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Geneva Cocktail for Cytochrome P450 and P-Glycoprotein Activity Assessment Using Dried Blood Spots

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The suitability of the capillary dried blood spot (DBS) sampling method was assessed for simultaneous phenotyping of cytochrome P450 (CYP) enzymes and P-glycoprotein (P-gp) using a cocktail approach. Ten volunteers received an oral cocktail capsule containing low doses of the probes bupropion (CYP2B6), flurbiprofen (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6), midazolam (CYP3A), and fexofenadine (P-gp) with coffee/Coke (CYP1A2) on four occasions. They received the cocktail alone (session 1), and with the CYP inhibitors fluvoxamine and voriconazole (session 2) and quinidine (session 3). In session 4, subjects received the cocktail after a 7-day pretreatment with the inducer rifampicin. The concentrations of probes/metabolites were determined in DBS and plasma using a single liquid chromatography–tandem mass spectrometry method. The pharmacokinetic profiles of the drugs were comparable in DBS and plasma. Important modulation of CYP and P-gp activities was observed in the presence of inhibitors and the inducer. Minimally invasive one- and three-point (at 2, 3, and 6 h) DBS-sampling methods were found to reliably reflect CYP and P-gp activities at each session.

Cytochrome P450 (CYP) enzymes comprise the major drug-metabolizing enzyme system in humans. Genetic polymorphisms or environmental factors such as dietary components, toxins, or drugs can affect the activity of these enzymes and result in interindividual variations in drug concentrations. In addition to metabolizing enzymes, drug influx and efflux proteins such as P-glycoprotein (P-gp) are important sources of pharmacokinetic variability in drug response, as has been underlined recently.[1] The pharmacokinetic variability and modifications in activities of CYP and/or P-gp can cause various pharmacological and toxicological consequences. It is therefore important to precisely and reliably evaluate their *in vivo* activity (phenotyping). A cocktail approach involving the administration of multiple CYP- or P-gp-specific probe drugs can be used to simultaneously assess the activities of these enzymes and the transporter. Many phenotyping cocktails have been developed and used over the past 2 decades.[2–7] The use of some of these cocktails is limited by the fact that a few probe drugs, such as mephenytoin[6] and debrisoquine,[2,6] are no longer available in many countries. Another limitation is the use of therapeutic doses or insufficiently validated probes that might provoke side effects,[8] especially if used in clinical practice in a more vulnerable population. This limitation may be overcome by the use of lower probe doses, but this alternative requires the development of sensitive analytical methods. Several currently available phenotyping procedures require tedious and multiple venous blood sampling.[2,4] For a few cocktails, limited sampling strategies and phenotyping indexes have been proposed, but these cocktails require the collection of both plasma and urine samples.[3,5,6] Moreover, phenotyping indexes are established based on normal CYP function, and it is often unknown whether the chosen indexes will be reliable in case of altered CYP activity.

A novel and promising approach for CYP and P-gp activity phenotyping is the use of dried blood spots (DBSs) as a sampling procedure. This sampling method has been successfully applied for therapeutic drug monitoring and in pharmacokinetic studies.[9–11] Recent studies have shown that DBS sampling could also be used for individual cytochrome phenotyping of CYP2C9 (ref. 12) or CYP3A[13] activities.

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The purpose of this study was to evaluate the usefulness and effectiveness of DBS sampling for simultaneous assessment of the activities of six CYP isoforms and P-gp using a low-dose phenotyping cocktail composed of caffeine (CYP1A2), bupropion (CYP2B6), flurbiprofen (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6), midazolam (CYP3A), and fexofenadine (P-gp). The reliability of the method for the assessment of the modulation of CYP and P-gp activities was examined both by administration of the cocktail alone and in the presence of known CYP and P-gp inhibitors and an inducer.

RESULTS

Ten volunteers participated in the study and successfully completed the four study sessions. None of the subjects reported side effects after cocktail administration. However, four volunteers reported short-lasting troubled vision (a well-known and common adverse effect of voriconazole)[14] immediately after administration of fluvoxamine and voriconazole at session 2.

Poor CYP2C9, CYP2C19, and CYP2D6 metabolizers were not included in the study (see Methods section). Three volunteers were identified as CYP2D6 intermediate metabolizers (IMs) (**5/*10*; **5/*41*; and **10/*41*), and the results for these volunteers were analyzed as a separate group (lower dextrorphan/dextromethorphan (dor/dem) area under the concentration–time curve (AUC) ratio; $P = 0.017$). A trend toward increased CYP2C19 metabolism (as determined by a lower omeprazole AUC and a higher 5′-hydroxyomeprazole/omeprazole (OH-opz/opz) AUC ratio) was noticed in five volunteers who were heterozygous carriers of *CYP2C19*17*, but this did not reach statistical significance ($P = 0.06$). The 4′-hydroxybupropion/bupropion (OH-bup/bup) AUC_{last} ratio tended to be lower in the two homozygous *CYP2B6*6* carriers, but due to the small subject number, the difference was not statistically significant. Two volunteers were heterozygous *CYP2C9*2* carriers, but flurbiprofen pharmacokinetics in these subjects did not differ from those of homozygous *CYP2C9*1* carriers. Fexofenadine pharmacokinetic data for three volunteers with the TT-TT haplotype for the transporter adenosine triphosphate–binding cassette (ABC) B1 G2677T and C3435T single-nucleotide polymorphisms did not differ from the data of other subjects.

Pharmacokinetic profiles of all the CYP-specific substrates and metabolites were comparable in DBS and plasma in terms of distribution and elimination (**Figure 1**). Caffeine AUCs for the four volunteers who received Coke were approximately four times lower than the AUCs of those who received coffee; however, the paraxanthine/caffeine AUC ratios were similar, and therefore these were analyzed together (**Table 1**). For flurbiprofen, midazolam, and omeprazole, maximum plasma concentration (C_{max}) and AUC were proportionally lower in DBS than in plasma, with an approximate mean ratio of 0.6 corresponding to the dilution factor due to the presence of blood cells. Bupropion or dextromethorphan C_{max} and AUC were higher in DBS in comparison with plasma, probably as a result of blood cell partitioning (**Figure 1** and **Table 1**). For fexofenadine, a similar pharmacokinetic profile was observed in DBS and plasma, with no statistically significant differences between the two matrices in terms of half-lives or time to maximum concentration (T_{max}) at each session, whereas C_{max} and AUC values were slightly higher in plasma, with a blood to plasma ratio of ~0.9 (**Figure 2** and **Table 2**).

As expected, fluvoxamine and voriconazole inhibited CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A activities, as shown by a significant increase in AUC_{last} for the administered probes and decrease of the AUC_{last} ratios of metabolite/probe. The mean decrease in the AUC_{last} ratios in DBS was 81% for paraxanthine/caffeine (par/caf), 93% for OH-bup/bup, 33% for 4-hydroxyflurbiprofen/flurbiprofen (OH-flb/flb), 88% for OH-opz/opz, and 66% for 1-hydroxymidazolam/midazolam (OH-mdz/mdz). Quinidine strongly inhibited CYP2D6, as shown by a mean AUC_{last} ratio decrease for dor/dem of 99% for extensive metabolizers and 93% for IMs. Coadministration of quinidine also resulted in a significant 2.8-fold increase in fexofenadine AUC_{last}. The same magnitude of interaction was observed in plasma, for which, at the inhibition sessions, the decrease in AUC_{last} ratio was 81% for par/caf, 93% for OH-bup/bup, 36% for OH-flb/flb, 86% for OH-opz/opz, 62% for OH-mdz/mdz, and 99% for dor/dem; in addition, fexofenadine AUC_{last} increase was 2.7-fold.

