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Simultaneous Determination of Metabolites from Multiple Cytochrome P450 Probe Substrates by Gradient Liquid Chromatography with UV Detection

A generic gradient LC/UV method has been developed for simultaneous and quantitative determination of cytochrome P450 (CYP) probe substrate metabolites (6-hydroxychlorzoxazone for CYP2E1, acetaminophen for CYP1A2, dextrorphan for CYP2D6, 4'-hydroxymephenytoin for CYP2C19, 4-hydroxytolbutamide for CYP2C9 and 6 -hydroxytestosterone for CYP3A4) from in vitro *microsomal incubations. The mobile phase consisted of 10 mM potassium phosphate buffer (pH 2.8) and acetonitrile. Separation of the analytes was achieved within 27 min on a 5 µm, 3.9 × 150 mm C8 column with a gradient elution. A wavelength of 230 nm was used for UV detection. The calibration curves showed good linearity with correlation coefficients greater than 0.999 for the analytes in the investigated concentration range. The lower limit of detection (LLOD) was 0.066 µM for acetaminophen, 0.049 µM for dextrorphan, 0.0054 µM for 6-hydroxychlorzoxazone, 0.021 µM for 4'-hydroxymephenytoin, 0.0087 µM for 4-hydroxytolbutamide and 0.0164 µM for 6 -hydroxytestosterone. The lower limit of quantitation (LLOQ) was 0.066 µM for acetaminophen, 0.049 µM for dextrorphan, 0.027 µM for 6-hydroxychlorzoxazone, 0.021 µM for 4'-hydroxymephenytoin, 0.035 µM for 4-hydroxytolbutamide and 0.033 µM for 6 -hydroxytestosterone, respectively. Michaelis-Menten kinetic parameters K_m and V_{max} for the probe substrates in rat liver microsomes were also obtained. This gradient LC/UV method can be used efficiently to improve throughput and cost effectiveness in preclinical drug metabolism studies.*

Introduction

The cytochrome P450 (CYP) enzyme family consists of multiple isoforms with different substrate specificities and catalyzes biotransformation of a vast number of drugs (1-4). Knowledge of the specific CYP isoform responsible for metabolism of a given drug is critical for predicting potential drug-drug interactions and genetically-based individual variation in drug metabolism (5). Suitable CYP isoform-selective substrates have been identified and are commonly used for probing the role of specific human CYP enzymes in drug metabolism (6,7). Examples of well-studied CYP probe substrates for *in vitro* drug metabolism studies are listed in ***T1***.

Evaluating CYP enzyme activities is traditionally performed for individual CYP isoforms. This approach is labor intensive, time consuming and not cost effective. Throughput can be increased by co-incubating a mixture of probe substrates with liver microsomes *in vitro*, and the activities of several CYP isoforms can be assessed simultaneously by monitoring metabolite formation (8-10). Success of this mixed-substrate incubation approach requires an analytical method that allows rapid quantitative determination of metabolites from multiple probe substrates, ideally in a single run.

Liquid chromatography (LC) with ultraviolet (UV) (7,11-13), fluorescence (14,15) or mass spectrometry (MS) (5,9,10,16-20) detection has been commonly used for quantitative determination of CYP probe substrates. Among different detection methods, only LC/MS has been used for simultaneous analysis of multiple CYP probe substrates (9,10,17-20). LC/MS has the advantages of high sensitivity, selectivity and speed. However, LC/MS instrumentation is costly and may not be available for routine analysis in every research laboratory. In addition, LC/MS-based assays often require use of different ionization and ion detection modes due to the diverse structure of CYP probe substrate, which creates difficulty and complexity in developing LC/MS methods for simultaneous analysis (18). It has been reported that two sample injections and two runs (one for positive ion and one for negative ion) are needed to analyze the common probe substrates for major drug-metabolizing human CYP enzymes (9,10,17). In our previous work, we developed a rapid and quantitative LC/MSMS method for determination of multiple probe metabolites in a single run in which aniline was used as a probe substrate for CYP2E1 isozyme (21).

