective inhibitor of the isoform. The $IC_{50}$ value (0.20 $\mu M$) measured by the new method is in good agreement with the literature value (0.22 $\mu M$). 

*Keywords:* Cassette probe-dosing; Cytochrome P450

## 1. Introduction

Integrated in vitro screening of metabolic stability [1], Caco-2 cell permeability [2] and cytochrome P450 (CYP) enzyme inhibition potential [3] of new chemical entities provides critical information for lead finding and optimization of drug candidates in the pharmaceutical industry. For the inhibition assessment, a common strategy is to monitor the effect of test compounds on the metabolism of CYP probe substrates using liver microsomes [3–5].

In an attempt to minimize cost while maximizing throughput of CYP inhibition screening, we have performed CYP inhibition studies via cassette [6,7] and individual dosing of CYP probe substrates in human microsomal incubations using a direct injection/on-line guard cartridge extraction–tandem mass spectrometry (DI/GCE–MS–MS) method [8].

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The CYP isoform–substrate–metabolite–inhibitor systems investigated in the studies included 1A2–ethoxyresorufin–resorufin–α-naphthoflavone, 2A6–coumarin – 7-hydroxycoumarin – tranylcypromine, 3A4 – midazolam – 1′-hydroxymidazolam – ketoconazole, 2C9–tolbutamide–4-hydroxytolbutamide–sulfaphenazole, 2C19–*S*-mephenytoin–4′-hydroxymephenytoin–tranylcypromine, 2D6–dextromethorphan–dextrorphan–quinidine, and 2E1–chlorzoxazone – 6-hydroxychiorzoxazone – 4-methylpyrazole. From these studies, no significant interference (analytical or metabolic) was observed and the extent of inhibition (e.g., $IC_{50}$) achieved by the cassette dosing approach was in good agreement with that obtained by the individual dosing regimen. Here, we exclusively present the validation of the DI/GCE–MS–MS method for high-throughput CYP2D6 inhibition assessment using human hepatic microsomes and 96-well microtiter plates. This assay involves *O*-demethylation of dextromethorphan to dextrorphan, an index reaction for CYP2D6 [9], and DI/GCE–MS–MS quantification of dextrorphan.

## 2. Experimental

### 2.1. Chemicals

Pooled human liver microsomes were received from the International Institute for the Advancement of Medicine (Exton, PA, USA). Pooled rat liver microsomes were prepared in-house at Covance Laboratories (Madison, WI, USA). Dextromethorphan (Dm) and dextrorphan (Dp) were obtained from ICN Biomedicals (Aurora, OH, USA). Phenacetin, quinidine, glucose-6-phosphate (G6P), $NADP^+$, and glucose-6-phosphate dehydrogenase (G6PD) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available.

### 2.2. Standards and quality control samples

Calibration standards (0.05, 0.1, 0.2, 0.5, 1, 2 and 5 μ*M* Dp, $n=2$) and quality control (QC) samples (0.15, 1.5 and 3.5 μ*M* Dp, $n=6$) were prepared in 96-well plates using rat liver microsomes. The samples (200 μl) contained 0.1 *M* potassium phosphate buffer (pH 7.4), 1 m*M* EDTA, 0.25 mg/ml microsomal protein, 20 μ*M* Dm, varying concentrations of Dp, and an NADPH-generating system (5 m*M* G6P, 1 m*M* $NADP^+$, 3 m*M* $MgCl_2$ and 1 U/ml G6PD). The NADPH-generating system was added after all other components had been preincubated for 10 min at 37°C in an air bath with gentle shaking and had been acidified with 50 μl of 1 *M* formic acid. Phenacetin (4 μ*M*, 30 μl) was added as internal standard. The resulting samples were centrifuged at 1600 *g* for 5 min and the supernatant (5 μl) was injected for DI/GCE–MS–MS analysis.

