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High-throughput cytochrome P450 (CYP) inhibition screening via cassette probe-dosing strategy: III. Validation of a direct injection/on-line guard cartridge extraction – tandem mass spectrometry method for CYP2C19 inhibition evaluation

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Abstract

A new 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) 2C19 inhibition potential using human hepatic microsomes and 96-well microtiter plates. Microsomal incubations were quenched with formic acid, centrifuged, and the resulting supernatants were injected for DI/GCE – MS – MS analysis. Due to the use of a C_{18} guard cartridge (4×2.0 mm I.D.), this method exhibits several advantages such as little sample preparation, rapid on-line extraction, short analysis time (2.5 min), and minimal source contamination and performance deterioration. The DI/GCE – MS – MS method demonstrates an inter-day accuracy ranged from 0.3 to 2.4% with precision ranging from 2.0 to 3.0% for the quantification of 4-hydroxymephenytoin, a marker metabolite of *S*-mephenytoin mediated by CYP2C19, in microsomal incubations. The CYP2C19 inhibition assay has been validated using tranylcypromine as a known inhibitor of the isoform. The IC_{50} value (43.5 μM) measured by the new method is in agreement with a reported literature value (~ 30 μM).

Keywords: Direct injection; On-line extraction; Mass spectrometry; High-throughput; Cytochrome P450; Inhibition

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].

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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 hepatic microsomal incubations using a direct injection/on-line guard cartridge extraction – tandem mass spectrometry (DI/GCE – MS – MS) method [8]. The CYP isoform/substrate/metabolite/inhibitor systems investigated in those 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-hydroxychlorzoxazone/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 agreement with that obtained by the individual dosing regimen. This report presents the validation of the DI/GCE – MS – MS method for high-throughput CYP2C19 inhibition assessment using human hepatic microsomes and 96-well microtiter plates. This assay involves 4′-hydroxylation of mephenytoin to 4′-hydroxymephenytoin, an index reaction for CYP2C19 [9], and DI/GCE – MS – MS quantification of 4′-hydroxymephenytoin.

2. Experimental

2.1. Chemicals

Pooled human Liver microsomes were received from the International Institute for the Advancement of Medicine (Exton, PA). Pooled rat liver microsomes were prepared in-house at Covance Laboratories (Madison, WI). *S*(+)-mephenytoin (Mep) and 4′-hydroxymephenytoin (OHMep) were supplied by Gentest (Woburn, MA). Chlorzoxazone, tranylcypromine, glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (NADP), and glucose-6-phosphate dehydrogenase (G6PD) were purchased from Sigma Chemical Co. (St. Louis, MO). 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 μM OHMep, $n = 2$) and quality control (QC) samples (0.15, 1.5 and 3.5 μM OHMep, $n = 6$) 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 mM EDTA, 1.0 mg/ml microsomal protein, 80 μM Mep, varying concentrations of OHMep, and an NADPH-generating system (5 mM G6P, 1 mM $NADP^+$, 3 mM $MgCl_2$ 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 in acetonitrile. Chlozoxazone (10 μM, 20 μl) was added as internal standard. The resulting samples were centrifuged at $1600 \times g$ for 5 min and the supernatant (20 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 mM EDTA, 0.5 – 1.5 mg/ml microsomal protein, 80 μM Mep, 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 – 120 min), the reactions were quenched by the addition of formic acid in acetonitrile (1 M, 50 μl). Chlorzoxazone (10 μM, 20 μl) was added as the internal standard. For IC_{50} measurement, microsomal protein concentration and incubation time were fixed at 1.0 mg/ml and 90 min, respectively, and inhibitor concentrations varied from 0.15- to 7-fold of the literature IC_{50} value. Formation of OHMep in all incubations was quantified using the DI/GCE – MS – MS method.

2.4. DI/GCE − MS − MS

A Hewlett Packard 1090 liquid chromatograph (Palo Alto, CA) was operated using a Phenomenex C_{18} guard cartridge (4 × 2.0 mm I.D., Torrance, CA). The mobile phase gradient conditions are specified in Table 1. The total analysis 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-SPRAY® interface (source temperature 100°C, desolvation temperature 350°C) of a Micromass Quattro II mass spectrometer (Manchester, 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 1×10^{-3} mbar for collision-induced dissociation. Multiple reaction monitoring (MRM, dwell time 0.1 s) of the precursor/product ions was performed at m/z 233/190 (cone voltage 30 V and collision energy 15 eV) for OHMep and m/z 168/132 (cone voltage 40 V and collision energy 20 eV) for chlorzoxazone in the negative ion mode. Quantification was carried out using an internal standard calibration method with peak area ratios and $1/x$ weighting. All raw data were processed with Micromass Masslynx version 3.1.