Fluorescence and UV are conventional and inexpensive detectors for LC. Fluorescence detectors are very

sensitive but respond only to the few analytes that fluoresce. In contrast, many compounds can absorb ultraviolet light. Therefore, LC with UV detection can be used for simultaneous analysis of multiple CYP probe substrates and metabolites. The drawback of UV detection is its relatively low sensitivity and selectivity. However, our preliminary results show that the sensitivity of LC/UV is sufficient for detection of CYP probe substrate metabolites resulting from normal microsomal incubations. Prior to this work, no report has been published using LC/UV for simultaneous analysis of metabolites from multiple CYP probe substrates. In this work, a generic gradient LC/UV method has been developed for simultaneous and quantitative determination of metabolites from several common CYP probe substrates for major drug-metabolizing CYP isozymes (including CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4) in a single run. This method has been used to quantify the probe substrate metabolites from *in vitro* human liver microsomal incubation of a mixture of probe substrates. The enzyme kinetic parameters K_m and V_{max} were also obtained for each probe substrate.

Experimental

Chemicals and reagents

Chlorzoxazone (CZ), 6-hydroxychlorzoxazone (OH-CZ), phenacetin (PA), acetaminophen (APAP), tolbutamide (TB), dextromethorphan hydrobromide monohydrate (DM), dextrorphan (DX) D-tartrate salt, (±)-4-hydroxy-mephenytoin (OH-MP), testosterone (TS), 6 -hydroxytestosterone (OH-TS), bucetin, and -NADPH were purchased from Sigma (St. Louis, MO, USA). S-(+)-mephenytoin (MP) and 4-hydroxytolbutamide (OH-TB) were received from Gentest (Beford, MA, USA). Potassium phosphate dibasic was supplied by Mallinckrodt Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile was from Burdick & Jackson (Muskegon, MI, USA). Distilled, deionized water was generated from a NANOpure ultrapure water system (Barnstead/Thermolyne, Dubuque, IA, USA). Pooled human and rat liver microsomes containing 20 mg/mL protein were obtained from XenoTech (Kansas City, KS, USA).

Chromatography

The chromatographic system consisted of a BASi PM-80 gradient pump, UV detector, and an autosampler (20 µL loop). Data acquisition was controlled through epsilon™ interface by ChromGraph® version 2200 software (BASi, West Lafayette, IN, USA). Separation was carried out using a 5 µm, 3.9 × 150 mm Symmetry C8 column (Waters, Milford, MA, USA) at a flow rate of 0.7 mL/min. The UV detection wavelength was 230 nm. The mobile phase consisted of 10 mM potassium phosphate buffer at pH 2.8 (A) and acetonitrile (B). In gradient elution, the mobile phase was eluted with 5% acetonitrile for 2 min, followed by a linear gradient to 25% acetonitrile at 3 min, to 30% acetonitrile at 18 min, to 50% acetonitrile at 19 min, and then with an isocratic elution for 7 min, followed by a linear gradient to 5% acetonitrile at 27 min. The column was allowed to equilibrate for 10 min between runs.

Microsomal incubations

Stock solutions of individual probe substrate PA, DM, CZ, MP, TB and TS in acetonitrile (100 mM) were prepared. A primary stock solution containing 300/200/600/300/2000/600 µM PA/DM/CZ/MP/TB/TS was prepared by diluting an appropriate amount of the individual substrate stock. Secondary stock solution of the mixed probe substrates was prepared by a 10-fold dilution of the primary stock with 50 mM potassium phosphate buffer (pH 7.4). The primary and secondary stock solutions were used to prepare the working solutions of different substrate concentration for rat liver microsomal incubations, in which the organic percentage of the solution was controlled below 1%. The stock solutions were stored at -20°C. Incubations of mixed CYP probe substrates with human and rat liver microsomes were carried out at 37°C in a bench-top Lab-Line shaker (Barnstead/Thermolyne, Dubuque, IA, USA). The incubation samples contained 50 mM potassium phosphate buffer (pH 7.4), 0.2-2 mg/mL microsomal proteins, 10 mM $MgCl_2$, 1.2 mM NADPH and 6/4/12/6/40/12 µM to 120/80/240/120/800/240 µM PA/DM/CZ/MP/TB/TS in a final volume of 100 µL. The reaction was initiated by adding NADPH after a 5-min preincubation at 37°C. After a given incubation time (0-60 min), the reaction was terminated by adding 50 µL acetonitrile. The samples were then centrifuged (Allegra™ 6R centrifuge, Beckman Coulter, Palo Alto, CA, USA) at 5°C for 10 min at 3000 rpm (2060 g) to separate and remove protein. The supernatant was directly injected for LC/UV analysis.