Pretreatment with rifampicin increased the mean metabolite/probe AUC_{last} ratios. The magnitude of this increase was 1.15- (nonsignificant), 4.82-, 1.30-, 3.29-, and 5.6-fold in DBS and 1.54-, 5.08-, 1.25-, 2.50-, and 5.3-fold in plasma for par/caf, OH-bup/bup, OH-flb/flb, OH-opz/opz, and OH-mdz/mdz, respectively. Fexofenadine AUC_{last} decreased by 45 and 51% in DBS and plasma, respectively.

The DBS concentration ratios of metabolite/probe (metabolic ratios (MRs)) at each sampling time point after oral

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ Modulation and interindividual variability in the activities of CYP and P-gp can lead to differences in drug concentrations. The *in vivo* activity of these enzymes and the transporter can be assessed using a cocktail approach.

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ This study evaluated the usefulness of dried blood spot (DBS) sampling for the assessment of CYP and P-gp activity using a new low-dose phenotyping cocktail.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ The activities of six CYP isoforms and P-gp can be measured using a low-dose cocktail approach with simple and minimally invasive capillary sampling. The degree of modulation of CYP and P-gp activity in the presence of inhibitors/inducers can be reliably assessed using three-point DBS samples.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

✓ The use of a low-dose cocktail accompanied by a minimally invasive DBS sampling can be easily implemented for the evaluation of CYP and P-gp activities in a clinical setting and can be used as a tool for therapeutic individualization.

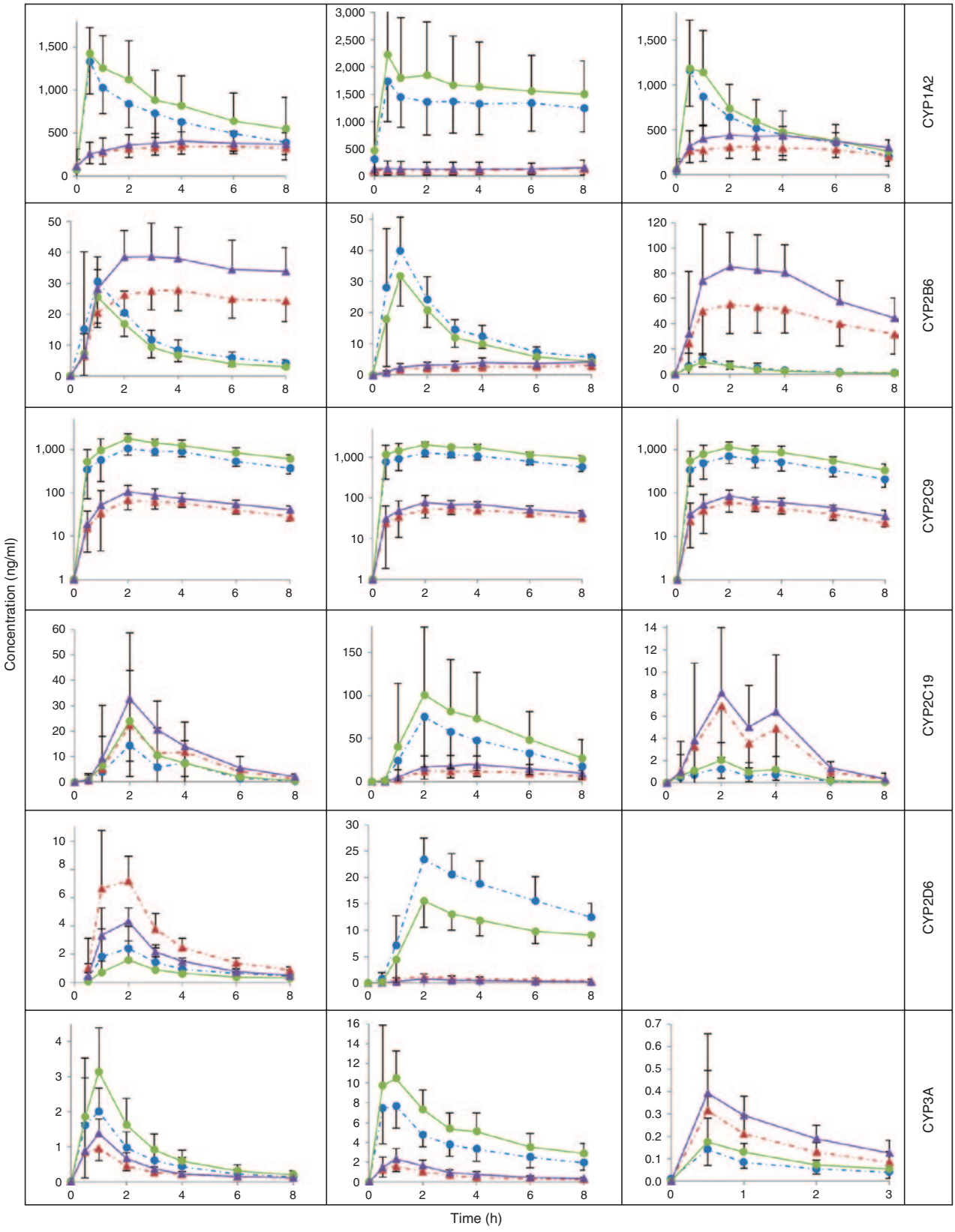

Figure 1 Concentration–time profiles for CYP probe substrates (circles) and their metabolites (triangles) obtained in 10 µl of capillary DBS (dashed lines) and venous plasma samples (continuous lines) after oral administration of cocktail alone (left column), in the presence of inhibitor(s) (middle), or in the presence of an inducer (right) in 10 healthy volunteers. CYP1A2 profile is presented only for volunteers who received coffee ($n = 6$); CYP2D6 profiles are presented only for EMs and UMs ($n = 7$). Error bars represent SD. CYP, cytochrome P450; DBS, dried blood spot; EM, extensive metabolizer; UM, ultrarapid metabolizer.

