A UPLC-MS/MS assay of the "Pittsburgh cocktail": six CYP probe-drug/metabolites from human plasma and urine using stable isotope dilution†

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The efficiency of drug metabolism by a single enzyme can be measured as the fractional metabolic clearance which can be used as a measure of whole body activity for that enzyme. Measurement of activity of multiple enzymes simultaneously is feasible using a cocktail approach, however, analytical approach using different assays for drug probes can be cumbersome. A quantitative ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) based method for the rapid measurement of six cytochrome P450 (CYP) probe drugs and their relevant metabolites is described. The six specific probe substrates/metabolites are caffeine/paraxanthine (CYP1A2), flurbiprofen/4′-hydroxyflurbiprofen (CYP2C9), mephenytoin/4′-hydroxymephenytoin (CYP2C19), debrisoquine/4-hydroxydebrisoquine (CYP2D6), chlorzoxazone/6′-hydroxychlorzoxazone (CYP2E1) and dapsone/*N*-monoacetyldapsone (NAT2). These probes were quantified by stable isotope dilution from plasma and urine. The present workflow provides a robust, fast and sensitive assay for the "Pittsburgh cocktail", and has been successfully applied to a clinical phenotyping study of liver disease. A representative group of 17 controls and patients with chronic liver disease were administered orally caffeine (100 mg), chlorzoxazone (250 mg), debrisoquine (10 mg), mephenytoin (100 mg), flurbiprofen (50 mg) and dapsone (100 mg). Urine (0 through 8 h) and plasma (4 and 8 h) samples were analyzed for drug/metabolite amounts by stable isotope dilution UPLC-MS/MS. The phenotypic activity of drug metabolizing enzymes was investigated with 17 patient samples. Selected reaction monitoring (SRM) was optimized for each drug and metabolite. In the method developed, analytes were resolved by reversed-phase by development of a gradient using a water/methanol solvent system. SRM of each analyte was performed in duplicate on a triple quadrupole mass spectrometer utilizing an 8 min analytical method each, one with the source operating in the positive mode and one in the negative mode, using the same solvent system. This method enabled quantification of each drug (caffeine, chlorzoxazone, debrisoquine, mephenytoin, flurbiprofen, and dapsone) and its resulting primary metabolite in urine or plasma in patient samples. The method developed and the data herein demonstrate a robust quantitative assay to examine changes in CYP enzymes both independently or as part of a cocktail. The clinical use of a combination of probe drugs with UPLC-MS/MS is a highly efficient tool for the assessment of CYP enzyme activity in liver disease.

Introduction

The liver is the primary site of drug metabolism and plays a pivotal role in the detoxification and elimination of substances from the body. Drug metabolism occurs mainly through the actions of the hepatic cytochrome P450 (CYP) enzymes.[1–3] The CYP family is an abundant and diverse group of metabolic enzymes, which in humans account for almost 75% of a drug's total metabolism (reviewed by Wang & Chou in 2010).[4]

Expression of CYP enzymes and their activities depend on genetic and environmental factors,[5–8] a consequence of which is a wide degree of inter-individual variability, resulting in large differences among the population in the rate and extent of drug metabolism. The substrates of CYP enzymes include metabolic intermediates, such as lipids and steroidal hormones as well as xenobiotics. The metabolite products of CYP turnover combine with other chemical moieties in secondary metabolic cascades for their excretion through the lungs, or bodily fluids such as saliva, sweat, breast milk, and urine, or through absorption by the intestines. CYP superfamily members exhibit substrate selectivity in the drugs that they metabolize though, often with overlap.[9] This substrate selectivity has enabled the identification of compounds to selectively probe for the activities of individual CYP enzymes, enabling development of approaches to measure enzyme activity both *in vitro* and *in vivo*.

In 1988, Breimer and Schellens were the first group to describe the administration of multiple compounds simultaneously as a "cocktail" strategy.[10–13] With the ever-expanding

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field of metabolomics, several investigators have begun to use this cocktail strategy. These studies include the 'Pittsburgh cocktail',[14,15] the 'GW cocktail',[16] the 'Cooperstown cocktail'[17,18] and the 'Karolinska cocktail',[19] among others (see review [20]). The use of a drug cocktail is advantageous because it allows for the comprehensive analyses of metabolic pathways obtained in single multiplexed experimental workflow. Typically, analytical methods for these cocktail are time consuming and are dependent upon either high performance liquid chromatography (HPLC), gas chromatography (GC) or liquid chromatography mass spectrometry (LC-MS).

We have previously used the cocktail approach to successfully characterize the effects of liver disease on multiple CYP enzymes in human blood and urine samples.[14,15] We have further characterized the effects of CYP enzymes in normal subjects and in patients with a variety of forms of cancer. For each drug, we selected an optimal dose which was able to minimize risk while providing sufficient concentration for its measurement, along with its metabolites. However, this approach utilized an HPLC method, which was laborious and inherently low throughput.