### 2.3. Incubations

Incubations were performed in 96-well plates using human liver microsomes. Incubation mixtures (200 μl) contained 0.1 *M* potassium phosphate buffer (pH 7.4), 1 m*M* EDTA, 0.125–0.5 mg/ml microsomal protein, 20 μ*M* Dm, and the NADPH-generating system. Reactions were initiated by adding the NADPH-generating system after a 10-min preincubation at 37°C. After a given incubation time (0–30 min), the reactions were terminated by the addition of formic acid (1 *M*, 50 μl). Phenacetin (4 μ*M*, 30 μl) was added as the internal standard. For $IC_{50}$ measurement, microsomal protein concentration and incubation time were fixed at 0.25 mg/ml and 30 min, and inhibitor concentrations varied from 0.2- to five-fold of the literature $IC_{50}$ value. Formation of Dp in all incubations was quantified by the DI/GCE–MS–MS method.

### 2.4. DI/GCE–MS–MS

A Hewlett Packard 1090 liquid chromatograph (Palo Alto, CA, USA) was operated using a Phenomenex $C_{18}$ guard cartridge (4×2.0 mm I.D., Torrance, CA, USA). The mobile-phase gradient conditions are specified in Table 1. The total run time was 2.5 min. When no data acquisition was taking placing, the post-cartridge effluent was diverted to waste using an electronic switching valve, to remove salts and other interfering species. In this method, all data were acquired from 1.2 to 2 min by diverting the flow to a Z-design electrospray interface (source temperature, 120°C; desolvation temperature, 350°C) of a Micromass Quattro II mass spectrometer (Manchester,

Table 1
Gradient conditions for the DI/GCE–MS–MS method

| Time (min) | Flow rate (ml/min) | A[a] (%) | B[a] (%) |
|---|---|---|---|
| Initial | 0.5 | 0 | 100 |
| 0.00 | 0.4 | 0 | 100 |
| 0.10 | 0.4 | 0 | 100 |
| 0.11 | 0.4 | 80 | 20 |
| 2.00 | 0.4 | 80 | 20 |
| 2.01 | 2.0 | 0 | 100 |
| 2.50 | 2.0 | 0 | 100 |

[a] A is acetonitrile–formic acid (100:0.01, v/v) and B is water–formic acid (100:0.01, v/v).

UK). Nitrogen served as the drying and nebulizing gas at flow rates of 400 and 20 l/h, respectively. Argon was used as the target gas at a pressure of $1\times10^{-3}$ mBar for collision-induced dissociation. Quantification was performed by multiple reaction monitoring (MRM; dwell time, 0.1 s) of the precursor/product ions at *m/z* 258/157 (cone voltage, 40 V and collision energy, 40 eV) for Dp and *m/z* 180/110 (cone voltage, 35 V and collision energy, 20 eV) for phenacetin, with positive ion mode. All raw data were processed with Micromass Masslynx Version 3.1.

### 2.5. Assay validation

A 3-day validation was performed to evaluate the DI/GCE–MS–MS method for the quantification of Dp in microsomal incubations. Intra- and inter-day accuracy and precision were determined by analyzing replicates of the QC samples. The microsomal matrix effect was cross-validated by quantifying QC samples prepared with human liver microsomes using the standard curves prepared with rat liver microsomes. Dm *O*-demethylation reaction linearity was evaluated by varying protein concentrations (0.125, 0.25 and 0.5 mg/ml, $n=3$) and incubation time (0, 10, 20 and 30 min, $n=3$) on three separate days. Inhibition of CYP2D6 by quinidine, a known competitive inhibitor for the isoform, was assessed by determination of $IC_{50}$ with quinidine concentrations at 0.05, 0.1, 0.2, 0.4, 0.6 and 1 μ*M*. $IC_{50}$ was calculated via curve fitting analysis using SigmaPlot software.

## 3. Results and discussion

### 3.1. DI/GCE–MS–MS optimization

During the development of the DI/GCE–MS–MS method, no attempt was made to chromatographically separate Dp from Dm, phenacetin, and other matrices due to the high selectivity of MRM detection. The extremely short $C_{18}$ guard cartridge allowed the utilization of high flow rates and step gradients (Table 1) to shorten run time without generation of high back pressure. The rapid passage of sample matrices (e.g., salts and proteins) through the cartridge led to efficient on-line extraction of the microsomal incubation samples via direct injection, minimizing possible matrix-induced electrospray ionization suppression. In addition, no visible source of contamination was observed and system performance (chromatographic and mass spectrometric) did not significantly deteriorate after 500 consecutive injections. Fig. 1 shows the representative MRM chromatograms of Dp and phenacetin in a rat liver microsomal incubation.