The reaction linearity was examined by incubating a mixture of

Human CYP probe substrates, enzyme reactions and metabolites. **T1.**

Isoform	Probe Substrate	Reaction	Metabolite
CYP1A2	Phenacetin (PA)	O-deethylation	Acetaminophen (APAP)
CYP2D6	Dextromethorphan (DM)	O-demethylation	Dextrorphan (DX)
CYP 2E1	Chlorzoxazone (CZ)	6-hydroxylation	6-hydroxychlorzoxazone (OH-CZ)
CYP2C19	S-mephenytoin (MP)	4'-hydroxylation	4'-hydroxymephenytoin (OH-MP)
CYP2C9	Tolbutamide (TB)	4-hydroxylation	4-hydroxytolbutamide (OH-TB)
CYP3A4	Testosterone (TS)	6 -hydroxylation	6 -hydroxytestosterone (OH-TS)

30/20/60/30/200/60 µM PA/DM/CZ/MP/TB/TS with 1 mg/mL microsomal protein for different lengths of time (5, 10, 15, 20, 25, 30, 45, and 60 min). The effect of microsomal protein concentration on enzyme reaction was investigated by varying protein concentration in the range of 0.2 to 2 mg/mL with an incubation time of 20 min. To obtain Michaelis-Menten enzyme kinetic parameters for the probe substrate metabolites, incubations were carried out with 1 mg/mL microsomal protein at different probe substrate concentrations for 20 min. The concentration-velocity data were fitted to the Lineweaver-Burk equation to obtain K_m and V_{max} values.

Working solutions for calibrations and quality controls

A standard metabolite mixture of 100/300/25/250/100/100 µM APAP/DX/OH-CZ/OH-MP/OH-TB/OH-TS was prepared by appropriately diluting 1 mM individual standard metabolite with phosphate buffer. The standard metabolite mixture was used as a stock to prepare working solutions for calibration with APAP/DX/OH-CZ/OH-MP/OH-TB/OH-TS ranging from 0.066/0.098/0.054/0.043/0.035/0.033 µM to 6.6/9.8/5.4/4.3/3.5/3.3 µM.

Quality control (QC) samples for validating the method were prepared independently (i.e., separate weighing) with concentrations of APAP/DX/OH-CZ/OH-MP/OH-TB/OH-TS at 0.198/0.294/0.215/0.128/0.0699/0.0658, 0.992/1.47/1.07/0.640/0.350/0.329 and 4.96/7.36/5.40/3.20/1.75/1.65 µM, respectively. The working solutions (100 µl) for calibration and quality control contained the same components as for incubation (i.e., 25/20/30/30/200/60 µM PA/DM/CZ/ MP/TB/TS, 50 mM potassium phosphate buffer [pH 7.4], 1 mg/mL microsomal protein and 10 mM $MgCl_2$). The samples were incubated at 37°C for 10 min without NADPH, followed by addition of 50 µL acetonitrile and NADPH to make the same exact matrix as from real microsomal incubations. The samples were centrifuged at 3000 rpm (2060 g) for 10 min at 5°C and the supernatant (20 µL) was directly injected for LC/UV analysis.

Results and Discussion

Simultaneous determination of multiple CYP probe substrate metabolites in a single run by LC/UV

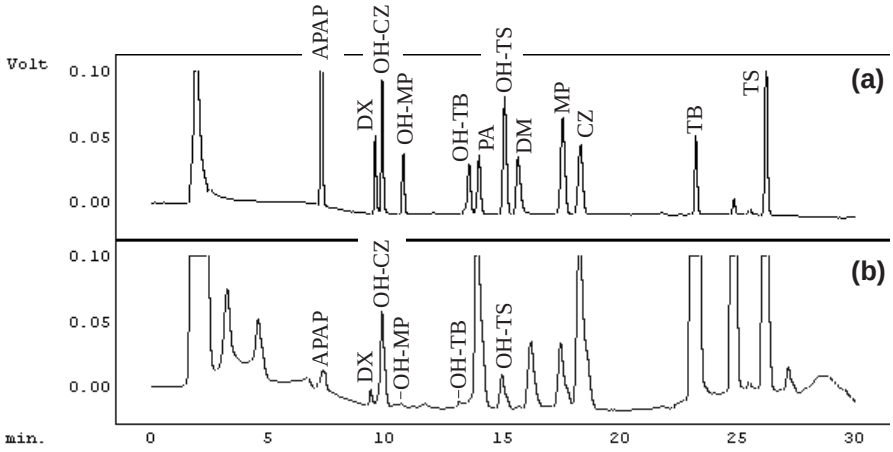
Prior to the LC/UV experiment, UV spectral scans were performed for all the probe substrates and metabolites used in this study. A survey of the UV spectra revealed that all analytes show UV absorbance at 230 nm. Therefore, UV detection was set at 230 nm in the LC/UV experiments. ***F1a*** shows a representative LC/UV chromatogram of a mixture of standard probe substrates and metabolites using gradient elution. The metabolites were generally eluted before the substrates, which is consistent with the more hydrophilic nature of the metabolites compared to the substrates. Separation of all compounds was achieved in 27 min, and all metabolites were eluted in 15 min. In subsequent analyses, only the metabolite peaks were quantified, since only metabolite formation is of interest. The data demonstrate that multiple probe substrates and metabolites can be monitored simultaneously in a single LC/UV run with single-wavelength detection.