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

Time (min)	Flow rate (ml/min)	A[a] (%)	B[a] (%)
Initial	0.4	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).

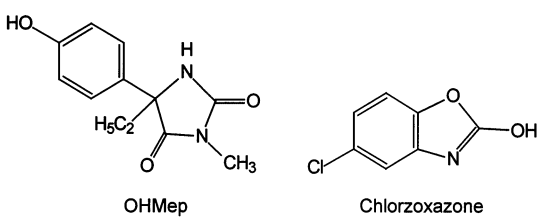

Fig. 1. Chemical structures of OHMep and chlorzoxazone.

2.5. Assay validation

A 3-day validation was performed to evaluate the DI/GCE − MS − MS method for the quantification of OHMep in microsomal incubations. Intra- and inter-day accuracy and precision were determined by analyzing replicates of the QC samples. Microsomal matrix effect was cross-validated by quantifying QC samples prepared with human liver microsomes using the calibration curves prepared with rat liver microsomes. Mep 4′-hydroxylation was evaluated by varying protein concentrations (0.5, 1.0 and 1.5 mg/ml, $n = 3$) and incubation time (0, 30, 60, 90 and 120 min, $n = 3$) on three separate days. Inhibition of CYP2C19 by tranylcypromine, a known inhibitor for the isoform, was assessed by determination of IC_{50} with tranylcypromine concentrations at 5, 10, 20, 50, 80, 120 and 200 μM. IC_{50} was calculated via a three-parameter curve fitting analysis using SigmaPlot software (version 5.0).

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 OHMep (Fig. 1) from Mep, chlorzoxazone (Fig. 1), 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 analysis time without generation of high back-pressure. The rapid passage of sample matrices (e.g. salts and proteins) through the cartridge led to sufficient on-line isolation of the analyte and internal standard from the matrices after direct injection, minimizing possible matrix-induced electrospray ionization suppres-

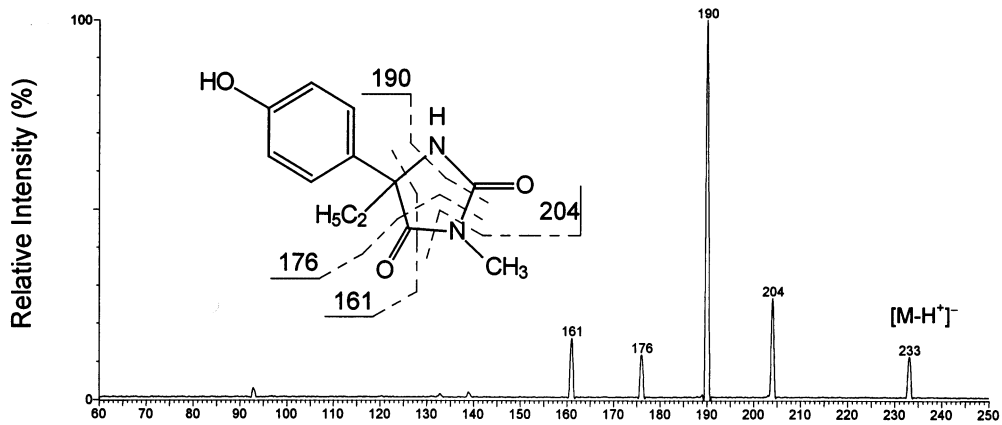

Fig. 2. Electrospray product ion mass spectrum of OHMep.

sion [8,10]. In addition, no visible source contamination was observed and system performance (chromatographic and mass spectrometric) did not significantly deteriorate after 500 consecutive injections. Fig. 2 shows the product ion mass spectrum of OHMep. Representative MRM chromatograms of OHMep and chlorzoxazone in rat liver microsomal incubations are showed in Fig. 3.

3.2. Calibration curve linearity

The calibration range of $0.05 - 5$ μM proved to be sufficient for the analysis of OHMep in microsomal incubations. Calibration curves were constructed with duplicate OHMep standards at each concentration. Excellent linearity was achieved with correlation coefficients greater than 0.999. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve for which an accuracy of $\pm 20\%$ and a precision of 20% were obtained. This method had an LLOQ of 0.05 μM OHMep.