### 3.2. Calibration curve linearity

The calibration range of 0.05–5 μ*M* proved to be sufficient for the analysis of Dp in microsomal incubations. Calibration curves were constructed with duplicate Dp standards at each concentration. Excellent linearity was achieved, with correlation coefficients greater than 0.997. The lower limit of quantification (LLOQ) was not measured in the validation process. However, it can be expected that the LLOQ value would be 5–10 n*M*, based on the large peak area and high signal-to-noise ratio generated at 0.05 μ*M* Dp (data not shown).

### 3.3. Method precision and accuracy

The intra-day method precision and accuracy were determined by analyzing six QC replicates at 0.15, 1.5 and 3.5 μ*M* Dp on each of three days. The method's accuracy was determined by calculating relative error (RE) and the precision by calculating relative standard deviation (RSD). Table 2 summarizes the intra- and inter-day accuracy and precision data for Dp in rat liver microsomal incubations. The

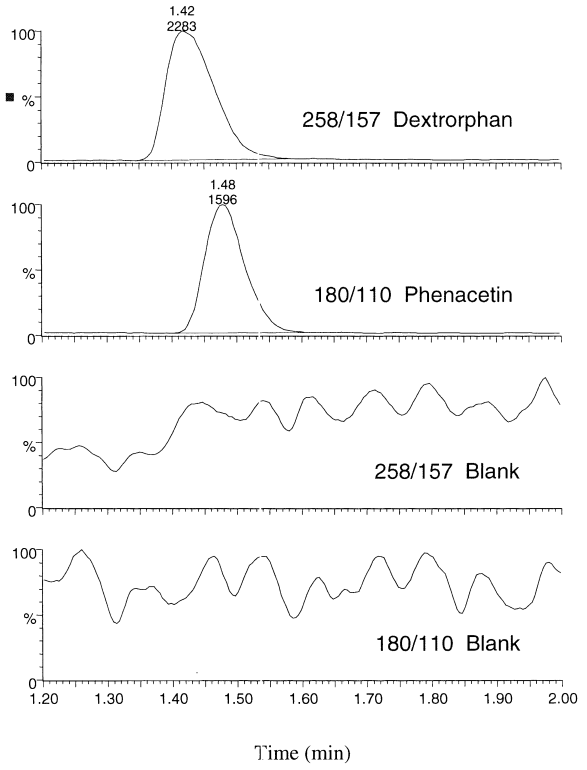


Fig. 1. Representative MRM chromatograms of dextrorphan and internal standard (phenacetin) in a rat liver microsomal incubation treated with 1 *M* formic acid.

inter-day accuracy ranged from −4.8 to 1.3%, with precision ranging from 4.0 to 5.9% over the three concentrations evaluated.

The influence of the microsomal matrix was examined by quantifying six QC replicates prepared with human liver microsomes at 0.15, 1.5 and 3.5 μ*M* Dp using calibration curves prepared with rat liver microsomes on each of two days. The intra-day accuracy ranged from −3.3 to 5.1%, with precision ranging from 2.8 to 9.6%. The results indicate that the matrix effect is insignificant. Therefore, all human microsomal incubation samples were analyzed using calibration curves prepared with rat liver microsomes in order to reduce the consumption of human liver microsomes.

### *3.4. Recovery*

Overall recovery of a compound was represented by the peak area ratio of the analyte in incubations containing 0.25 mg/ml rat liver microsomal protein versus in water ($n=3$). All of the samples contained