In the present work, we describe a novel ultra-performance LC (UPLC)-tandem mass spectrometry (MS/MS) assay of human urine or plasma by stable isotope dilution (SID) to evaluate the six CYP probe drugs in the 'Pittsburgh cocktail' and their relevant metabolites. The structures of the six specific probe substrates/metabolites are shown in Fig. 1 and these include caffeine/paraxanthine (CYP1A2), flurbiprofen/4′-hydroxyflurbiprofen (CYP2C9), mephenytoin/4′-hydroxymephenytoin (CYP2C19), debrisoquine/4-hydroxydebrisoquine (CYP2D6), chlorzoxazone/6′-hydroxychlorzoxazone (CYP2E1) and dapsone/mono-*N*-acetyl dapsone (NAT2).

Experimental

Chemicals and reagents

Standards caffeine, paraxanthine, debrisoquine sulfate, (±) 4-hydroxydebrisoquine, chlorzoxazone, 6-hydroxychlorzoxazone, flurbiprofen, (±) mephenytoin, (±) 4′-hydroxymephenytoin and dapsone were purchased from Sigma (St Louis, MO, USA). 4′-Hydroxyflurbiprofen and mono-*N*-acetyl dapsone (MAD) were obtained from Toronto Research Chemicals Inc. (TRC, Toronto, ON, Canada). Heavy labeled internal standards: paraxanthine-1-methyl-$^{2}H_3$, debrisoquine-^{13}C,$^{15}N_2$ hemisulfate, (±) 4-hydroxydebrisoquine-^{13}C,$^{15}N_2$ hemisulfate, chlorzoxazone-4,6,7-$^{2}H_3$, 6-hydroxychlorzoxazone-$^{13}C_6$, (±) mephenytoin-$^{2}H_3$, (±) 4′-hydroxymephenytoin-$^{2}H_3$, flurbiprofen-$^{2}H_3$, hydroxyflurbiprofen-$^{2}H_3$, MAD-$^{2}H_8$ were obtained from TRC. Dapsone-$^{13}C_{12}$ was synthesized in-house using the method to synthesize the ^{14}C-labeled analog[21,22] starting from aniline-$^{13}C_6$ (Isotec, Sigma) and purified to >99% by RP-HPLC. $^{13}C_3$-Caffeine and β-glucuronidase (Type H-1; from *Helix pomatia*, 449 000 units g^{-1}) were obtained from Sigma.

Methanol, acetonitrile, trifluoroacetic acid (TFA) and all other chemicals used in this study were HPLC or reagent grade.

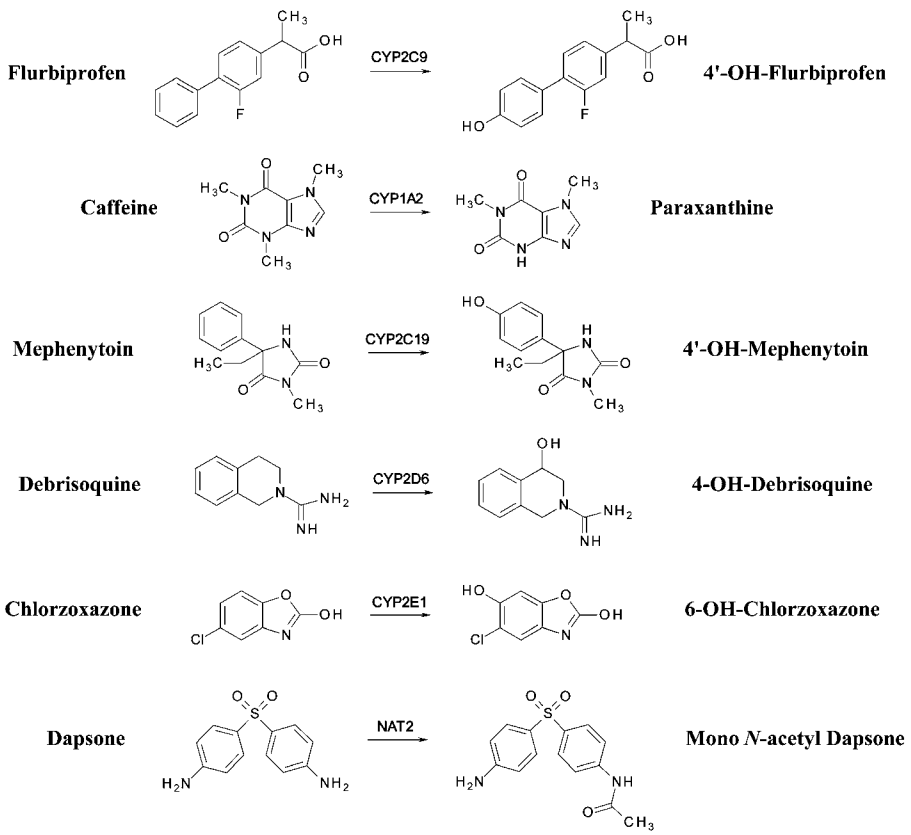

Fig. 1 Chemical structures of the six probe drugs used in the Pittsburgh cocktail and their *in vivo* metabolic conversion product.

