A High-Throughput Assay Using Liquid Chromatography–Tandem Mass Spectrometry for Simultaneous In Vivo Phenotyping of 5 Major Cytochrome P450 Enzymes in Patients

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Abstract: The phenotyping cocktail is a practical approach for phenotyping of cytochrome P450 (CYP) enzymes in vivo. In this study, a liquid chromatography–tandem mass spectrometry method using a dual-extraction approach was developed and validated to quantify 5 selective substrates and their metabolites for the simultaneous phenotyping CYPs 1A2, 2C19, 2C9, 2D6, and 3A4 in patient blood samples. The assay was applied in a pilot study of 11 patients with schizophrenia. Five blood samples were collected before and at 1, 2, 4, and 6 hours after administration of a phenotyping cocktail consisting of 100 mg caffeine, 20 mg omeprazole, 25 mg losartan, 30 mg dextromethorphan, and 2 mg midazolam. The method successfully quantitated the CYP enzyme activities without serious side effects in patients. The ratios of metabolite to parent area under the concentration–time curve values were calculated over the 6-hour postdosage to reflect CYP2D6, CYP3A4, and CYP2C9 activities. The ratios of metabolite to parent plasma concentrations were calculated at 4-hour postdosage for CYP1A2 and at 4- or 6-hour postdose for CYP2C19, respectively. The plasma concentration of midazolam at 4 hours was also estimated as another phenotyping index for CYP3A4 activity. The simultaneous assay of all these analytes in a single matrix (plasma) will increase the feasibility of CYP phenotyping in patients.

Key Words: phenotyping cocktail, cytochrome P450 activity, LC/MS/MS, mass spectrometry

(*Ther Drug Monit* 2009;31:239–246)

INTRODUCTION

The cytochrome P450 (CYP) enzymes are responsible for oxidative metabolism of a large number of endogenous substrates and xenobiotics.[1] The in vivo phenotyping of CYP activity in an individual can provide insights into the impact of genetics, disease, concomitant therapy, and environmental factors on drug metabolism.[2] This requires the use of "selective" substrates for specific CYPs and an appropriate metric or phenotyping index.[3] The mixed phenotyping or drug "cocktail" is a practical approach for in vivo phenotyping.[3] Several phenotyping cocktails have been developed that have utilized different combinations of substrates for specific CYPs (Table 1). Some methods are becoming more widely used, especially for the evaluation of pharmacokinetic drug–drug interactions, although the complexity of the analytical approach remains a significant impediment.[4–6] The development of efficient and more convenient analytical methods could increase the application of in vivo CYP phenotyping, which has the potential to assist in individualizing drug therapy.

The ideal phenotyping cocktail should include substrates that are CYP selective, that do not interact, that are well tolerated when used at low doses, and that are easy to administer (eg, orally). The phenotyping index (as influenced by the sample collection protocol) should be practical, convenient, and precise.[13] Although many of the existing cocktails meet most of these requirements, the analytical aspects are generally inconvenient and involve the separate quantification of drug/metabolite pairs and often require the validation and use of multiple analytical methodologies. This has contributed to the slow uptake of in vivo CYP phenotyping into the clinical setting. The concept that multiple CYP substrates and their metabolites may be analyzed simultaneously has been suggested,[9,12] but this has not yet been achieved in patients.

The aim of the present study was to develop and validate a rapid and convenient analytical method for simultaneous quantification of caffeine, omeprazole, losartan, midazolam, dextromethorphan, and their corresponding metabolites in patient plasma samples. A further aim was to establish the utility of the cocktail and the analytical method in a pilot clinical study in patients.

MATERIALS AND METHODS

Chemicals and Reagents

All calibrators used in this study were of analytical reagent grade with a purity of at least 98%. Caffeine,

Received for publication June 26, 2008; accepted December 8, 2008.
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Supported in part by grants from the Australian National Health and Medical Research Council, The Rebecca Cooper Foundation, and Mayne Pharmaceuticals. S.G. was supported by an International Postgraduate Research Scholarship from The University of Sydney.
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TABLE 1. Summary of Selected Phenotyping Cocktails Reported in the Literature