Table 2
Intra-day ($n=6$) and inter-day ($n=18$) validation data for dextrorphan in rat liver microsomal incubations

| Day | Parameter | QC sample level (μ*M*) | | |
|---|---|---|---|---|
| | | 0.15 | 1.50 | 3.50 |
| 1 | Mean (μ*M*) | 0.151 | 1.55 | 3.46 |
| | SD (μ*M*) | 0.0033 | 0.042 | 0.114 |
| | Accuracy (RE, %) | 0.66 | 3.38 | −1.22 |
| | Precision (RSD, %) | 2.22 | 2.69 | 3.31 |
| 2 | Mean (μ*M*) | 0.155 | 1.55 | 3.29 |
| | SD (μ*M*) | 0.0074 | 0.137 | 0.142 |
| | Accuracy (RE, %) | 3.47 | 3.26 | −6.27 |
| | Precision (RSD, %) | 4.79 | 8.82 | 4.32 |
| 3 | Mean (μ*M*) | 0.150 | 1.46 | 3.27 |
| | SD (μ*M*) | 0.0071 | 0.080 | 0.167 |
| | Accuracy (RE, %) | −0.22 | −2.42 | −6.88 |
| | Precision (RSD, %) | 4.76 | 5.48 | 5.10 |
| Inter- | Mean (μ*M*) | 0.152 | 1.52 | 3.34 |
| | SD (μ*M*) | 0.0063 | 0.091 | 0.159 |
| | Accuracy (RE, %) | 1.20 | 1.25 | −4.73 |
| | Precision (RSD, %) | 4.04 | 5.87 | 4.89 |

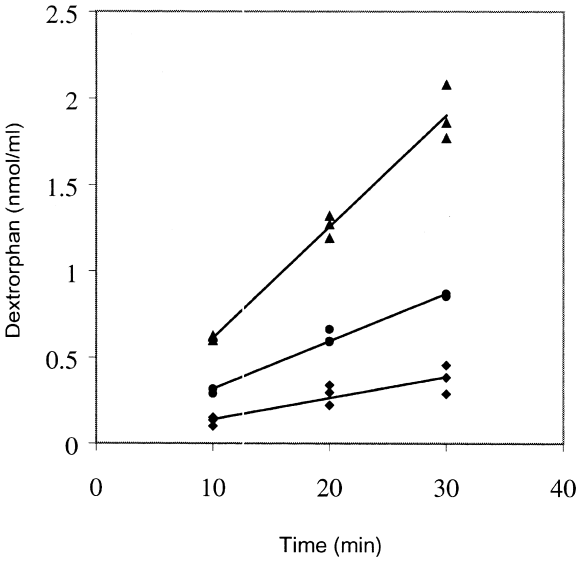


Fig. 2. Dextrorphan formation versus incubation time at microsomal protein concentrations of 0.125 (♦), 0.25 (●) and 0.5 (▲) mg/ml with 20 μ*M* dextromethorphan. Each data point is a mean value of three replicates performed on a single day.

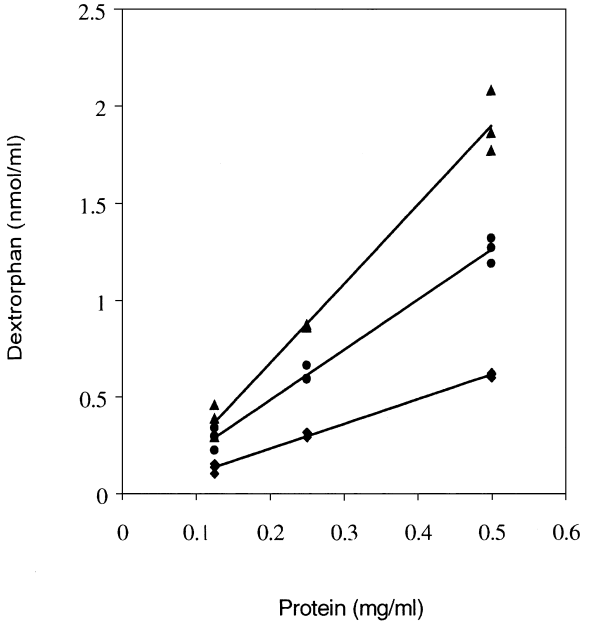


Fig. 3. Dextrorphan formation versus microsomal protein concentration at incubation times of 10 (♦), 20 (●) and 30 (▲) min with 20 μ*M* dextromethorphan. Each data point is a mean value of three replicates performed on a single day.

0.2 μ*M* analyte and were equally treated with 1 *M* formic acid. The overall recoveries of Dp and phenacetin were determined to be 70 and 55%, respectively.