Subject samples

Subjects were administered orally, in tablet form, caffeine (100 mg), chlorzoxazone (250 mg), debrisoquine (10 mg), mephenytoin (100 mg), flurbiprofen (50 mg) and dapsone (100 mg), as previously described.[15] Three sample types per subject were required to assess the cocktail. Urine was collected from 0–8 h and plasma was collected at 4 h and 8 h following drug administration. The three samples per subject were analyzed in triplicate against calibration curves.

Preparation of standards

Primary stock solutions of each analyte/standard were prepared at a concentration of 1 mg mL^{-1} in methanol except for caffeine, paraxanthine, debrisoquine and hydroxydebrisoquine which were prepared in water. Further dilutions were prepared in 50% methanol.

A stock solution containing all heavy standards was prepared by combining aliquots from individual stock solutions. This mixture was lyophilized under centrifugation and dissolved in 1000 μL 50% methanol with the final concentration for each as follows: caffeine (100 ng mL^{-1}), paraxanthine (500 ng mL^{-1}), debrisoquine (200 ng mL^{-1}), OH-debrisoquine (1.00 μg mL^{-1}), mephenytoin (2.00 μg mL^{-1}), OH-mephenytoin (2.00 μg mL^{-1}), dapsone (100 ng mL^{-1}), MAD (200 ng mL^{-1}), chlorzoxazone (500 ng mL^{-1}), OH-chlorzoxazone (2.00 μg mL^{-1}), flurbiprofen (10 μg mL^{-1}), and OH-flurbiprofen (2.00 μg mL^{-1}).

Similarly calibration standards were prepared at varying concentrations ranging from 0, 50, 100, 200, 500 and 1000 pg mL^{-1}, and 1.5, 2.0, 5.0, 10.0, 20, 50, 100, 200, 500, 1000, and 2000 ng mL in a final volume of 1000 μL prior to injection.

Standard curves were constructed in 100 μL pooled plasma and 10 μL pooled urine. Internal stable isotope standards (20 μL) were spiked into sample prior to digestion/processing.

β-Glucuronidase treatment

Urine samples and standards (10 μL) were diluted to 100 μL with addition of water prior to enzymatic treatment. Plasma samples and standards (100 μL) were treated directly with glucuronidase (100 μL, 0.2 M Na acetate pH 4.3, 1000 units mL^{-1}) for 4 h in a water bath at 37 °C. Phenolphthalein glucuronide was used as a positive control, with the appearance of a pink color with the addition of 1 M sodium hydroxide, as an indication for digestion.

Protein precipitation and sample processing

Protein from the samples was precipitated by the addition of acetonitrile (800 μL) and centrifuged at 14 000 rpm on tabletop centrifuge (Eppendorf) at 4 °C for 5 min. The supernatants (~1 mL) were transferred to microcentrifuge tubes and lyophilized under centrifugation (SpeedVac, Savant). Samples and standards were reconstituted in 1000 μL (0.1% TFA) and transferred to glass autosampler vials.

Calibration curves for each standard were constructed in pooled urine or plasma from pre-cocktail administration from five replicate injections.

UPLC selected reaction monitoring (SRM) MS/MS

Compounds were separated by C18 reverse phase (Gold C_{18}, 50 × 2.1 mm, 1.9 μm particle size, Thermo Fisher Scientific) equipped with a guard column using a water/methanol solvent gradient at a flow rate of 1 mL min^{-1} (Accela, ThermoFisher Scientific, San Jose). Sample flow was diverted to waste for the first 0.5 min. The gradient was as follows: (0–0.8 min) 2% methanol, (0.8–1.5 min) 2–30% methanol, (1.5–5.0 min) 30–75% methanol, (5.0–5.2 min) 100% methanol for 1 min, back to 2% methanol in 30 s held constant for 1.5 min for a total 8 min LC run. The autosampler was equipped with a 25 μL sample loop and injections were performed using the full loop injection program. Column, guard column and sample loop were kept at 55 °C. Samples were kept at 4 °C. SRM of each analyte was performed on a triple quadrupole mass spectrometer (TSQ Ultra, ThermoFisher Scientific, San Jose) using the atmospheric pressure chemical ionization (APCI) source with two runs of 8 min each, one with the source operating in the positive mode and one operating in the negative mode, using the same solvent system and gradient. SRM was optimized for each analyte/standard by direct infusion using the APCI source. A scan width of 0.100 u, scan time of 0.010 s, a Q1 peak width (PW) of 0.70 u full width at half-maximum (FWHM) and a Q3 PW of 0.70 u FWHM were used for spectra acquisition. The APCI source was operated with a discharge current of 12.0 μA, vaporizer temperature set at 350 °C, sheath gas pressure of 40 AUs (arbitrary units) and auxiliary gas pressure of 20 AUs. The capillary temperature was set at 280 °C, the capillary offset at 50 V and the skimmer offset at 10 V. Collision pressure was set at 1.5 mTorr and tube lens values were determined from the tune file.