F1b shows a LC/UV chromatogram of a sample from a mixed-substrate incubation with human liver microsomes. All the metabolites were detected at the expected retention time. The OH-MP and OH-TB peaks are small, but still visible. It was calculated that only ~0.25% TB and ~1.4% MP were converted into the monitored metabolites in a 30-min incubation, while the conversion efficiency was ~62% for PA, 47% for DM, 7.6% for CZ, 4.8% for TS. The difference in substrate conversion efficiency can be attributed to the difference in the relative amounts and activities of the respective CYP enzymes in human liver microsomes.

Calibration curves

The calibration curves show good linearity with correlation coefficients greater than 0.999 for all the metabolites. The lower limit of detection (LLOD) was ~66.0 nM for APAP, 49.0 nM for DX, 21.3 nM for OH-MP, 8.7 nM for OH-TB and 16.4 nM for OH-TS. The LLOD for OH-CZ was below 5.4 nM and was not pursued further because it was below the lowest concentration of the working solutions for calibrations. The lower limit of quantification (LLOQ) was ~66.0 nM for APAP, 49.0 nM for DX, 26.9 nM for OH-CZ, 21.3 nM for OH-MP, 35.0 nM for OH-TB and 32.9 nM for OH-TS, based on the lowest concentration on the calibration curves that gives accuracy and precision better than ±20%. The linear concentration range of APAP/DX/OH-CZ/OH-MP/OH-TB/OH-TS from 0.066/0.049/0.027/0.021/0.035/0.033 µM to 6.6/9.8/5.4/

F1.
LC/UV chromatogram of (a) a standard metabolite mixture; (b) a human liver microsomal incubate.



4.3/3.5/3.3 µM was shown to be sufficient for analysis of the metabolites in microsomal incubations.

Method precision and accuracy

Precision and accuracy of the LC/UV method were investigated using three QC samples at different standard metabolite concentrations. Six measurements were taken for each sample. Precision was calculated as the relative standard deviation (RSD), and accuracy as the relative error (RE). The results are summarized in ***T2***. The values of RE and RSD are typically better than 8% for the analytes under study, indicating good accuracy and precision of the LC/UV method.

Analyte recovery from microsomal matrix

Recovery of analytes from the microsomal matrix was obtained by calculating the ratio of peak areas of the standard metabolites at several different concentrations in the microsomal matrix, relative to peak areas in the potassium phosphate buffer. The microsomal matrix contained the same components (i.e., protein, NADPH, $MgCl_2$ and phosphate buffer, etc.) at the concentrations used for incubation. The average recovery was ~87.6% for APAP, 97.9% for DX, 81.3% for OH-CZ, 94.5% for OH-MP, 104.3% for OH-TB and 96.4% for OH-TS.

Reaction linearity

Linearity of enzyme reactions in *in vitro* rat liver microsomal incubations was assessed by monitoring the effect of incubation time (from 5 to 60 min) and protein concentration (from 0.2 to 2 mg/mL) on probe substrate metabolite formation. Formation of APAP, DX and OH-CZ showed good linearity up to 35 min, while formation of OH-TB and OH-MP was linear up to 60 min. Formation of OH-TS was linear up to 20 min. Enzyme reactions were linear with protein concentrations in the range of 0.2-1.2 mg/mL. For subsequent analysis, an incubation time of 20 min and 1.0 mg/mL protein concentration were used. Under these conditions, the reaction is linear with respect to both time and protein concentration.

T2.

Method accuracy and precision data (n = 6) for QC samples at different concentrations.