Table 1 Pharmacokinetic parameters of CYP probe drugs after oral administration of cocktail with and without inhibitors and inducer

		DBS					Plasma				
CYP		Cocktail alone	Cocktail + inhibitor	Ratio (95% CI)	Cocktail + inducer	Ratio (95% CI)	Cocktail alone	Cocktail + inhibitor	Ratio (95% CI)	Cocktail + inducer	Ratio (95% CI)
1A2 (given with coffee)	Caffeine										
	AUC_8 (h·ng/ml)	5,351 ± 1,847	9,724 ± 2,716	1.87 (1.04–3.36)	3,986 ± 1,571	0.74 (0.51–1.09)	6,738 ± 2,628	13,168 ± 6,405	1.92 (0.92–4.05)	4,527 ± 1,819	0.67 (0.50–0.91)
	C_{max} (ng/ml)	1,335 ± 376	1,593 ± 476	1.20 (0.76–1.89)	1,193 ± 393	0.88 (0.73–1.08)	1,450 ± 306	2,223 ± 1,075	1.43 (0.86–2.38)	1,293 ± 514	0.84 (0.62–1.15)
	AUC_8par/AUC_8caf	0.54 ± 0.26	0.08 ± 0.03	0.15 (0.12–0.20)	0.58 ± 0.20	1.13 (0.75–1.69)	0.48 ± 0.25	0.08 ± 0.04	0.16 (0.11–0.22)	0.74 ± 0.30	1.59 (0.99–2.56)
1A2 (given with Coke)	AUC_8 (h·ng/ml)	1,585 ± 296	4,359 ± 2,485	2.42 (1.07–5.48)	1,093 ± 184	0.69 (0.63–0.75)	1,911 ± 217	6,100 ± 2,904	2.89 (1.11–7.53)	1,293 ± 160	0.68 (0.50–0.90)
	C_{max} (ng/ml)	416 ± 57	697 ± 319	1.55 (0.83–2.90)	354 ± 72	0.84 (0.74–0.96)	464 ± 64	952 ± 404	1.92 (0.78–4.76)	334 ± 30	0.72 (0.65–0.80)
	AUC_8par/AUC_8caf	0.64 ± 0.11	0.19 ± 0.10	0.27 (0.09–0.75)	0.77 ± 0.19	1.18 (0.81–1.71)	0.66 ± 0.20	0.18 ± 0.07	0.27 (0.10–0.70)	0.94 ± 0.10	1.45 (1.05–2.01)
1A2 Total	AUC_8par/AUC_8caf	0.58 ± 0.21	0.12 ± 0.08	0.19 (0.13–0.28)	0.66 ± 0.21	1.15 (0.92–1.44)	0.56 ± 0.24	0.12 ± 0.07	0.19 (0.13–0.28)	0.82 ± 0.25	1.54 (1.19–1.99)
2B6	Bupropion										
	AUC_8 (h·ng/ml)	91.0 ± 28.6	121.6 ± 27.2	1.37 (1.11–1.69)	33.9 ± 13.3	0.37 (0.28–0.48)	70.6 ± 16.1	96.2 ± 16.5	1.38 (1.15–1.65)	28.2 ± 10.7	0.38 (0.29–0.51)
	C_{max} (ng/ml)	35.5 ± 15.7	43.7 ± 9.3	1.30 (0.98–1.73)	13.7 ± 7.1	0.38 (0.30–0.48)	27.2 ± 7.0	34.9 ± 10.3	1.26 (0.93–1.72)	11.8 ± 7.8	0.39 (0.26–0.58)
	AUC_8OH-bup/ AUC_8bup	2.26 ± 0.87	0.16 ± 0.07	0.07 (0.06–0.08)	10.7 ± 3.3	4.82 (3.62–6.41)	3.95 ± 1.43	0.28 ± 0.14	0.07 (0.06–0.08)	19.9 ± 6.6	5.08 (3.99–6.48)
2C9	Flurbiprofen										
	AUC_8	5,319 ± 847	7,316 ± 796	1.38 (1.26–1.52)	3,480 ± 630	0.65 (0.59–0.72)	8,279 ± 1,399	11,309 ± 953	1.38 (1.20–1.58)	5,631 ± 857	0.68 (0.59–0.78)
	C_{max}	1,141 ± 237	1,427 ± 238	1.26 (1.09–1.46)	841 ± 211	0.73 (0.63–0.85)	1,991 ± 532	2,206 ± 304	1.14 (0.94–1.38)	1,335 ± 346	0.67 (0.55–0.83)
	AUC_8OH-flb/AUC_8flb	0.068 ± 0.016	0.046 ± 0.009	0.67 (0.63–0.72)	0.088 ± 0.019	1.30 (1.16–1.45)	0.062 ± 0.014	0.040 ± 0.010	0.64 (0.59–0.71)	0.076 ± 0.013	1.25 (1.14–1.37)
2C19	Omeprazole										
	AUC_8	37.4 ± 30.1	308.1 ± 208.4	8.7 (6.2–12.4)	3.9 ± 2.4	0.11 (0.08–0.15)	61.1 ± 42.7	479.1 ± 323.5	7.7 (5.7–10.4)	6.4 ± 4.0	0.10 (0.07–0.15)
	C_{max}	17.8 ± 10.9	84.5 ± 53.8	4.6 (3.7–5.8)	1.95 ± 1.36	0.10 (0.07–0.15)	28.6 ± 17.5	112.7 ± 71.6	3.9 (3.3–4.5)	2.96 ± 1.81	0.09 (0.06–0.15)
	AUC_8OH-opz/ AUC_8opz	2.63 ± 1.88	0.31 ± 0.17	0.12 (0.10–0.15)	8.60 ± 7.30	3.29 (2.59–4.16)	2.40 ± 1.56	0.31 ± 0.16	0.14 (0.11–0.16)	5.31 ± 1.83	2.50 (1.72–3.63)
2D6 EM	Dextromethorphan										
	AUC_8 (h·ng/ml)	8.7 ± 6.1	117 ± 23	16.5 (10.0–27.2)	—	—	5.3 ± 4.5	76 ± 23	19.2 (10.0–36.8)	—	—
	C_{max} (ng/ml)	2.7 ± 2.0	23.8 ± 4.3	11.0 (6.3–19.3)	—	—	1.6 ± 1.4	16.3 ± 5.4	12.6 (7.3–21.6)	—	—
	AUC_8dor/AUC_8dem	3.76 ± 1.90	0.05 ± 0.02	0.015 (0.010–0.024)	—	—	4.40 ± 3.11	0.06 ± 0.02	0.016 (0.008–0.029)	—	—
2D6 IM	AUC_8 (h·ng/ml)	62 ± 32	132 ± 28	2.3 (0.9–5.7)	—	—	36 ± 19	87 ± 2	2.7 (0.7–10.4)	—	—
	C_{max} (ng/ml)	15.2 ± 8.1	24.4 ± 4.8	1.7 (0.6–4.9)	—	—	8.5 ± 4.4	15.1 ± 2.0	2.0 (0.6–6.7)	—	—
	AUC_8dor/AUC_8dem	0.48 ± 0.43	0.024 ± 0.005	0.07 (0.002–0.33)	—	—	0.40 ± 0.32	0.005 ± 0.002	0.02 (0.001–0.25)	—	—
3A	Midazolam										
	AUC_8	5.1 ± 2.4	29.9 ± 9.3	6.1 (4.9–7.6)	0.21 ± 0.08	0.04 (0.03–0.06)	7.5 ± 3.1	43.1 ± 11.8	6.0 (4.6–7.7)	0.33 ± 0.12	0.04 (0.03–0.06)
	C_{max}	2.3 ± 1.0	9.1 ± 2.5	4.0 (3.2–5.0)	0.13 ± 0.06	0.05 (0.04–0.07)	3.4 ± 1.3	12.3 ± 4.2	3.7 (2.7–5.2)	0.17 ± 0.10	0.05 (0.03–0.07)
	AUC_8OH-mdz/ AUC_8mdz	0.55 ± 0.25	0.18 ± 0.05	0.34 (0.24–0.48)	3.29 ± 1.89	5.6 (3.2–9.8)	0.48 ± 0.16	0.18 ± 0.06	0.38 (0.30–0.47)	2.49 ± 0.62	5.3 (4.2–6.8)

Data are presented as mean values ± SD and geometric mean ratios with 95% CIs.