Data processing

Instrument control and processing of the SRM chromatograms were accomplished using the processing software Quan Browser in Xcalibur (2.0.6). Two processing files, one for positive and one for negative ionization mode, were used for analysis of both plasma and urine samples. The genesis peak detection algorithm was used for peak generation with an expected peak width of 20 s.

Results and discussion

Herein we describe a robust and high throughput approach to study the Pittsburgh CYP phenotyping cocktail. The chemical structures of each of the six probe drugs and their metabolites are depicted in Fig. 1. This assay is advantageous over the HPLC assay because it is less time consuming, reproducible and allows for the use of only two processing files.

Collision induced dissociation spectra were obtained for each probe drug, metabolite and standard by direct infusion by APCI (Fig. 2). The transition ion of highest intensity was chosen and its collision energy was optimized for each compound. Table 1 lists the selected precursor ions and respective selected transition ions with their corresponding collision energies under the appropriate ionization mode chosen for quantitation. We have successfully transferred the preexisting HPLC-based workflow[14] to a SRM MS/MS based assay. Typical chromatograms of this assay are shown in Fig. 3.

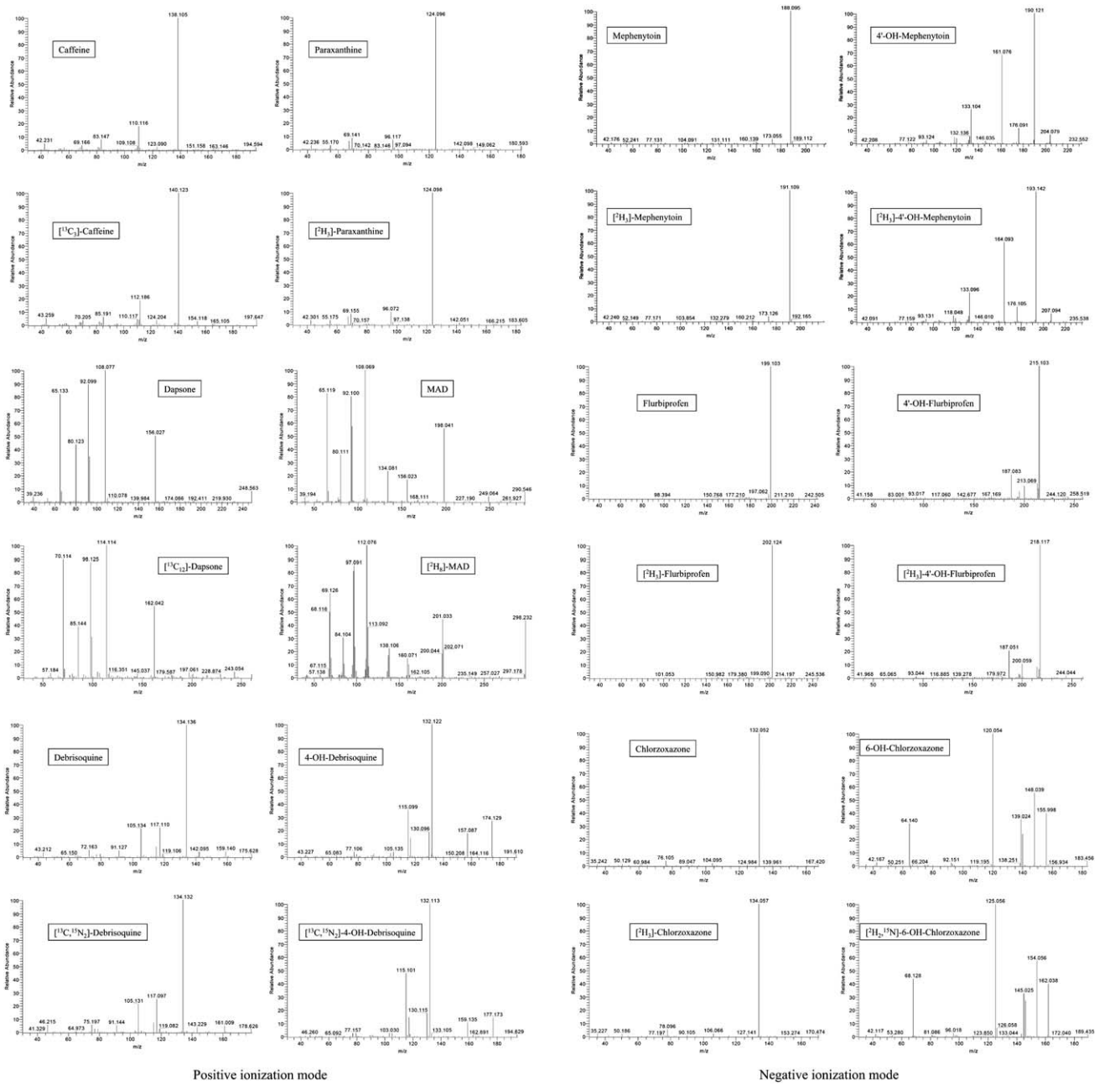

Fig. 2 Collision induced dissociation product ion mass spectra of the probe drugs, metabolites and stable isotope internal standards. The APCI spectra were recorded by direct infusion using a CE of 5 V.