APAP			
Nominal conc. (µM)	0.198	0.992	4.96
Measured conc. (µM)	0.208	1.000	5.00
Accuracy (RE, %)	5.0	1.3	0.8
Precision (RSD, %)	4.5	1.5	1.0
DX			
Nominal conc. (µM)	0.294	1.47	7.36
Measured conc. (µM)	0.268	1.60	8.05
Accuracy (RE, %)	-8.8	8.7	9.3
Precision (RSD, %)	5.3	3.0	6.7
OH-CZ			
Nominal conc. (µM)	0.215	1.07	5.40
Measured conc. (µM)	0.205	0.97	5.16
Accuracy (RE, %)	-4.6	-9.3	-4.2
Precision (RSD, %)	6.5	5.5	2.6
OH-MP			
Nominal conc. (µM)	0.128	0.640	3.20
Measured conc. (µM)	0.120	0.674	3.41
Accuracy (RE, %)	-6.2	5.3	6.5
Precision (RSD, %)	4.5	5.8	4.0
OH-TB			
Nominal conc. (µM)	0.0699	0.350	1.75
Measured conc. (µM)	0.0730	0.363	1.67
Accuracy (RE, %)	4.4	3.7	-4.6
Precision (RSD, %)	7.2	5.5	3.7
OH-TS			
Nominal conc. (µM)	0.0658	0.329	1.65
Measured conc. (µM)	0.0612	0.313	1.59
Accuracy (RE, %)	-7.0	-4.9	3.0
Precision (RSD, %)	4.8	5.2	7.2

T3.

K_m and V_{max} values determined in rat liver microsomes.

CYP enzyme	Probe substrate	Km (µM)	V_{max} (nmol/min/mg protein)
1A2/2C6	Phenacetin (PA)	32.10	1.56
2D6	Tolbutamide (TB)	202.00	0.058
Multiple CYP	S-mephenytoin (MP)	22.90	0.039
2D2	Dextromethorphan (DM)	6.32	0.48
2E1	Chlorzoxazone (CZ)	60.10	1.99
3A1/2	Testosterone (TS)	71.90	0.00029

Determinating K_m and V_{max} for probe substrates in rat liver microsomes

New drugs are typically tested on animals such as rats before they are applied to humans. *In vitro* studies such as liver microsomal incubations are a valuable tool for the study of drug metabolism and drug-drug interactions. Although many probe substrates have been developed for human CYPs (6,7), very few have been designed to estimate enzyme activities in rat liver microsomes. As a result, the same human CYP-based probe substrates are also used for *in vitro* studies with rat liver microsomes (22). However, little enzyme kinetic data are available in the literature for reactions of the probe substrates in rat liver microsomes. In this work, the Michaelis-Menten kinetic parameters K_m and V_{max} for reactions of the probe substrates in rat liver microsomes were determined by using the LC/UV method in conjunction with a mixed-substrate incubation approach.

The K_m and V_{max} values were determined by fitting the substrate concentration ([S]) and velocity (V) data into Lineweaver-Burke equation ($1/V = K_m/V_{max} \cdot 1/[S] + 1/V_{max}$). The results are summarized in ***T3***. K_m reflects the substrate affinity of the enzyme and V_{max} reflects the intrahepatic concentration of the enzyme. The K_m and V_{max} values may vary significantly among different species, or different sources for the same species, and the K_m data are relatively more comparable than the V_{max} data (7). It should be noted that the probe substrate specificity can be different for rat CYP isoforms (23). Formation of OH-CZ is catalyzed by CYP2E1 enzyme in both rat and human liver microsomes, and therefore the measured K_m for CZ in rat liver microsomes is in agreement with the reported K_m (59.2 µM) in human liver microsomes. For other probe substrates such as PA, TB, MP and TS, the measured K_m values are also close to values reported from human liver microsomes (7). However, it has been reported that the CYP enzymes responsible for PA, TB, MP and TS reactions are different in rats and humans (23), as listed in ***T1*** and ***T3***. Therefore, the measured K_m and V_{max} values for these probe substrates may reflect different CYP enzyme activities in rats and should not be compared to kinetic data from human liver microsomes.

Conclusions

A LC/UV method utilizing gradient elution and single-wavelength detection has been developed for simultaneous and quantitative determination of metabolites from multiple probe substrates in *in vitro* microsomal incubations. This method has been validated, and has been used to obtain the enzyme kinetic parameters of the probe substrates in rat liver microsomes using a mixed-substrate incubation approach. By monitoring the metabolites from probe substrates for major drug-metabolizing CYP enzymes in a single run, we demonstrate that LC/UV can be used as an effective and inexpensive analytical tool with increased throughput in preclinical drug metabolism studies.

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