AUC, area under the plasma concentration–time curve; bup, bupropion; caf, caffeine; CI, confidence interval; C_{max}, maximum plasma concentration; CYP, cytochrome P450; DBS, dried blood spot; dem, dextromethorphan; dor, dextrorphan; EM, extensive metabolizer; IM, intermediate metabolizer; mdz, midazolam; OH-bup, 4′-hydroxybupropion; OH-flb, 4′-hydroxyflurbiprofen; OH-mdz, 1′-hydroxymidazolam; OH-opz, 5′-hydroxyomeprazole; opz, omeprazole; par, paraxanthine.

administration of the cocktail, alone and with pretreatment, are shown in Figure 3. Statistically significant differences among the sessions for every sampling point were observed for OH-bup/bup, OH-flb/flb, and dor/dem MRs. OH-mdz/mdz MRs at the inhibition and induction sessions statistically differed from those of the cocktail-alone session at every sampling point for which the substances could be quantified (0.5–3 h). Most significant statistical differences ($P < 0.01$) in OH-opz/opz MRs were observed 2, 3, and 4 h after cocktail administration. MRs of par/caf significantly decreased at each time point for the inhibition session. At the induction session, a tendency toward an increase in MRs was observed, but the difference was statistically significant ($P < 0.05$) only 4 h after cocktail administration.

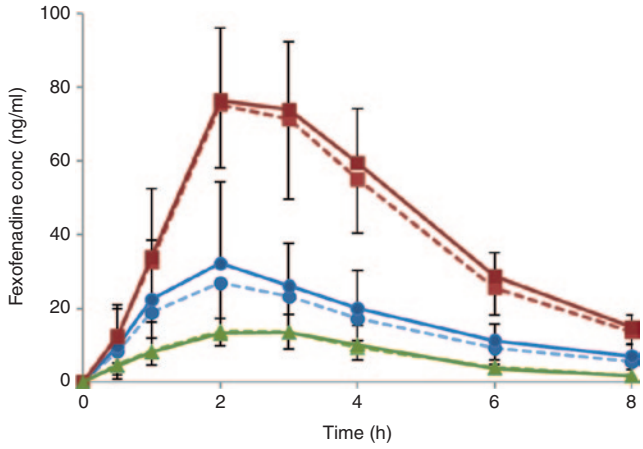

Figure 2 Concentration–time profile of fexofenadine in capillary DBS (dashed lines) and venous plasma samples (continuous lines) after administration of the cocktail capsule alone (circles), with a P-gp inhibitor (squares), or with a P-gp inducer (triangles) in 10 healthy volunteers. Error bars represent SD. DBS, dried blood spot; P-gp, P-glycoprotein.

The Spearman rank correlation coefficients (ρ_s) between the MRs at each time point and the AUC_{last} ratios for each session are shown in Table 3. The MRs of par/caf (0.5–8 h), OH-bup/bup (1–8 h), OH-flb/flb (2–8 h), and dor/dem (1–8 h) were highly correlated with their respective AUC_{last} ratios at each separate session ($\rho_s > 0.74$; $P < 0.015$). The OH-mdz/mdz MRs at 0.5, 1, and 2 h of each session significantly correlated with the AUC_{last} ratio ($\rho_s > 0.64$; $P \leq 0.043$). For OH-opz/opz, the correlation was significant at 2, 3, and 4 h of the cocktail-alone session and at the inhibition session ($\rho_s > 0.69$; $P \leq 0.025$), but no correlation was observed at the induction session.

Limited (three- or four-point) sampling strategy was evaluated for P-gp activity assessment, and the best correlation ($\rho_s \geq 0.964$; $P < 0.001$) for each session was observed between AUC_{last} and $AUC_{2,3,6}$ (Table 4). Limited $AUC_{2,3,6}$ sampling perfectly predicted the magnitude of interactions, as shown by the mean 3.0-fold $AUC_{2,3,6}$ (vs. 2.8-fold for AUC_{last}) increase after quinidine administration and 48% $AUC_{2,3,6}$ (vs. 45% for AUC_{last}) decrease after rifampicin pretreatment in the DBS method (Table 2).

DISCUSSION

We evaluated the utility of DBS sampling for simultaneous assessment of P-gp and CYP function using a seven-drug phenotyping cocktail. All the currently available cocktails require venous blood sampling, often accompanied by urinary sampling. Although this seems acceptable for clinical research studies, a less invasive method, using, for instance, capillary blood after a small finger prick, would enhance the acceptance of phenotyping in a clinical setting. Because plasma is the historically accepted matrix for drug analysis, it is important to evaluate the correlation between drug concentrations in plasma and capillary blood. We have previously shown that a great correlation

Table 2 Pharmacokinetic parameters of P-gp probe drug fexofenadine after oral administration of cocktail with and without quinidine and rifampicin

	DBS					Plasma				
Parameter	Cocktail alone	Cocktail + inhibitor	Ratio (95% CI)	Cocktail + inducer	Ratio (95% CI)	Cocktail alone	Cocktail + inhibitor	Ratio (95% CI)	Cocktail + inducer	Ratio (95% CI)
C_{max} (ng/ml)	28.1 ± 15.0	79.4 ± 18.6	3.14 (2.35–4.21)	14.4 ± 4.2	0.55 (0.42–0.73)	34.4 ± 21.2	82.4 ± 19.5	2.75 (1.84–4.12)	15.1 ± 4.7	0.49 (0.35–0.69)
T_{max} (h)	2.2 ± 0.8	2.4 ± 0.5	—	2.4 ± 0.7	—	2.5 ± 0.7	2.3 ± 0.5	—	2.8 ± 0.8	—
AUC_8 (h·ng/ml)	118.5 ± 49.8	316.8 ± 64.5	2.84 (2.15–3.75)	56.6 ± 15.1	0.50 (0.38–0.65)	139.6 ± 68.0	343.7 ± 69.1	2.66 (1.86–3.82)	59.2 ± 15.8	0.45 (0.34–0.60)
AUC_∞ (h·ng/ml)	145.2 ± 57.2	357.6 ± 71.8	2.69 (1.94–3.73)	61.2 ± 15.2	0.44 (0.33–0.60)	181.3 ± 78.0	388.2 ± 77.2	2.31 (1.51–3.53)	62.7 ± 12.0	0.41 (0.28–0.62)
$AUC_{2,3,6}$ (h·ng/ml)	100.9 ± 47.5	285.5 ± 67.1	3.05 (2.27–4.09)	50.4 ± 15.3	0.52 (0.39–0.71)	117.7 ± 58.3	306.3 ± 64.7	2.82 (2.00–3.99)	52.3 ± 15.1	0.47 (0.34–0.65)
$t_{1/2}$ (h)	2.6 ± 0.8	2.1 ± 0.3	—	1.8 ± 0.5	—	3.1 ± 1.5	2.1 ± 0.3	—	1.6 ± 0.1	—
Cl/F (L/h)	198.3 ± 81.3	72.5 ± 14.4	0.37 (0.27–0.52)	435.4 ± 124.6	2.25 (1.65–3.07)	161.3 ± 64.8	66.9 ± 14.4	0.43 (0.28–0.66)	411.0 ± 72.5	2.42 (1.62–3.62)

Data are presented as mean values ± SD and geometric mean ratios with 95% CIs.