Calibration curves

The calibration curves of area ratios *vs.* concentration are given in Table 2. The calibration curves for each showed good linearity with the lowest correlation coefficient of 0.9957 for debrisoquine. The lower limit of quantitation (LLOQ) defined by the lowest concentration within the linear range of the calibration curves that gives an acceptable accuracy of <20% and a precision of <20% ($S/N > 8$) is 100 pg mL^{-1} for chlorzoxazone, 200 pg mL^{-1} for hydroxychlorzoxazone, 2 ng mL^{-1} for flurbiprofen, 5 ng mL^{-1} for hydroxyflurbiprofen, 2 ng mL^{-1} for hydroxymephenytoin, 500 pg mL^{-1} for caffeine, 500 pg mL^{-1} for paraxanthine, 100 pg mL^{-1} for dapsone, 100 pg mL^{-1} for MAD, 20 ng mL^{-1} for hydroxydebrisoquine, and 20 ng mL^{-1} for debrisoquine. Based on the calibration curves of area ratios (*i.e.* light/heavy) *vs.* concentration, the method described herein is highly sensitive and selective for all drugs and their metabolites. Most notably, caffeine and dapsone are those with the lowest LLOQ whereas flurbiprofen possesses the highest.

Accuracy and precision

Accuracy and precision were determined from 3 quality control samples of low, mid, and high concentration values ($n = 5$) (Table 3). The values for each analyte are acceptable (%CV < 15%) within the linear range of the assay.

Table 1 List of precursor ions, selected transition ions and corresponding collision energies used for quantitation

Probe drug	Q1 *m/z*	Q3 *m/z*	CE V	Metabolite	Q1 *m/z*	Q3 *m/z*	CE V	Ionization mode	Matrix
Chlorzoxazone	168.0	132.0	22	6-OH-Chlorzoxazone	184.0	120.1	21	–	Plasma
[$^{2}H_3$]-Chlorzoxazone	171.0	134.0	22	[$^{2}H_2$,^{15}N]-6-OH-Chlorzoxazone	190.0	125.0	21	–	Plasma
Mephenytoin	217.1	188.1	19	4′-OH-Mephenytoin	233.1	190.1	17	–	Urine
[$^{2}H_3$]-Mephenytoin	220.1	191.1	19	[$^{2}H_3$]-4′-OH-Mephenytoin	236.1	193.1	17	–	Urine
Flurbiprofen	243.1	199.1	14	4′-OH-Flurbiprofen	259.0	215.1	18	–	Urine
[$^{2}H_3$]-Flurbiprofen	246.1	202.1	14	[$^{2}H_3$]-4′-OH-Flurbiprofen	262.1	218.1	18	–	Urine
Debrisoquine	176.1	134.1	20	OH-Debrisoquine	192.1	132.1	20	+	Urine
[^{13}C,$^{15}N_2$]-Debrisoquine	179.1	134.1	20	[^{13}C,$^{15}N_2$]-OH-Debrisoquine	195.1	132.1	20	+	Urine
Caffeine	195.05	138.1	20	Paraxanthine	181.1	124.1	20	+	Plasma
[$^{13}C_3$]-Caffeine	198.2	140.1	20	[$^{2}H_3$]-Paraxanthine	184.1	124.1	20	+	Plasma
Dapsone	249.1	156.0	14	MAD	291.1	108.1	28	+	Plasma
[$^{13}C_{12}$]-Dapsone	261.1	162.0	14	[$^{2}H_8$]-MAD	299.1	112.0	28	+	Plasma

CYP phenotyping indices

CYP1A2 activity is assessed by dividing the concentration of paraxanthine by the concentration of caffeine in the 8 hour plasma sample. CYP2E1 activity is estimated by the 6-hydroxychlorzoxazone to chlorzoxazone plasma ratio at 4 hours. The activity of CYP2D6 is estimated by the debrisoquine recovery ratio, which is calculated as the urinary recovery of 4-hydroxydebrisoquine divided by the sum total of 4-hydroxydebrisoquine and debrisoquine recovery. The total urinary recovery of 4′-hydroxymephenytoin (expressed in μmol) is used as the phenotypic index of CYP2C19 activity.[23] The acetylation phenotype (NAT2) is defined as the ratio of MAD to dapsone in the 8 hour plasma sample. The activity of CYP2C9 is assessed by the 8 hour urinary flurbiprofen recovery ratio, which is calculated as the urinary recovery of 4′-hydroxyflurbiprofen divided by the sum total of 4′-hydroxyflurbiprofen and flurbiprofen recovery. Using the SID SRM MS/MS approach we analyzed 17 subject samples in triplicate and typical results are shown in Table 4. As expected, inter-subject variation was wide and based on these quantitative data, two of the subjects are apparently poor metabolizers of debrisoquine. These results will be integrated with a larger sample of controls and patients with well characterized liver disease to further explore regulation of drug metabolizing enzyme activity in liver disease.[24]