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 μM OHMep on each of three days. The method 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 OHMep in rat liver microsomal incubations. The inter-day accuracy ranged from 0.3 to 2.4% with precision ranging from 2.0 to 3.0% over the three concentrations evaluated.

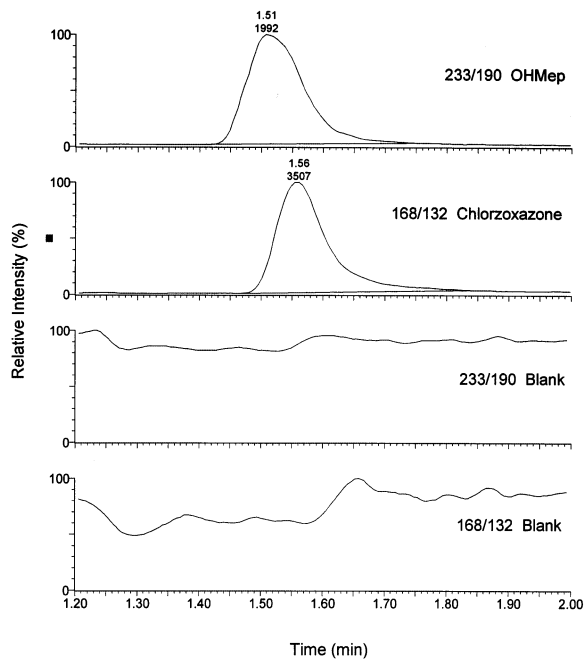

Fig. 3. Representative MRM chromatograms of OHMep and internal standard (chlorzoxazone) in rat liver microsomal incubations containing 80 μM Mep treated with 1 M formic acid.

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

Day	Parameter	QC sample level (μM)		
		0.15	1.50	3.50
1	Mean (μM)	0.152	1.52	3.51
	SD (μM)	0.0019	0.028	0.038
	Accuracy (RE, %)	1.21	1.56	0.37
	Precision (RSD, %)	1.23	1.84	1.08
2	Mean (μM)	0.153	1.53	3.56
	SD (μM)	0.0028	0.017	0.039
	Accuracy (RE, %)	1.70	2.04	1.80
	Precision (RSD, %)	1.86	1.14	1.11
3	Mean (μM)	0.149	1.55	3.46
	SD (μM)	0.0036	0.032	0.168
	Accuracy (RE, %)	−0.55	3.33	−1.07
	Precision (RSD, %)	2.40	2.06	4.86
Inter-	Mean (μM)	0.151	1.54	3.51
	SD (μM)	0.0031	0.028	0.105
	Accuracy (RE, %)	0.80	2.32	0.38
	Precision (RSD, %)	2.03	1.81	2.99

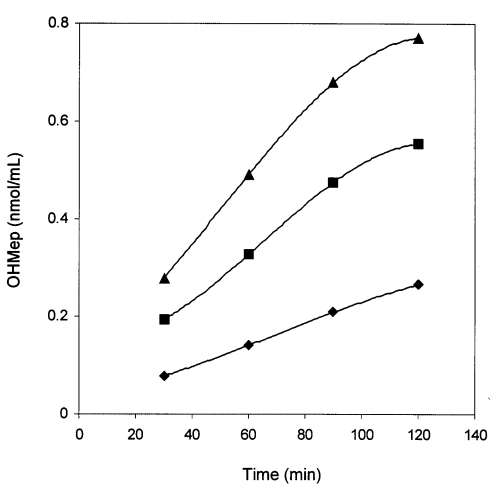

Fig. 4. OHMep formation vs. incubation time at microsomal protein concentrations of 0.5 (◆), 1.0 (■) and 1.5 (▲) mg/ml with 80 μM Mep. Each data point is a mean value of nine replicates performed on 3 days (three replicates/day).

Microsomal matrix influence was examined by quantifying six QC replicates prepared with human liver microsomes at 0.15, 1.5 and 3.5 μM OHMep using calibration curves prepared with rat liver microsomes. The intra-day accuracy ranged from −11.5 to −8.2% with precision ranging from 1.1 to 3.3%. The results indicate 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 1.0 mg/ml rat liver microsomal protein versus the analyte in water ($n = 3$). All the samples contained 0.2 μM analyte and were treated identically with 1 M formic acid. The overall recoveries of OHMep and chlorzoxazone were determined to be 40 and 42%, respectively [8].