AUC, area under the plasma concentration–time curve; CI, confidence interval; Cl/F, apparent clearance; C_{max}, maximum plasma concentration; DBS, dried blood spot; P-gp, P-glycoprotein; $t_{1/2}$, half-life; T_{max}, time to maximum plasma concentration.

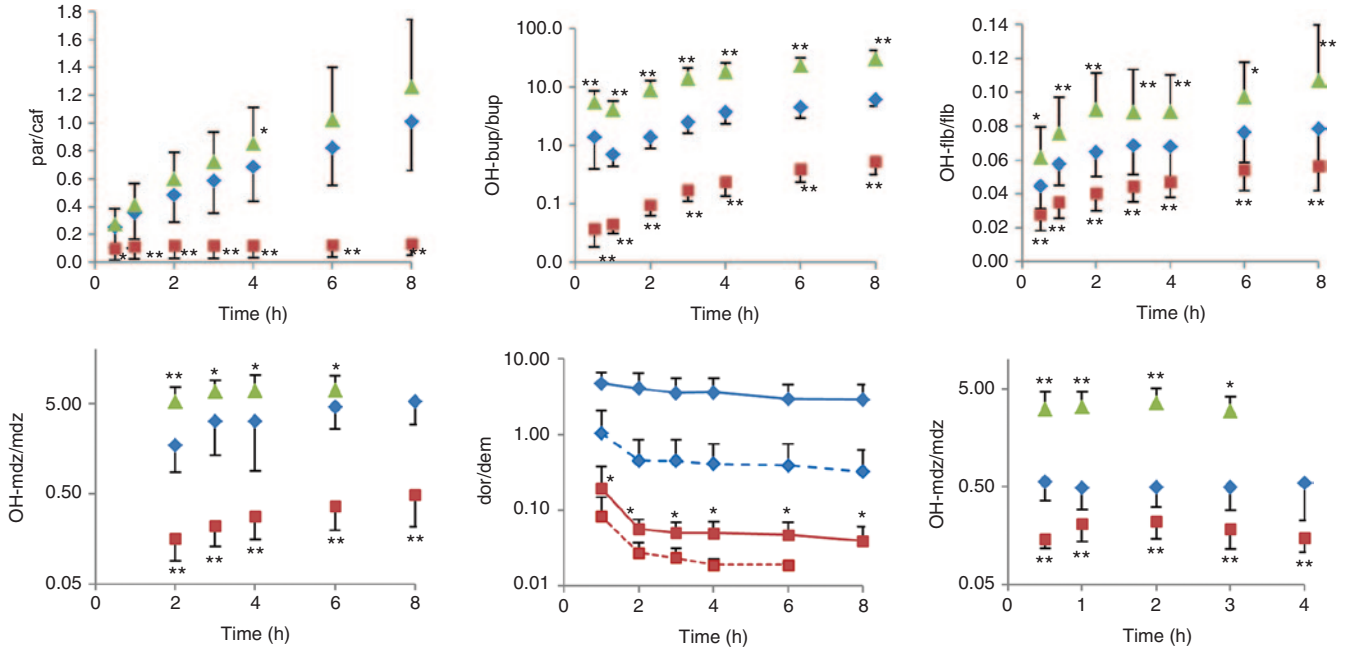

Figure 3 Metabolic ratio profiles after oral administration of cocktail capsule alone (diamonds), with an inhibitor (squares), or with an inducer (triangles) in dried blood spots. *$P < 0.05$; **$P < 0.01$. dor/dem: continuous lines are used for extensive metabolizers (EMs) and dashed lines for intermediate metabolizers (IMs). bup, bupropion; caf, caffeine; dem, dextromethorphan; dor, dextrorphan; mdz, midazolam; OH-bup, 4-hydroxybupropion; OH-flb, 4-hydroxyflurbiprofen; flb, flurbiprofen; OH-mdz, 1-hydroxymidazolam; OH-opz, 5-hydroxyomeprazole; opz, omeprazole; par, paraxanthine.

existed between the plasma and DBS concentrations of all probe drugs and their metabolites ($R^2 = 0.843–0.985$).[15] As a result, the pharmacokinetic profiles observed in this study and the magnitude of modulation of CYP and P-gp activities in the presence of inhibitors or the inducer were similar in the two matrices (see **Supplementary Figure S1 online** for details).

To further enhance phenotyping acceptance and to minimize the potential pharmacodynamic effect of the probe drugs, the probe doses used in this cocktail were at least two times lower than the lowest commercialized doses. Probe and metabolite concentrations were assessed using a single analytical method after a minimal sample pretreatment,[15] which represents a great advantage in terms of time and costs in comparison to previously published cocktails for which individual methods with complex sample preparation were used.[3–5]

The use of different matrices and the complicated sampling procedures are considered major drawbacks of many phenotyping cocktails. To overcome this limitation, several cocktails propose the use of single–time point plasma MRs or urinary MRs as phenotyping metrics. Although changes in metrics in the presence of inhibitors/inducers comprise one of the main validation criteria for phenotyping metrics, this question has been rarely investigated in the previously proposed cocktails.[16]

Almost all of the previously published cocktails included caffeine as a probe drug to evaluate CYP1A2 activity. The paraxanthine/caffeine ratio in plasma or urine is often used as a phenotyping index. Caffeine is only weakly bound to plasma proteins and enters into erythrocytes but does not bind to proteins there.[17] Therefore, its plasma concentrations were only slightly higher than the DBS concentrations, and the observed pharmacokinetic profiles were similar in the two matrices.

As expected,[18,19] fluvoxamine potently inhibited CYP1A2 activity, as shown by a decrease in the AUC_{last} ratio of par/caf and the MRs at different sampling times in DBS. Rifampicin is known to be only a weak inducer of CYP1A2. The administration of rifampicin slightly, but not significantly, increased paraxanthine/caffeine AUC_{last} ratio in DBS. These results are consistent with previously published data in which rifampicin increased the mean par/caf ratio 1.2- to 1.3-fold (4 h[20] and 6 h[21] plasma samples, respectively). In the current study, 4- and 6-h DBS MRs increased by 1.25-fold. Ryu *et al.*[3] have shown that an excellent correlation existed between the AUC_{last} ratio and the single- point MRs in plasma. These results were confirmed for DBS samples in our study, which also showed that an important correlation existed between these parameters even when CYP activity was modulated. The best correlation was observed at 2 h ($\rho_s \geq 0.927$; $P < 0.001$).