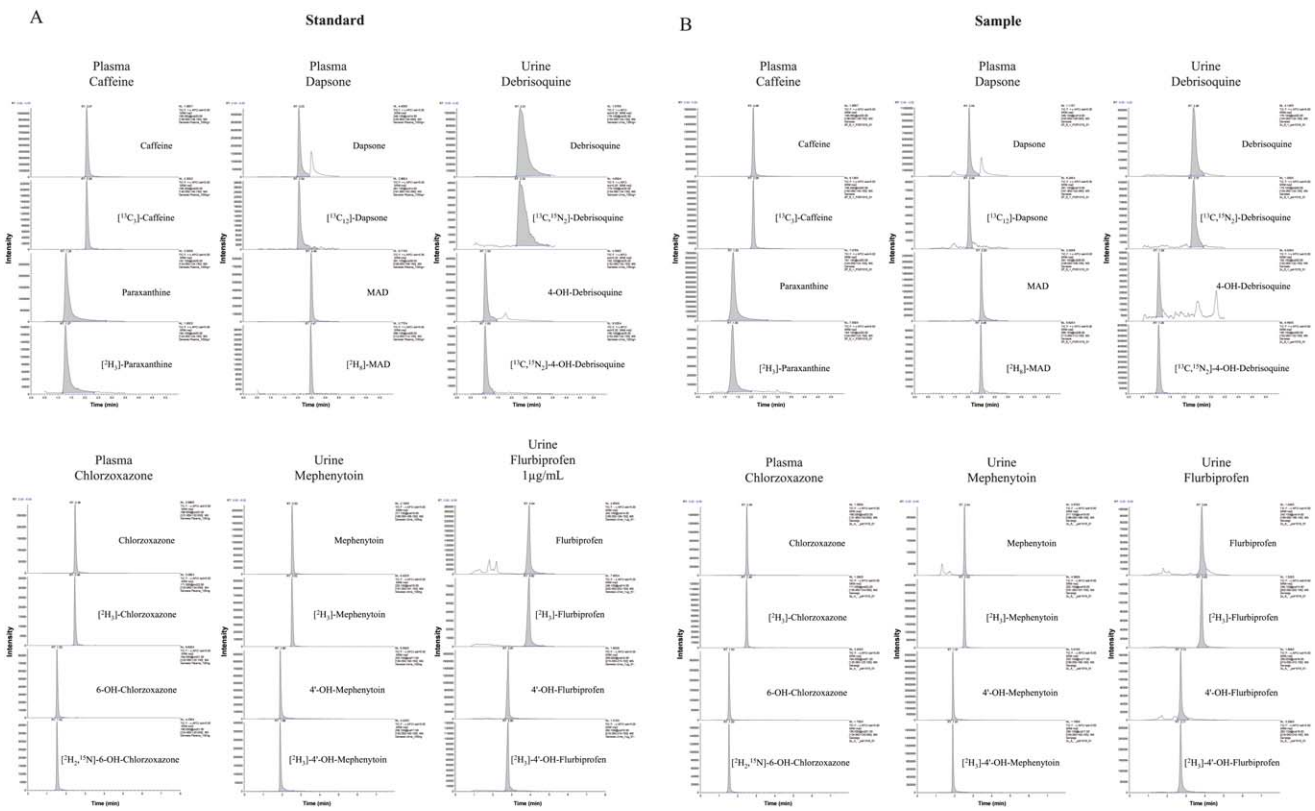

Fig. 3 SRM chromatograms for the complete Pittsburgh cocktail from (A) urine and plasma standards 100 ng mL^{-1} (1μg mL^{-1} for flurbiprofen/hydroxyflurbiprofen) and (B) subject urine and plasma standards.