Substrate (Pathways)	Matrix	Dose	Analytical Method
Inje cocktail[7]			
Caffeine (CYP1A2), omeprazole (CYP2C19), dextromethorphan (CYP2D6), losartan (CYP2C9), and midazolam (CYP3A4)	Plasma and urine	Caffeine 93 mg, omeprazole 20 mg, dextromethorphan 30 mg, losartan 30 mg, and midazolam 2 mg	HPLC and LC/MC
Pittsburgh[8]			
Caffeine (CYP1A2), mephenytoin (CYP2C19), debrisoquine (CYP2D6), chloroxazone (CYP2E1), dapsone (NAT2), and flurbiprofen (CYP2C9)	Plasma and urine	Caffeine 100 mg, mephenytoin 100 mg, debrisoquine 10 mg, chloroxazone 250 mg, dapsone 100 mg, and flurbiprofen 50 mg	HPLC
Yin et al[9] cocktail			
Caffeine (CYP1A2), tolbutamide (CYP2C9), omeprazole (CYP2C19), debrisoquine (CYP2D6), and midazolam (CYP3A)	Plasma and urine	Caffeine 100 mg, tolbutamide 500 mg, omeprazole 40 mg, debrisoquine 10 mg, and midazolam 3.75 mg	LC/MS
Cooperstown 5 + 1[10]			
Caffeine (CYP1A2, NAT2, XO), dextromethorphan (CYP2D6), omeprazole (CYP2C19), midazolam (CYP3A4), and S-warfarin (plus vitamin K)	Plasma and urine	Caffeine 2 mg/kg, dextromethorphan 30 mg, omeprazole 40 mg, midazolam 0.025 mg/kg, and S-warfarin 10 mg	HPLC
Karolinska[11]			
Caffeine (CYP1A2), losartan (CYP2C9), omeprazole (CYP2C19), debrisoquine (CYP2D6), and quinine (CYP3A4)	Plasma and urine	Caffeine 100 mg, losartan 25 mg, omeprazole 20 mg, debrisoquine 10 mg, and quinine 250 mg	HPLC
GW cocktail[12]			
Caffeine (CYP1A2), mephenytoin (CYP2C19), diclofenac (CYP2C9), debrisoquine (CYP2D6), chlorozoxazone (CYP2E1), and midazolam (CYP3A4)	Plasma and urine	Caffeine 100 mg, mephenytoin 25 mg, diclofenac 10 mg, debrisoquine 250 mg, and midazolam 5 mg	Fast gradient LC/MS

HPLC, high-performance liquid chromatography, LC/MC, liquid chromatography-mass spectrometry.

paraxanthine, dextromethorphan, dextrorphan, and phenacetin were purchased from Sigma-Aldrich (Sydney, Australia). Midazolam was kindly donated by Dr John Vine from Racing Analytical Services (Flemington, Victoria, Australia). Losartan was donated by Merck Sharp & Dohme (Guildford, New South Wales, Australia). Omeprazole was purchased from Trapeze Associates Pty (Rhenochem AG, Basel, Switzerland), and 5-hydroxyomeprazole was obtained from Ramidus AB (Lund, Sweden). 1′-hydroxymidazolam was purchased from Toronto Research Chemicals (Pickering, Ontario), and EXP-3174 (losartan carboxy acid) was obtained from SynFine Research, Inc (Richmond Hills, Ontario). High-performance liquid chromatography grade acetonitrile and methanol were purchased from LabScan (Lomb Scientific, Taren Point, New South Wales, Australia). Formic acid was purchased from Fluka Chemicals (Castle Hill, New South Wales, Australia). Analytical reagent–grade chloroform and propan-2-ol were from Univar (Crown Scientific, Minto, New South Wales, Australia). Drug-free human plasma was purchased from the Australian Red Cross Blood Service (Melbourne, Australia). Oasis HLB cartridges were supplied by Waters Corporation, Milford.

Solid-Phase Extraction for Dextromethorphan, Dextrorphan, Midazolam, 1′-Hydroxymidazolam, Omeprazole, 5-Hydroxyomeprazole, Losartan, and EXP-3174

All glasswares used for the preparation of calibrations and analysis of drugs and metabolites in plasma were silanized before use. The internal standard (IS) phenacetin (1 μg) was added to each plasma sample (1 mL). Acetonitrile (3 mL) was added to each sample followed by vigorous shaking and centrifugation for 15 minutes at 4000*g*. After drying under nitrogen at 40°C, water (2 mL) was added to the samples and 1.5-mL aliquots of the aqueous phase were loaded onto Oasis HLB solid-phase extraction (SPE) cartridges (3 mL, 60 mg). The following sequence was used for cartridge conditioning: methanol (1 mL) followed by water (1 mL), sample loading (1.5 mL at 0.5 mL/min), 2 washes with double-distilled water (2 mL) followed by 2 washes with 10% methanol in water (2 mL) at 0.5 mL/min, and sample elution with 3 washes of methanol at 0.5 mL/min. The methanol was evaporated to dryness under nitrogen at 40°C. SPE was performed with a Gilson ASPEC XL4 instrument (Villiers-le-Bel, France) using 735 Sampler software package Version 6.0.

Liquid–Liquid Extraction for Caffeine and Paraxanthine

SPE cartridges proved unsuitable for analysis of paraxanthine, and a separate liquid–liquid extraction procedure for both caffeine and paraxanthine was established. Plasma samples (200 μL) that had been spiked with IS (250 ng) were mixed with acetonitrile (600 μL) and vortexed vigorously to precipitate plasma proteins. After centrifugation at 4000*g*, the acetonitrile layer was removed and evaporated under a stream of nitrogen at 40°C. Double-distilled water (200 μL) was then added, followed by chloroform: isopropanol (85:15; 3 mL). The mixture was shaken for 15 minutes and centrifuged for 15 minutes at 4000*g*. The organic

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phase was removed and evaporated to dryness under nitrogen at 40°C.