The CYP2B6 enzyme is involved in the metabolism of ~8% of clinically used drugs, including bupropion, efavirenz, methadone, propofol, cyclophosphamide, and tamoxifen.[22] Despite its importance in drug metabolism, most previously published cocktails did not include a probe for assessment of CYP2B6 activity. As suggested by individual CYP2B6-phenotyping studies[23,24] and by the European Medicines Agency,[25] bupropion was used as a probe drug in our cocktail. As expected, administration of voriconazole potently inhibited CYP2B6 and resulted in a 1.3-fold increase in bupropion AUC, which is in agreement with a previously published study.[26] Rifampicin-mediated induction led to an approximately threefold decrease in bupropion AUC, as

Table 3 Spearman rank correlations (ρ_s) between the AUC_{last} ratios of metabolite/probe and the metabolic ratios in DBS at various time points at different sessions

			Cocktail alone		Cocktail + inhibitor(s)		Cocktail + inducer	
CYP	Phenotyping indexes	Sampling time (h)	ρ_s	*P* value	ρ_s	*P* value	ρ_s	*P* value
1A2	[par]/[caf]	0.5	0.964	<0.001	0.973	<0.001	0.860	0.001
		1	0.960	<0.001	0.972	<0.001	0.927	<0.001
		2	**0.985**	**<0.001**	**0.982**	**<0.001**	**0.927**	**<0.001**
		3	0.988	<0.001	0.960	<0.001	0.900	<0.001
		4	0.960	<0.001	0.963	<0.001	0.927	<0.001
		6	0.815	0.004	0.979	<0.001	0.782	0.008
		8	0.794	0.006	0.884	0.001	0.742	0.014
2B6	[OH-bup]/[bup]	1	0.964	<0.001	0.910	<0.001	0.952	<0.001
		2	0.782	0.008	0.756	0.011	0.879	0.001
		3	**0.903**	**<0.001**	**0.827**	**0.003**	**0.927**	**<0.001**
		4	0.939	<0.001	0.930	<0.001	0.927	<0.001
		6	0.842	0.002	0.952	<0.001	0.952	<0.001
		8	0.745	0.013	0.869	0.001	0.830	0.003
2C9	[OH-flb]/[flb]	1	0.997	<0.001	0.402	0.249	0.927	<0.001
		2	0.982	<0.001	0.778	0.008	0.903	<0.001
		3	**0.979**	**<0.001**	**0.939**	**<0.001**	**0.964**	**<0.001**
		4	0.976	<0.001	0.982	<0.001	0.921	<0.001
		6	0.927	<0.001	1.000	<0.001	0.855	0.002
		8	0.930	<0.001	0.915	<0.001	0.857	0.002
2C19	[OH-opz]/[opz]	2	0.809	0.005	0.869	0.001	0.285	0.425
		3	0.806	0.005	0.867	0.001	0.479	0.162
		4	0.697	0.025	0.888	0.001	0.418	0.229
		6	0.612	0.060	0.891	0.001	0.750	0.052
		8	0.511	0.132	0.924	<0.001	0.200	0.747
	$\frac{AUC_{2,3,6\ OH\text{-}opz}}{AUC_{2,3,6\ opz}}$	**2, 3, 6**	**0.855**	**0.002**	**0.964**	**<0.001**	**0.855**	**0.002**
2D6	dor/dem	1	0.891	<0.001	0.745	0.013	—	—
		2	1.000	<0.001	0.948	<0.001	—	—
		3	**1.000**	**<0.001**	**0.964**	**<0.001**	—	—
		4	0.988	<0.001	0.979	<0.001	—	—
		6	0.976	<0.001	0.918	<0.001	—	—
		8	0.976	<0.001	0.821	0.023	—	—
3A4	[OH-mdz]/[mdz]	0.5	0.830	0.003	0.862	0.001	0.697	0.025
		1	0.915	<0.001	0.760	0.011	0.647	0.043
		2	**0.745**	**0.013**	**0.742**	**0.014**	**0.673**	**0.033**
		3	0.745	0.013	0.762	0.010	0.500	0.391
		4	0.442	0.200	0.738	0.015	—	—
		6	0.895	0.001	0.911	<0.001	—	—

Chosen phenotyping indexes are shown in bold.

AUC_{last}, AUC from 0 to 8 h, except for mdz and OH-mdz at the induction session, when AUC_{last} = AUC from 0 to 3 h.

AUC, area under the plasma concentration–time curve; bup, bupropion; caf, caffeine; CYP, cytochrome P450; DBS, dried blood spot; dem, dextromethorphan; dor, dextrorphan; mdz, midazolam; OH-bup, 4′-hydroxybupropion; OH-flb, 4′-hydroxyflurbiprofen; OH-mdz, 1′-hydroxymidazolam; OH-opz, 5′-hydroxyomeprazole; opz, omeprazole; par, paraxanthine.

Table 4 Spearman rank correlations (ρ_s) between fexofenadine AUC_{0-8} and different three- or four-point fexofenadine AUCs in DBS at different sessions

Sampling points used for AUC determination (h)	Cocktail alone		Cocktail + inhibitor(s)		Cocktail + inducer	
	ρ_s	*P* value	ρ_s	*P* value	ρ_s	*P* value
1, 2, 4	0.952	<0.001	0.685	0.029	0.842	0.002
2, 3, 4	0.976	<0.001	0.806	0.005	0.939	<0.001
2, 3, 6	**0.976**	**<0.001**	**0.964**	**<0.001**	**0.964**	**<0.001**
2, 4, 6	0.976	<0.001	0.915	<0.001	1.000	<0.001
1, 2, 4, 6	0.988	<0.001	0.939	<0.001	1.000	<0.001

Chosen limited-sampling AUC is shown in bold.

AUC, area under the plasma concentration–time curve; DBS, dried blood spot.

previously observed.[23] The inhibition and induction were much more obvious when OH-bup/bup AUC_{last} ratio or single-point MRs were used as phenotyping indexes (**Table 1** and **Figure 3**). At each session, AUC_{last} ratios most strongly correlated with DBS MRs at 4 h ($\rho_s \geq 0.927$; $P < 0.001$), but important and significant correlations were also observed at 1, 3, and 6 h.

Several drugs have been suggested as potentially useful probes for CYP2C9 phenotyping, including tolbutamide, phenytoin, warfarin, and losartan. To palliate the limitations of each of these drugs, flurbiprofen was recently validated as a reliable probe for CYP2C9 phenotyping.[27] A previous study has demonstrated the utility of DBS sampling for individual CYP2C9 activity assessment.[12] The pharmacokinetic profiles of flb and OH-flb in both DBS and plasma were similar to those observed in our study. Despite the use of different inhibitors (fluconazole vs. voriconazole + fluvoxamine), the magnitude of inhibition was comparable in the two studies. The magnitude of CYP2C9 induction by rifampicin was in agreement with previously published studies in which flurbiprofen[12] or losartan[20,28] was used as the probe drug.

Omeprazole is frequently used as a probe to assess CYP2C19 activity. It is metabolized by both CYP3A and CYP2C19, with the transformation of omeprazole to 5-hydroxyomeprazole being predominantly mediated by CYP2C19. Omeprazole hydroxylation (opz/OH-opz) as a phenotyping index has been shown to correlate with the hydroxylation of *S*-mephenytoin and also reliably predicts *CYP2C19* genotypes.[29] Although *in vitro* studies indicate an inhibitory effect of omeprazole on CYP3A activity, previous cocktail studies have shown that omeprazole does not influence the pharmacokinetic parameters of midazolam *in vivo*. An inhibitory effect of omeprazole on CYP2C19 activity is suspected, but this is not expected to occur at a 5-mg dose. However, one should be aware of this risk if higher omeprazole doses are used for phenotyping.