Table 2 Calibration curves and sensitivity of the assay

Analyte	Slope area ratios *vs.* conc./ng mL^{-1}	r^2	LLOQ/ng mL^{-1}
Caffeine	$y = 0.200308x + 3.01874$	0.9975	0.5
Paraxanthine	$y = 0.0803823x + 0.232653$	0.9990	0.5
Dapsone	$y = 0.721520x + 5.45995$	0.9975	0.1
MAD	$y = 0.157119x + 0.258403$	0.9959	0.1
Debrisoquine	$y = 0.0542820x + 1.21180$	0.9957	20
4-OH-Debrisoquine	$y = 0.0121179x + 0.0814633$	0.9977	20
Mephenytoin	$y = 1.52682 \times 10^{-5}x + 1.15142 \times 10^{-5}$	0.9986	2
4′-OH-Mephenytoin	$y = 1.61011 \times 10^{-5}x - 3.06084 \times 10^{-6}$	0.9969	2
Flurbiprofen	$y = 2.65709 \times 10^{-6}x + 1.28192 \times 10^{-4}$	0.9974	2
4′-OH-Flurbiprofen	$y = 1.08998 \times 10^{-5}x + 3.52417 \times 10^{-5}$	0.9964	5
Chlorzoxazone	$y = 5.80009 \times 10^{-5}x + 6.48346 \times 10^{-5}$	0.9992	0.1
6-OH-Chlorzoxazone	$y = 1.48571 \times 10^{-5}x + 3.71915 \times 10^{-6}$	0.9987	0.2

Although mephenytoin can be analyzed in either positive or negative ionization mode, we selected to use the negative mode, which enabled its detection at lower LLOQ. In the urine standard, an earlier eluting peak is observed with flurbiprofen (light) but because it was not the major peak in the sample, and its relative retention time is earlier, it did not interfere with the

Table 3 Accuracy and precision of method from QC samples ($n = 5$)

	Caffeine			Paraxanthine		
Nominal/ng mL^{-1}	20	100	1000	10	100	1000
Measured/ng mL^{-1}	16.3	111.3	1054.9	8.3	104.3	1001.0
Std. dev./ng mL^{-1}	0.5	7.7	49.9	0.4	7.8	36.9
%CV	3.2	6.9	4.7	5.4	7.4	3.7
Accuracy (% bias)	−18.5	11.3	5.5	−16.8	4.3	0.1
	Debrisoquine			4-OH-Debrisoquine		
Nominal/ng mL^{-1}	20	100	1000	20	100	1000
Measured/ng mL^{-1}	16.5	100.4	979.8	16.1	98.0	997.0
Std. dev./ng mL^{-1}	0.8	5.0	89.3	0.9	3.6	54.5
%CV	4.6	5.0	9.1	5.5	3.7	5.5
Accuracy (% bias)	−17.3	0.4	−2.0	−19.5	−2.0	−0.3
	Dapsone			MAD		
Nominal/ng mL^{-1}	20	100	1000	10	100	1000
Measured/ng mL	18.6	106.9	1010.8	9.8	107.1	971.8
Std. dev./ng mL	1.0	12.2	32.1	0.6	6.0	73.7
%CV	5.2	11.4	3.2	6.1	5.6	7.6
Accuracy (% bias)	−6.9	6.9	1.1	−1.9	7.1	−2.8
	Flurbiprofen			4′-OH-Flurbiprofen		
Nominal/ng mL^{-1}	20	500	2000	20	100	1000
Measured/ng mL^{-1}	22.3	480.2	2048.8	18.1	92.4	1008.2
Std. dev./ng mL^{-1}	3.1	43.9	78.1	0.3	5.2	46.0
%CV	13.8	9.2	3.8	1.7	5.6	4.6
Accuracy (% bias)	11.5	−4.0	2.4	−9.3	−7.6	0.8
	Chlorzoxazone			6-OH-Chlorzoxazone		
Nominal/ng mL^{-1}	10	100	1000	10	100	1000
Measured/ng mL^{-1}	9.9	107.8	1016.6	11.2	108.8	1020.0
Std. dev./ng mL^{-1}	0.7	4.6	33.6	0.3	2.1	42.1
%CV	7.2	4.3	3.3	2.8	1.9	4.1
Accuracy (% bias)	−0.7	7.8	−3.8	11.7	8.8	2.0
		4′-OH-Mephenytoin				
Nominal/ng mL^{-1}		20		100		1000
Measured/ng mL^{-1}		17.1		107.2		938.6
Std. dev./ng mL^{-1}		1.7		5.4		53.1
%CV		9.9		5.1		5.7
Accuracy (% bias)		−14.4		7.2		−6.1