Extracts from both SPE (100 μL) and liquid–liquid (100 μL) extraction methods were reconstituted in a mixture of water and acetonitrile (50:50) from which 20 μL was injected separately into liquid chromatography–tandem mass spectrometry.

Instrumentation

Liquid chromatography with electrospray ionization–tandem mass spectrometry was used to analyze the drugs and metabolites of interest. Separation was achieved on an Agilent HP 1090 liquid chromatograph (Agilent Technologies, Sydney, Australia) using an Altima C_{18} 5 μm (150 × 2.1 mm inner diameter) narrow-bore column (Alltech Associates, Castle Hill, Australia). The mobile phase consisted of acetonitrile: MilliQ water (1:1), containing 0.1% formic acid that was pumped at a flow rate of 300 μL/min. This facilitated separation of the drugs and metabolites over a 7-minute run time. Analytes were detected on a Thermo Finnigan TSQ 7000 liquid chromatography–mass spectrometry (LC/MS) system (San Jose, CA) using electrospray ionization (ESI) operated in positive ionization mode, with argon as the collisional gas at 2.2 mTorr. The temperature of the heated capillary was set to 275°C. Selected reaction monitoring was used for quantification using the product ion with the highest sensitivity. The software, Xcalibur Revision 1.2 Core Data System, was used for the analysis of the data.

Calibration and Method Validation

The stock solutions of all probes and their metabolites and phenacetin (IS) were prepared at 1 mg/mL in methanol and stored at −20°C. Calibrators consisted of 6 concentrations for caffeine and paraxanthine and 9 for other compounds and were prepared by spiking drug-free plasma with the working solutions. Quality control (QC) samples were prepared at 3 different concentrations designated within the low-, medium-, and high-concentration ranges. The internal QC samples were prepared from a different stock solution from which the calibrators were prepared. The method validation fulfilled the Food and Drug Administration Guidelines for Bioanalytical Methods (May 2001) and is detailed below.

Linearity and Sensitivity

Calibration curves for compounds in plasma were generated in replicate analyses on at least 3 different days. Least squares regression confirmed linear relationships between the peak area ratios of each analyte to the IS with the concentration of standards. To assess assay precision, deviations of the mean calculated concentrations over 3 runs were set at ±15% of nominal concentrations, except for the lower limit of quantification (LLOQ) where a deviation of ±20% was permitted. The LLOQ was determined based on the criterion that the analyte response at LLOQ was 5 times the baseline noise and could be determined with precision of ≤20% and accuracy of 80%–120%.

Recovery

The extraction recoveries of analytes from plasma samples were compared with postextracted spiked samples. For each analyte, the recovery was evaluated using 3 QC concentrations (low, medium, and high) and triplicate determinations at each concentration.

Precision and Accuracy

The intraday precision and accuracy were determined by analysis of 5 replicates of QC samples. Samples were analyzed in triplicate on 3 different days to assess the interday precision and accuracy of the assay. Precision was calculated as the coefficient of variation (CV) of the replicate analysis, whereas accuracy is the percent difference between nominal and observed values.

Specificity

Six different samples of drug-free plasma were analyzed to investigate the potential interference in the chromatograms for each analyte. Cocktail drug-free plasma samples from the 11 patients with schizophrenia in the clinical study were also evaluated for potential interfering chromatographic peaks that may be related to concomitant drug therapy.

Potential Interference From Plasma Matrix Components

Plasma was tested for the capacity to interfere with the analysis of drugs and metabolites. Six different postextraction plasma samples were spiked with QC concentrations of calibrators after extraction. "Neat" samples were prepared by direct addition of the same concentrations of calibrators to mobile phase. To assess the absolute matrix effect, the following equation was used:

$$\text{Matrix Effect (\%)} = (\text{Response Postextracted Spiked Sample} / \text{Response Nonextracted Neat Samples} - 1) \times 100$$

A negative value is suggestive of ion suppression, whereas a positive value is consistent with ion enhancement. These analyses were performed at low- and high-QC concentrations. The CV of the ratios of the peak areas of each analyte to the IS was used to assess interlot matrix variability.

Stability of Analytes

Three freeze/thaw cycles were applied to plasma samples that had been spiked with high and low concentrations of analytes to assess stability. The analyte was considered stable when the percent difference between nominal and observed concentrations was within 15%.

Clinical Study

The assay was applied to samples collected in a pilot study of people with schizophrenia who were receiving clozapine. This study was approved by the Northern Sydney Central Coast Human Research Ethics Committee. Participants were selected on the basis of having no history of chronic diseases such as diabetes, asthma, and cancer, and women could not be pregnant at the time of study. After informed consent was obtained, subjects abstained from caffeine-containing products for 48 hours before and during

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the period of the study. Subjects were provided with caffeine-free beverages during this period.