Important modulation of CYP2C19 activity occurred in the presence of inhibitors (fluvoxamine and voriconazole) and an inducer (rifampicin), as seen by modification of OH-opz/opz AUC ratio. Previous studies have reported a similar extent of CYP2C19 induction by rifampicin (4.2- and 2.6-fold).[20,28] These and other studies[2,3] have used a single-point opz/OH-opz MR at 3 or 4 h as a phenotyping index. Ryu *et al.*[3] have shown a good correlation between the AUC_{last} ratio and single-point MRs in plasma. In our study, good correlation between these parameters was observed in both plasma and DBS at both the cocktail-alone and inhibition sessions, but the correlation was nonsignificant at the induction session. Despite the lack of correlation, the 2-, 3-, and 4-h MRs at the induction session were significantly higher than those of the cocktail-alone session, indicating that induction of CYP2C19 activity could be predicted by a single-point MR. However, the use of limited-sample (2-, 3-, and 6-h) AUC ratios, which significantly correlate with AUC_{last} ratios at every session ($\rho_s \geq 0.855$, $P \leq 0.002$), might constitute a better phenotyping index than the use of single-point MRs, especially if CYP2C19 induction is suspected.

In a review comparing several CYP2D6-phenotyping drugs, dextromethorphan and debrisoquine were considered the best probes, with debrisoquine having a very limited availability.[30] The urinary MR of dem/dor has been frequently used as a phenotyping index. This metric is sensitive to phenomena such as urinary pH changes and may therefore contribute to an increased variability, which can be overcome by the use of plasma AUC ratios. Moreover, AUC ratios showed a much better correlation with dem oral clearance than did urinary MRs.[31] Excellent correlation between 3-h plasma MR and AUC ratios was previously reported.[32] We have further confirmed a strong correlation with DBS MRs at 2, 3, or 4 h ($\rho_s \geq 0.948$, $P < 0.001$), both when the enzymatic function was normal and when it was inhibited by quinidine. As expected, quinidine strongly and almost completely inhibited CYP2D6 function, as shown by decreases in AUC ratios and single-point MRs. In addition to the modulation in the presence of inhibitor, DBS single-point MR was able to reliably distinguish genotypic extensive metabolizers from IMs(**Figure 3**). The MRs and AUC ratios in IMs were approximately eight times lower in comparison with those of extensive metabolizers at the cocktail-alone session. At the inhibition session, the production of dextromethorphan was also lower in IMs.

The majority of studies evaluating CYP3A activity have used midazolam as a probe drug. Many studies have evaluated the possibility of using limited-sampling methods with either single-point mdz concentration or single-point OH-mdz/mdz MRs.[3,33,34] In this study, MRs from 0.5 to 2 h correlated with AUC ratios at every session. Voriconazole significantly inhibited CYP3A and lowered OH-mdz/mdz AUC ratio to 0.34-fold of the baseline AUC ratio. The inhibition was slightly lower than that reported in a previous study (0.26-fold reduction in AUC ratio) in which voriconazole was administered for 2 days (400 mg b.i.d. the first day and 200 mg b.i.d. the second day).[35] The difference in dose and duration of voriconazole administration can explain the slight difference in the results. At the induction session, OH-mdz and mdz could be reliably quantified until 3 h after cocktail administration. Differences exist regarding the magnitude of CYP3A induction by rifampicin in previously published studies. A 3.3-fold increase in AUC ratio has been reported by Inui *et al.*,[28] whereas Backman *et al.*[36] found a 9.5-fold increase. The increase observed in the current study is between these two

values. The use of 1- or 2-h DBS MRs instead of AUC ratios resulted in the same interaction magnitude for both induction and inhibition.

Digoxin, which has a narrow therapeutic index, and fexofenadine are the most commonly used and currently recommended *in vivo* P-gp phenotyping probes. Whereas digoxin is mostly used for renal P-gp function assessment, a drug with lower oral bioavailability such as fexofenadine is recommended for intestinal P-gp function assessment.[25] The inhibition of the efflux transporter P-gp by quinidine leads to an increase in fexofenadine absorption, hence increasing its AUC and C_{max}, as observed. The opposite effect is observed in the case of P-gp induction by rifampicin, with a twofold decrease in AUC and C_{max} and an increase in oral clearance. This is in agreement with a previously published study in which rifampicin and fexofenadine were administered in a staggered manner.[37] Full fexofenadine AUC determination can be tedious and requires multiple sampling. In this study, we have shown that a three-point fexofenadine $AUC_{2,3,6}$ strongly correlated with AUC_{last} and could reliably predict the interaction magnitudes. It can thus be used as a limited-sampling strategy for P-gp activity assessment after administration of 25 mg of fexofenadine.

An ideal phenotyping probe is a substrate for a transporter or an enzyme which is completely specific. Because currently we do not dispose of completely specific probes, it is essential to choose probes with a degree of selectivity sufficient to reflect enzyme/transporter activity in various settings.[16] All of the probes included in this cocktail, with the exception of flurbiprofen, have been listed as sensitive CYP/P-gp substrates by the US Food and Drug Administration.[38] The validity of these probes/metrics has been evaluated in previous studies using different criteria such as correlation of the metrics to *in vivo* expression and activity of the proteins, accurate reflection of known genetic polymorphisms, or modulation of the metric in the presence of known inhibitors/inducers.[12,16,23,29,30,39,40] The use of metabolic or AUC ratios is advantageous, especially for probes having more than one metabolizing path, such as omeprazole or dextromethorphan, because they give information on the activity of a single CYP implicated in one specific pathway. If the metabolite formed is further metabolized, i.e., conjugated, one should be aware of the effect a modification in conjugating enzyme activities can have on the MRs, although the activity of these enzymes is often less affected by various disease states or drugs.

In the current study, the main limitation lies in the fact that concomitant use of the probe substrates has not been previously validated. Several cocktail studies have demonstrated the lack of interaction between caffeine, omeprazole, dextromethorphan, and midazolam.[3,41] We have previously validated the use of flurbiprofen with the above-mentioned probes[42] and this drug has been successfully incorporated into other cocktails.[6] Fexofenadine has been used in combination with some of the probes and is not expected to be influenced or to influence the pharmacokinetics of the other probes.[43,44] A few studies have shown that bupropion at steady state (150 mg b.i.d. for >2 weeks) can inhibit CYP2D6.[45,46] Nevertheless, no evidence exists that bupropion would inhibit CYP2D6 when administered as a single dose. It is considered that CYP2D6 inhibition is mainly mediated by bupropion's metabolites threohydrobupropion and erythrohydrobupropion, which accumulate after multiple administrations. The inhibition constant (K_i) values for bup, OH-bup, threohydrobupropion, and erythrohydrobupropion are 21, 13.3, 5.4, and 1.7 μmol/l, respectively.[47] After a single 75-mg bupropion dose, the maximal plasma concentrations were 0.49, 0.52, 0.24, and 0.03 μmol/l, respectively.[48] In our study, the highest plasma concentrations were observed at the inhibition session for bup (C_{max} = 0.15 μmol/l) and at the induction session for OH-bup (C_{max} = 0.35 μmol/l). When taking into account the free plasma fraction (0.16–0.58) and a liver/plasma ratio of 5.5–9.4,[47] the maximal estimated liver concentrations for bup, OH-bup, threohydrobupropion, and erythrohydrobupropion would be 0.73, 1.13, 1.31, and 0.16 μmol/l after a 75-mg single dose and would be approximately 3 times less after a single 25-mg dose (0.24, 0.38, 0.44, and 0.05 μmol/l). These concentrations are >10 times lower than the K_i values, indicating that a single dose of 25-mg bupropion should not have an inhibitory effect on CYP2D6. However, further validation is necessary to confirm the lack of interactions within the cocktail.