3.5. Reaction linearity

Hydroxylation of Mep was investigated with respective to varying incubation time and microsomal protein concentration. The dependence of OHMep formation on incubation time and protein concentration is shown in Figs. 4 and 5, respectively. The formation of OHMep in human liver microsomal incubations was linear with protein concentration up to 1.5 mg/ml and with incubation time up to 90 min. Mep 4′-hydroxylase activities in the human liver microsomes ranged from 4.2 to 6.5 pmol/min per mg protein. Note that conversion of Mep to OHMep was less than 1% for all incubations performed in this study. A microsomal protein concentration of 1.0 mg/ml and an incubation time of 90 min were chosen as the experimental conditions for human CYP2C19 inhibition assessment.

3.6. Inhibition study

Mep 4′-hydroxylation activity was inhibited by tranylcypromine, a potent inhibitor of CYP2C19

[9]. Fig. 6 displays the inhibition curve of percent control activity versus logarithm of tranylcypromine concentration. The IC_{50} value for tranylcypromine inhibition of CYP2C19 was calculated to be 43.5 μM, which is in agreement with the literature value of ~30 μM [9]. The percent control activity was reduced to ~25% by tranylcypromine at 120 μM. This concentration of tranylcypromine was chosen as a positive control for human CYP2C19 inhibition used in the evaluation of the potential for drug-drug interactions.

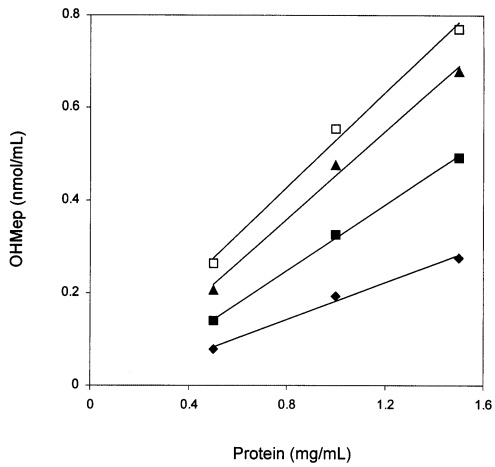

Fig. 5. OHMep formation vs. microsomal protein concentration at incubation times of 30 (◆), 60 (■), 90 (▲) and 120 (□) min with 80 μM Mep. Each data point is a mean value of nine replicates performed on 3 days (three replicates/day).

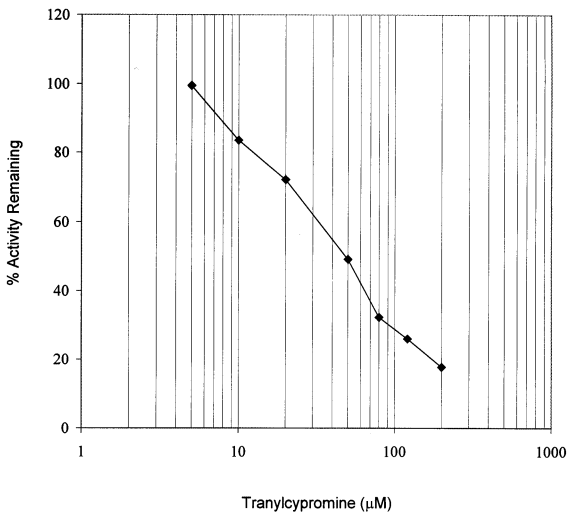

Fig. 6. Inhibition of Mep 4′-hydroxylation by tranylcypromine in incubations containing 1.0 mg/ml microsomal protein and 80 μM Mep with an incubation time of 90 min. Each data point denotes a mean value of six replicates performed on different days (three replicates/day).

4. Conclusion

The DI/GCE – MS – MS method proved to be rapid, accurate and precise in the quantification of OHMep from microsomal incubations. The overall advantage of the method is attributed to the use of the extremely short C_{18} guard cartridge. In addition, this method appears to be directly used 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 the CYP2C19 inhibition assessment using human liver microsomes and 96-well plates. The validation results indicate the CYP2C19 assay was fast and reliable. This assay has been routinely used in our laboratory for high-throughput CYP2C19 inhibition evaluation of drugs and new chemical entities.

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