Each study participant received the combination of 100 mg caffeine, 20 mg omeprazole, 25 mg losartan, 30 mg dextromethorphan (as an oral liquid), and an oral dose of 2 mg midazolam (in 5% dextrose solution). Venous blood samples (10 mL) were collected before administration of the cocktail and at 1-, 2-, 4-, and 6-hour postadministration. Blood samples were immediately centrifuged (4000*g* for 15 minutes), and plasma aliquots were stored at −80°C until analyzed. The maximum period between sample collection and analysis was 3 months.

Data Analysis

Concentration–time data for each analyte (parent and metabolite) were used to calculate the area under the concentration–time curve (AUC) up to the last sample using the linear trapezoidal rule. The ratios of metabolite to parent AUC values were calculated over the 6-hour postdosage to reflect CYP2D6, CYP3A4, and CYP2C9 activities. The ratios of metabolite to parent plasma concentrations were calculated at 4-hour postdosage for CYP1A2 and at 4- or 6-hour postdose for CYP2C19, respectively. The plasma concentration of midazolam at 4 hours was also estimated as another phenotyping index for CYP3A4 activity.

RESULTS

Mass Spectrometry and Chromatography

The product ions providing the highest analytical sensitivity are summarized in Table 2 and were selected for future study. All analytes were separated optimally within a 7-minute run time (Fig. 1). Analyte peaks were free of interference. It was also confirmed that drug-free plasma samples from patients and blank plasma provided by 6 different sources were free from interfering ions that corresponded to the analytes (data not shown). The matrix effects for most of the compounds were negative, except for losartan that had a positive matrix effect. The highest signal suppression was related to low concentration of 5-hydroxyomeprazole (−29.1%); however, the lot-to-lot variation (CV%) of the mean peak areas of 3 days was less than 15% for all analytes.

TABLE 2. Retention Times and Fragmentation Parameters for the Analytes and IS

Analyte	Retention Time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (eV)
Paraxanthine	1.28	181	123.9	20
Dextrorphan	1.29	258	157.0	35
5-hydroxyomeprazole	1.30	362	214.0	15
Caffeine	1.44	195	138.0	27
Omeprazole	1.46	346	198.2	13
1′-hydroxymidazolam	1.59	342	324.0	25
Midazolam	1.59	326	291.0	30
Dextromethorphan	1.60	272	171.0	35
Phenacetin (IS)	2.39	180	109.9	22
Losartan	2.95	423	207.0	17
EXP-3174	4.14	437	207.0	20

Assay Performance

The concentration ranges of each analyte were linearly related to peak area ratio (r^2 = 0.9873–0.9988 in all cases; Table 3). LLOQ values for all compounds are also shown in Table 3. The extraction methods used in this study yielded mean recoveries in the range of 83.5%–105.4%; recovery of the CYP2C9-derived losartan metabolite, EXP-3174, and the CYP1A2-derived paraxanthine was slightly lower at 76% and 73%, respectively. The recoveries were consistent over the indicated calibration ranges (Table 3). Precision and accuracy data for each of the probe drugs and metabolites are summarized in Table 4. The accuracies, calculated as percent deviations from the nominal concentrations, were within the guideline limits (Table 4). Precision (presented as CV%) ranged from 10.8% to 20.0% and 6.5% to 20.0% for interday and intraday measurements, respectively. All analytes were found to be stable in plasma after 3 freeze/thaw cycles with mean deviations of less than 15%.

Pilot Clinical Study

Eleven people with schizophrenia who were receiving clozapine provided informed consent, participated, and successfully completed the pilot clinical study (Table 5). None of the subjects experienced serious side effects after administration of the drug cocktail. Subject 3 experienced transient sedation but recovered by the time that the second blood sample was taken. The blood pressure, pulse, blood glucose concentration, and body temperature were monitored and remained within normal range throughout the course of the study.

Figure 2 shows the plasma concentration–time profiles for the different CYP substrates and their metabolites that were measured in 5 of the participants in the pilot clinical study. The phenotyping index for each of the different CYP pathways for each participant is presented in Table 5. The plasma concentrations of midazolam at 4-hour postdose were well correlated with the ratio of 1′-hydroxymidazolam to midazolam $AUC_{0\text{-}6h}$ (Spearman $r = -0.87$, $P < 0.01$, GraphPad Prism Version 4.00 for Windows, GraphPad Software, Inc., San Diego, CA).