### *3.5. Reaction linearity*

Dm *O*-demethylation was investigated with respect to varying incubation time and microsomal protein concentration. The dependence of Dp formation on incubation time and protein concentration is shown in Figs. 2 and 3, respectively. The formation of Dp in human liver microsomal incubations exhibits a good linearity with both incubation time and protein concentration under all conditions tested. Dm *O*-demethylase activities in human liver microsomes ranged from 100 to 130 pmol/min/mg protein. Note that substrate (i.e., Dm) consumption was less than 10% for all incubations performed in this study. A microsomal protein concentration of 0.25 mg/ml and an incubation time of 30 min were chosen as the experimental conditions for assessment of human CYP2D6 inhibition.

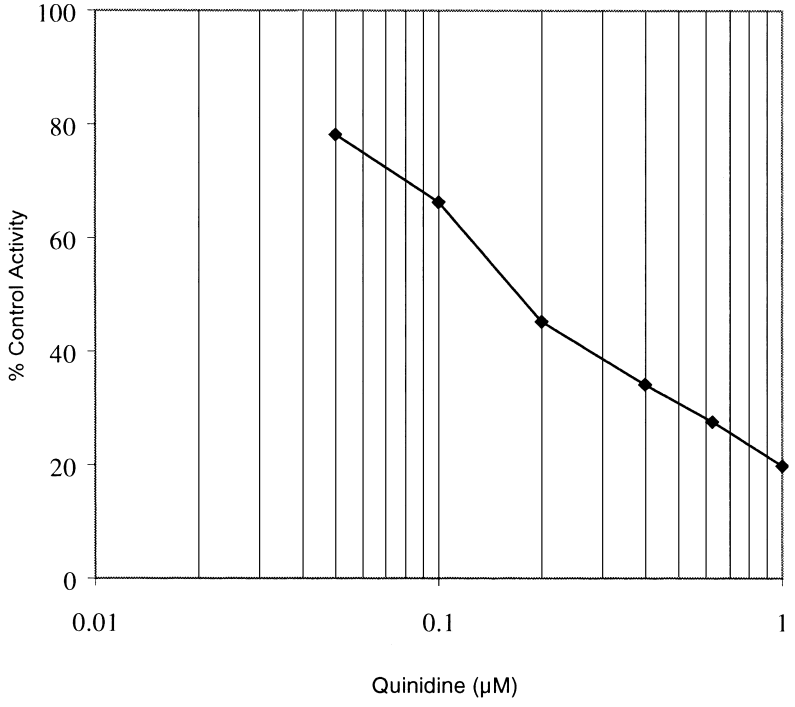


Fig. 4. Inhibition of dextromethorphan *O*-demethylation by quinidine in incubations containing 0.25 mg/ml microsomal protein and 20 μ*M* dextromethorphan, with an incubation time of 30 min. Each data point denotes a mean value of nine replicates performed on 3 days (three replicates/day).

### 3.6. Inhibition study

Dm *O*-demethylation activity was inhibited by quinidine, a potent and selective inhibitor of CYP2D6 [10]. Fig. 4 displays the inhibition curve of percent control activity versus logarithm of quinidine concentration. The $IC_{50}$ value for quinidine inhibition of CYP2D6 was calculated to be 0.20 μ*M*, which was in good agreement with the literature value of 0.22 μ*M* [11]. The percent control activity was reduced to ~20% by quinidine at 1 μ*M*. This concentration of quinidine was chosen as a positive control for human CYP2D6 inhibition and was used in the evaluation of the potential for drug–drug interactions.

## 4. Conclusion

The DI/GCE–MS–MS method proved to be highly efficient, accurate, and precise in the quantification of Dp from microsomal incubations. The overall advantage of the method is attributed to the novel use of the extremely short $C_{18}$ guard cartridge. In addition, this method can be used directly or easily modified to perform the analysis of other CYP probe substrates and their marker metabolites. The DI/GCE–MS–MS method was validated for CYP2D6 inhibition assessment using human liver microsomes and 96-well plates. The validation results indicate that the CYP2D6 assay was fast and reliable. This assay has been routinely used in our laboratory for high-throughput CYP2D6 inhibition evaluation of drugs and new chemical entities.

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