After this pilot study, larger population studies should be conducted to evaluate the distribution of CYP and P-gp activities and to establish normal activity ranges, allowing the prediction of an individual's phenotype. The clinical utility of phenotyping can be evaluated in studies comparing the clinical outcomes of phenotype-guided vs. non–phenotype guided therapeutic dosing.

In conclusion, this study demonstrated the utility of the DBS-sampling technique for assessment of the activities of six CYP isoforms and P-gp using a low-dose phenotyping cocktail. MRs at 2 (CYP1A2 and CYP3A) and 3 h (CYP2B6, CYP2C9, and CYP2D6), limited-sampling $AUC_{2,3,6}$ ratio (CYP2C19), or limited-sampling $AUC_{2,3,6}$ (P-gp) can be used as phenotyping metrics. These metrics using three DBS samples 2, 3, and 6 h after dosing were chosen based on their reliability for predicting both normal and altered CYP and P-gp activities. Once validated for the lack of interactions, this low-dose, high-throughput cocktail method can be applied as a tool in studies of drug–drug interactions or can be used in clinical practice.

METHODS

Subjects. This study (registration NCT01731067) was approved by the ethics committee of Geneva University Hospitals (ID: 12–085) and the Swiss Agency for Therapeutic Products (Swissmedic).

After giving written informed consent, 10 healthy male volunteers were included in the study. Their median age was 23 years (range: 20–36 years) and median body mass index was 22.0 (range: 19.9–24.4) (see **Supplementary Table S1** online for details). One subject was from North Africa, one was of mixed Caucasian and African origin, and all the others were Caucasians. All of the subjects were nonsmokers and had normal results on physical examination, electrocardiogram, and liver function test and were not taking any medications. Poor CYP2D6 (**4/*4*; **4/*5*; **5/*5*), CYP2C9 (**2/*2*; **2/*3*; **3/*3*), or CYP2C19 (**2/*2*) metabolizers were not included in the study. Subjects were not permitted to drink grapefruit juice for at least 1 week before and throughout the study period

and were required to abstain from alcohol and caffeine-containing products at least 48 h before each study session.

Study design. This open-label study was performed in four sessions with at least 1-week washout period between each session. Cocktail capsules containing low-dose bupropion (25 mg), flurbiprofen (25 mg), dextromethorphan (10 mg), omeprazole (5 mg), midazolam (1 mg), and fexofenadine (25 mg) were produced by the pharmacy of Geneva University Hospitals under good manufacturing practice conditions. At each session, after an overnight fast, volunteers received an oral cocktail capsule together with a cup of coffee (caffeine: 100 mg) or Coke (caffeine: 25 mg). They were given their first meal 1 h after cocktail administration and a second meal 3 h later. All the volunteers received the treatments in the same order. At session 1, volunteers received only the cocktail capsule with coffee or Coke. At session 2, volunteers received one dose of fluvoxamine (50 mg) 12 h before cocktail administration, followed by a second dose of fluvoxamine (50 mg) together with voriconazole (400 mg) 2 h before cocktail administration (CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A inhibition). At session 3, each subject was given 200 mg of quinidine (CYP2D6 and P-gp inhibitor) 2 h before cocktail administration. The CYP and P-gp inhibition was divided in two sessions due to the potential interaction (QT interval prolongation) between voriconazole and quinidine. Seven days before the fourth session, volunteers were given seven tablets of rifampicin (600 mg) and were asked to take one tablet every evening until the evening before the session (induction of CYP isoforms and P-gp). They were specifically asked about their compliance with the rifampicin regimen.

At each session, capillary and venous blood samples were collected simultaneously before and at 0.5, 1, 2, 3, 4, 6, and 8 h after cocktail administration. Capillary whole blood (10 µl) was collected on a Whatman 903 filter paper card (Westborough, MA) after a small finger prick (BD Microtainer; Contact-Activated Lancet, Plymouth, UK) using a volumetric micropipette (Rainin, Oakland, CA), whereas venous blood was collected into tubes containing EDTA (BD Vacutainer, Plymouth, UK). Plasma from venous blood was obtained after centrifugation at 2,500 rpm for 10 min and stored together with dried DBS cards at −20 °C.

Analytical methods. The cocktail substrates and their CYP-specific metabolites were quantified in plasma and DBS using a single reverse-phase high-performance liquid chromatography–tandem mass spectrometry method operating in dual electrospray ionization mode, as previously described.[15] The substances of interest were extracted from DBS samples using methanol, whereas protein precipitation using acetonitrile was used for plasma extraction. Both methods (those for DBS and plasma) were fully validated according to international criteria.[15]

Genotyping. Genomic DNA was extracted from whole blood (200 µl) using the QIAamp DNA Blood Mini Kit (Qiagen, Switzerland). The detection of *CYP2C19* (alleles **2* and **3*) and 33 *CYP2D6* alleles was performed using the AmpliChip CYP450 test (Roche Diagnostics, Rotkreuz, Switzerland) as previously described.[49] *CYP2B6*6* and *CYP2C19*17* genotypes were determined using commercially available TaqMan SNP genotyping assays. *CYP2C9*2* and *CYP2C9*3* genotypes, as well as *ABCB1* G2677T/A and C3435T polymorphisms, were determined by means of multiplex PCR with fluorescent probes (Lightmix; TibMolbiol, Berlin, Germany) and melting curve analysis on a LightCycler480 (Roche Diagnostics).

Data analysis. Pharmacokinetic parameters were estimated by standard noncompartmental methods using WinNonlin version 6.2.1 (Pharsight, Mountain View, CA). The results are presented as mean values (± SD) and geometric mean ratios with 95% confidence intervals. Single-point MRs were determined as the concentration ratio between the metabolite and the administered substance, i.e., par/caf, OH-bup/bup, OH-flb/flb, OH-opz/opz, dor/dem, and OH-mdz/mdz, at different time points. MRs at the inhibition or induction sessions were compared with those of the cocktail-alone session using a nonparametric Wilcoxon signed-rank test. Correlation between AUC ratios and single-point MRs was established using Spearman's rank correlation coefficient. All statistical analyses were performed using SPSS software version 21 (Chicago, IL). P values ≤ 0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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AUTHOR CONTRIBUTIONS

M.B., Y.D., C.F.S., J.A.D., and M.R. wrote the manuscript; M.B., C.F.S., B.W., P.D., J.A.D., and Y.D. designed the research; M.B., C.F.S., and Y.D. performed the research; M.B., J.D., M.R., and Y.D. analyzed the data; J.D. and C.S. contributed new reagents/analytical tools.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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