DISCUSSION

This study has developed, validated, and applied an analytical method, which can be used for the rapid determination of substrates and metabolites for measuring the in vivo activity of CYP enzymes in patients. A phenotyping cocktail of selective CYP substrates that was reported previously[7] has been enhanced with a convenient liquid chromatography–tandem mass spectrometry assay to rapidly quantitate the substrates for and metabolites generated from 5 important drug-metabolizing CYPs. The principal CYPs that are involved in drug metabolism are CYP1A2, CYP2C19, CYP2C9, CYP3A, and CYP2D6.[14] CYP2E1 has a relatively minor role in drug metabolism and was not selected for inclusion in this drug phenotyping cocktail. Moreover, the most widely accepted selective substrate for CYP2E1 (chlorzoxazone) has been shown to interact with CYP3A4 substrates, such as midazolam,[8] and with the CYP1A2

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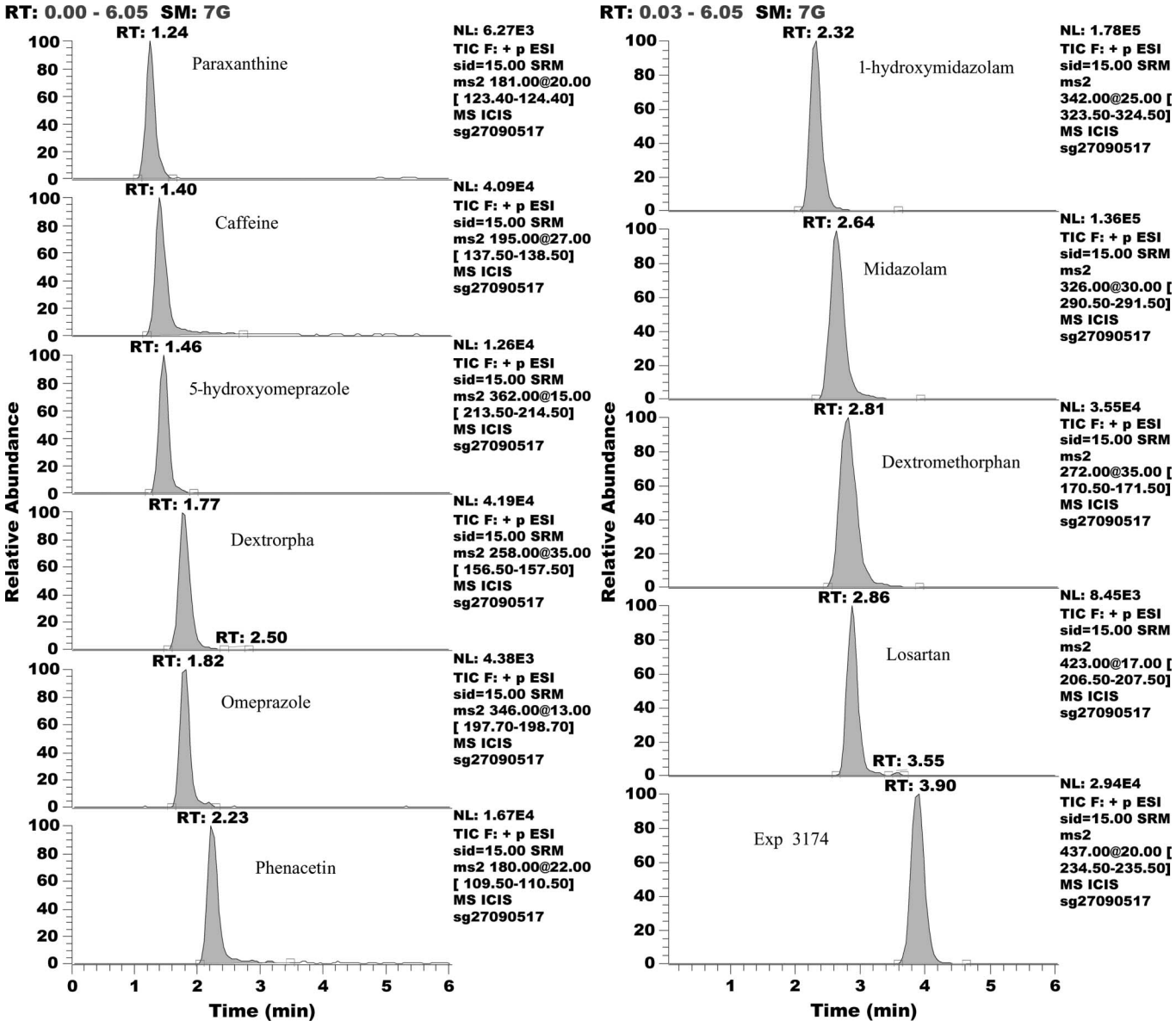

FIGURE 1. Liquid chromatography–tandem mass spectrometry separation of multiple analytes in a mixture of analytical standards including: caffeine and paraxanthine: 1500 ng/mL, dextromethorphan and dextrorphan: 100 ng/mL, omeprazole and 5-hydroxyomeprazole: 250 ng/mL, losartan and EXP-3174: 125 ng/mL, and midazolam and metabolites: 25 ng/mL.

substrate, caffeine.[15] Furthermore, ethical concerns remain over the use of chlorzoxazone because it is not an approved agent for use in humans.