Table 4 Phenotyping indices results from 17 subjects

Subject sample	Caffeine 8 h plasma ratio	Std. dev.	%CV	Dapsone 8 h plasma ratio	Std. dev.	%CV	Chlorzoxazone 4 h plasma ratio	Std. dev.	%CV	Flurbiprofen urine ratio	Std. dev.	%CV	OH-Meph./ µmol	Std. dev.	%CV	Debrisoquine urine recovery ratio	Std. dev.	%CV
1	1.05	0.05	5.0	0.83	0.07	8.7	0.60	0.06	9.3	0.128	0.009	6.8	29.6	0.9	3.1	0.54	0.028	5.1
2	0.71	0.05	6.5	0.66	0.04	6.4	0.57	0.04	6.6	0.174	0.042	23.8	42.2	1.6	3.9	0.67	0.029	4.3
3	0.41	0.03	7.3	1.04	0.06	5.4	0.61	0.06	9.2	0.573	0.068	11.9	183.2	8.0	4.4	OH Deb ND	—	—
4	0.72	0.04	5.3	0.16	0.02	15.4	0.37	0.01	2.8	0.370	0.053	14.3	73.5	1.8	2.4	0.80	0.027	3.3
5	0.85	0.10	11.5	0.57	0.05	8.7	1.33	0.07	5.4	0.407	0.096	23.6	87.8	2.0	2.2	0.79	0.07	9.3
6	0.80	0.08	10.1	1.00	0.12	11.6	0.70	0.08	11.8	0.452	0.065	14.4	93.5	1.8	1.9	0.87	0.036	4.2
7	0.79	0.10	12.9	0.62	0.08	13.6	0.95	0.05	4.9	0.330	0.011	3.2	53.3	0.8	1.5	0.56	0.022	4.0
8	0.26	0.02	9.5	0.19	0.04	20.0	0.98	0.07	7.5	0.257	0.012	4.9	33.2	0.7	2.1	OH Deb ND	—	—
9	1.19	0.04	3.8	0.16	0.03	17.9	0.91	0.15	16.3	0.466	0.070	14.9	175.8	6.6	3.8	0.66	0.037	5.6
10	0.62	0.03	5.2	0.82	0.03	4.2	1.12	0.07	5.9	0.484	0.073	15.0	250.9	10.0	4.0	0.92	0.099	10.8
11	1.06	0.06	5.6	0.99	0.09	9.2	1.15	0.04	3.3	0.345	0.012	3.6	73.6	3.7	5.0	0.62	0.022	3.5
12	0.72	0.06	7.9	0.22	0.02	7.4	0.59	0.03	5.4	0.161	0.020	12.5	59.9	3.6	6.1	0.78	0.034	4.4
13	0.68	0.06	9.2	0.23	0.04	16.8	0.99	0.06	6.2	0.136	0.018	12.9	21.1	0.1	0.5	0.81	0.026	3.1
14	0.49	0.05	10.6	0.66	0.11	17.3	0.87	0.04	4.7	0.982	0.024	2.4	289.4	4.2	1.5	0.82	0.05	6.1
15	1.13	0.11	9.5	0.17	0.02	10.5	1.10	0.08	6.9	0.373	0.009	2.5	137.5	5.2	3.8	0.68	0.008	1.2
16	1.05	0.11	10.9	0.43	0.05	11.6	0.45	0.02	4.9	0.309	0.037	12.1	63.0	1.4	2.2	0.32	0.025	7.9
17	0.88	0.09	10.6	0.86	0.14	16.3	0.63	0.05	8.1	0.141	0.004	2.9	29.3	0.8	2.7	0.01	0.0004	3.7

quantitative determination of flurbiprofen. Of note, in the dapsone SRM chromatogram (249.1 $m/z \rightarrow 156.0$ m/z), MAD gives rise to a peak eluting after that of dapsone. This is not an impurity in MAD but is due to the "in source" fragmentation of MAD producing dapsone from the loss of the acetyl group. MAD elutes later in the chromatogram relative to dapsone and the production of dapsone from this compound does not interfere with the quantitation of the latter.

In addition, OH-debrisoquine and debrisoquine experience the greatest matrix effects, most probably attributable to high hydrophilicity. This was offset by the use of TFA in the sample as ion pairing agent which greatly enhanced peak shape. In our laboratory, under the current conditions, column integrity was maintained for greater than 500 injections.

The Pittsburgh cocktail minimally requires three samples from each subject/patient namely: 0 through 8 hour urine, and 4 and 8 hour plasmas. Ideally, a cocktail that only requires one sample type at one time point for all CYP isoforms would be advantageous. Also, it would be wise to consider the instrument used for analysis (*i.e.* MS) in the selection of drug probes. From an analytical point of view it would be ideal if the probe set could be analyze in one run using one ionization mode with all species chromatographically resolved. Although feasible with the wide choice of drugs[25] this would require extensive clinical validation.

One minor issue we found was that, although subjects were requested to avoid caffeine intake, in the majority of our subject samples, caffeine/paraxanthine was clearly evident ($t = 0$) prior to administration. However, the presence of caffeine did not affect recovery ratios as the amounts of caffeine or paraxanthine in plasma were significantly increased by orders of magnitude when given as the drug.

In conclusion, the presently developed and validated UPLC SRM-MS/MS assay will enable on-going and future studies on the effect of liver disease on drug metabolism and furthermore, the use of a combination of probe drugs can be applied to other malignancies with altered CYP activities such as renal disease, neurological diseases and cancer, including liver cancer.

Abbreviations

CYP	cytochrome P450
MS	Mass Spectrometry
SRM	Selected Reaction Monitoring
SID	Stable Isotope Dilution
UPLC	Ultra-Performance Liquid Chromatography.

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