The "Inje cocktail" (Table 1) emerges as an optimal combination of widely available and well-tolerated substrates that interact minimally and may be administered orally. The major limitation of phenotyping methods is the requirement for individual analysis of each drug substrate and its metabolite. The present approach differs from that used in the Inje cocktail approach, only in the doses of caffeine (100 mg compared with 93 mg) and losartan (25 mg instead of 30 mg), and utilizes blood (for plasma) rather than urine sampling. Thus, the present study utilizes a combination of substrates that do not interact. Ryu et al[7] have shown that the plasma concentrations of midazolam at 3, 4, 5, and 6 hours after administration correlate with the apparent clearance of the drug ($r > 0.9$). Thus, it was suggested that the 4-hour plasma concentration is an appropriate phenotyping index for CYP3A4 activity. In the present study, the plasma concentrations of midazolam at 4 hours were used to present the activity of CYP3A4 (Table 5). The injectable dosage form of the drug was prepared for oral administration in dextrose 5% solution as described by Wong et al.[16] In the Inje cocktail, it was found that the ratios of plasma concentrations of omeprazole to 5-hydroxyomeprazole at 3, 4, 5, and 6 hours were correlated with the ratios of the AUCs of the drug to

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TABLE 3. Calibration Range, Recovery, and LLOQ for Each Analyte

Analyte	Calibration Range (ng/mL)	r^2 (Mean ± SD)	Recovery (n = 9)	LLOQ (ng/mL)
Caffeine	190–6000	0.9950 ± 0.0022	102.4 ± 7.5	95
Paraxanthine	190–6000	0.9958 ± 0.0050	73.0 ± 5.1	95
Dextromethorphan	3–400	0.9980 ± 0.0023	90.8 ± 9.0	3
Dextrorphan	1.5–400	0.9959 ± 0.0028	87.4 ± 14	1.5
Omeprazole	7.8–1000	0.9910 ± 0.0044	98.7 ± 3.3	7.8
5-hydroxyomeprazole	7.8–1000	0.9873 ± 0.0093	105.4 ± 10.9	7.8
Midazolam	0.78–100	0.9977 ± 0.0016	94.3 ± 10.8	0.39
1-hydroxymidazolam	0.78–100	0.9985 ± 0.0026	100.5 ± 6.7	0.39
Losartan	4–500	0.9988 ± 0.0008	83.5 ± 6.7	4
EXP-3174	4–500	0.9983 ± 0.0006	76.0 ± 5.8	4

metabolite ($r > 0.9$). Omeprazole is an enteric-coated tablet, and its absorption has a long lag time.[7] Food consumption can also impact the absorption of omeprazole from the gastrointestinal tract, and therefore, the subjects should be fasting during phenotyping for CYP2C19.[2] In the present study, quantification of omeprazole and 5-hydroxyomeprazole was done using 4-hour plasma samples. However, in some patients, neither omeprazole nor its metabolite was quantifiable at 4 hours, which might be related to the consumption of foods during the study and the variable absorption of the drug from the gastrointestinal tract. In these patients, 6 hours was used as the alternative sampling time.

The consumption of same drugs as the phenotyping cocktail might cause an increase in the baseline concentration of the substrates and their metabolites. As it can be seen in Figure 2, patient 10 has consumed medications containing dextromethorphan before the study and traces of dextromethorphan and dextrorphan have been detected in time zero of blood sampling.

A summary of previous cocktails is presented in Table 1. The cocktails reported by Yin et al[9] and Christensen et al[11] also monitor the same CYPs as the Inje cocktail: with caffeine, dextromethorphan, and omeprazole also used as substrates for CYP1A2, CYP2D6, and CYP2C19, respectively, although there were some differences in the doses employed. Caffeine is a widely used substrate for CYP1A2 phenotyping, and plasma is the optimal matrix for analysis of caffeine because multiple metabolites are excreted in urine, and several factors may influence the renal excretion of the metabolites.[2] Omeprazole and dextromethorphan are 2 of the most widely used substrates for CYP2C19 and CYP2D6, respectively, in reported phenotyping cocktails (Table 1). Omeprazole and dextromethorphan are well tolerated and readily available. Although omeprazole may induce or inhibit CYP1A2 in certain clinical situation,[13] this is unlikely to be important after a single low dose of the drug.

With the exception of the Karolinska cocktail, which contains quinine, all other cocktails used midazolam for measuring CYP3A4 activity. The 3-hydroxy metabolite of quinine is not readily available, which detracts from the wider use of that cocktail. The route of administration, dose, and the phenotyping index of midazolam may vary between the cocktails. Oral administration of midazolam is preferable to intravenous administration because, although the drug may undergo oxidation by both hepatic and intestinal CYP3A4,[13] patient adherence and acceptability are greater. The most suitable phenotyping index for midazolam remains unresolved. The apparent clearance of midazolam is possibly the most accurate metric, but typically, this requires 8–10 blood samples to be collected.[17] This relatively large number of blood samples is tedious and also requires sensitive analytical methods to detect low midazolam concentrations in the early and late periods after dosage. Instead, a limited sampling strategy with fewer blood samples[18] and a suitable pharmacokinetic model may be used to estimate midazolam clearance. Lin et al[19] have suggested that the ratio of 1'-hydroxymidazolam to midazolam plasma concentrations at 4 hours can predict CYP3A4 activity. In the present study, the ratios of the AUC of 1'-hydroxymidazolam to midazolam up to 6 hours after administration were also estimated.

TABLE 4. The Interday and Intraday Precision and Accuracy of 5 CYP Probe Drugs and Their Metabolites

Analyte	Nominal Concentration (ng/mL)	Accuracy (% of Deviation), n = 5	Intraday Precision (CV%), n = 5	Interday Precision (CV%), n = 3*
Caffeine	187.5	−16.7	9.8	16.4
	1500	−11.3	11.9	15.0
	6000	−2.0	11.6	14.8
Paraxanthine	187.5	−17.0	13.0	14.3
	1500	−5.5	12.0	15.5
	6000	1.0	10.7	15.3
Dextromethorphan	3.1	15.9	17.5	20.0
	100	−8.7	10.9	14.2
	400	1.3	7.9	12.0
Dextrorphan	1.56	−15.0	14.2	20.0
	100	−4.0	13.2	13.9
	400	1.0	12.0	13.0
Omeprazole	7.8	14.4	18.7	17.6
	250	7.6	7.7	16.2
	1000	0.4	12.1	15.8
5-hydroxyomeprazole	7.8	5.0	16.5	17.9
	250	16.0	14.5	15.0
	1000	2.7	14.8	15.0
Midazolam	0.78	20.0	9.2	18.8
	25	8.9	9.7	15.0
	100	−1.2	7.7	10.8
1′-hydroxymidazolam	0.78	20.0	12.5	18.3
	25	11.7	9.4	12.8
	100	−0.6	6.5	15.0
Losartan	3.9	8.2	19.3	20.0
	125	3.3	11.1	15.5
	500	−0.8	10.0	15.6
EXP-3174	3.9	1.5	20.0	19.9
	125	−0.7	12.0	14.5
	500	−3.0	14.6	14.9

*The interday precision has been calculated in 3 different days (n = 3).

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TABLE 5. CYP Phenotypic Indices and Demographic Information of the Study Subjects

Patient	Age (yrs)	Sex	CYP1A2*	CYP2C19†	CYP3A4‡	CYP3A4§	CYP2D6¶	CYP2C9‖
1	37	M	0.15	1.26	12.58	0.30	0.04	1.69
2	25	M	0.23	0.56	10.00	0.33	0.79	1.13
3	53	M	0.38	0.11	2.15	1.66	1.43	0.59
4	62	M	0.38	0.14	13.21	0.17	0.73	0.36
5	66	F	0.31	0.51	19.00	0.26	0.16	0.98
6	55	M	0.20	2.69	7.26	0.57	0.37	0.56
7	30	F	0.08	0.38	3.21	1.13	6.97	0.27
8	36	M	0.32	3.66	4.34	0.30	0.01	0.98
9	36	M	0.47	2.31	21.31	0.11	0.31	2.53
10	22	M	1.11	3.02	12.85	0.13	0.94	2.00
11	30	M	0.75	1.04	13.03	0.03	0.10	2.57
Median	36	—	0.32	1.15	10.00	0.30	0.37	0.98
Range	22–66	—	0.08–1.11	0.11–3.66	2.15–21.31	0.11–1.66	0.01–6.97	0.27–2.57

F, female; M, male.
*The ratio of paraxanthine to caffeine plasma concentrations (μmol/L) at 4-hour postdose.
†The ratio of 5-hydroxyomeprazole to omeprazole plasma concentrations (nmol/L) at 4- or 6-hour postdoses.
‡The plasma concentrations of midazolam (nmol/L) at 4 hours.
§The ratio of AUC $_{0-6h}$ of 1′-hydroxymidazolam to midazolam.
¶The ratio of AUC $_{0-6h}$ of dextrorphan to dextromethorphan.
‖The ratio of AUC $_{0-6h}$ of EXP-3174 to losartan.

Mephenytoin is another CYP2C19 probe drug that has been used in the Pittsburgh[8] and GW[12] cocktails but has limited availability in many countries and requires urine sampling for an extended period (more than 8 hours).[13] Debrisoquine is employed in the Pittsburgh,[8] Yin et al,[9] and GW[12] cocktails as a probe for CYP2D6 but is inferior to dextromethorphan in terms of its availability and tolerability.[13] CYP2C9 probes, other than losartan, include flurbiprofen,

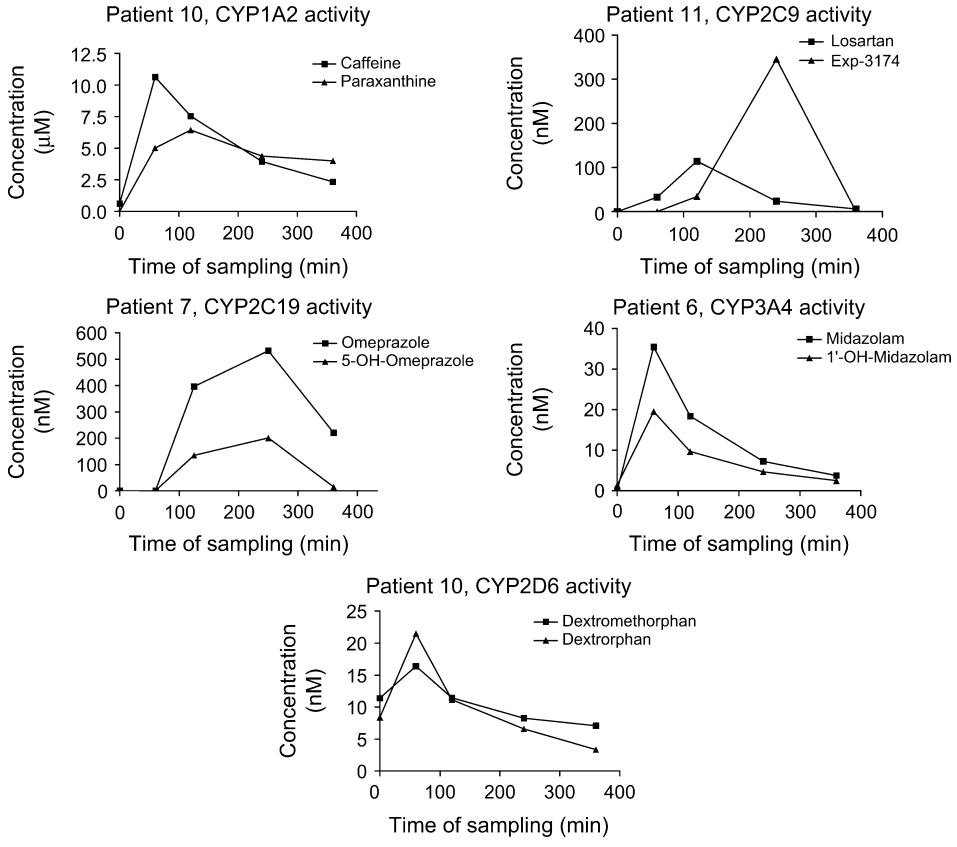

FIGURE 2. Representative plasma concentration–time profiles of CYP substrates and metabolites detected in participants after cocktail administration.

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which has limited dosage forms in some countries, S-warfarin, and tolbutamide. S-warfarin requires a relatively long blood sampling period (72 hours), analytical complexity to separate the enantiomers,[13] and the risk of anticoagulation, despite the coadministered vitamin K; this has reduced its utility as a component of phenotyping cocktails. Tolbutamide is no longer widely available for clinical use, which may also create ethical obstacles.

Some important steps were included in the present analytical methodology to optimize efficiency. Plasma proteins were precipitated to decrease the viscosity of the sample and to diminish the matrix effect during the mass spectrometry step. The use of SPE of multiple phenotyping drugs and metabolites enhances the speed and efficiency of the analysis. The Oasis HLB cartridges that were used in the present study were appropriate for the extraction of acidic (such as losartan and EXP-3174), basic (omeprazole, midazolam, dextromethorphan, and their metabolites), and the neutral compound (caffeine), but paraxanthine was not extracted efficiently. In the study of Yin et al,[9] all the compounds including paraxanthine were extracted by the same type of SPE cartridge as was used in this study. However, it is noteworthy that the recovery of caffeine and paraxanthine was estimated from 5% human serum albumin solution, which may not reflect the situation in plasma. The present study incorporated a protein precipitation step, and the wash volumes were increased to 2 mL of water and 2 mL of 10% methanol rather than 1 mL of water and 1 mL of 5% methanol in the study of Yin et al.[9] To improve the recovery of paraxanthine, a modified liquid–liquid extraction was used.[20] This additional step before the LC/MS analysis slightly complicated sample handling but markedly enhanced the analytical sensitivity and reliability. Although the matrix effect was high for omeprazole and 5-hydroxyomeprazole, it was reproducible at both high and low analyte concentrations. The interday assay precision observed in this study was higher than expected (10%–20%) for an LC/MS assay. The reason for this observation is unclear but may be related to the wide range of physicochemical properties of the analytes quantitated in this assay. In principle, simultaneous assays of the present type may lead to a decrease in analytical sensitivity for some of the test compounds. However, in the present method, the use of LC/MS adequately compensated for this.

CONCLUSIONS

The use of CYP phenotyping cocktails is an extremely attractive approach for the determination of optimal doses of drugs that are problematic in terms of efficacy and toxicity. However, the need for collection of both urine and plasma and the separate analysis of drug/metabolite pairs is inconvenient and adds to the time required for the provision of information on dosage adjustment to clinicians. The present method has decreased the time of analysis to less than 3 hours for each sample, which is significantly shorter than the analytical methods used in previous cocktails. The present adaptation of a limited sampling approach in plasma, combined with a rapid and convenient determination of multiple analytes, offers the opportunity for wider uptake of CYP phenotyping in the clinic.

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