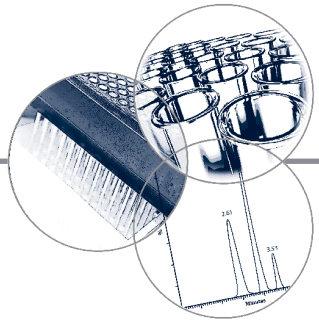

Simultaneous LC–MS/MS quantification of P-glycoprotein and cytochrome P450 probe substrates and their metabolites in DBS and plasma

Background: An LC–MS/MS method has been developed for the simultaneous quantification of P-glycoprotein (P-gp) and cytochrome P450 (CYP) probe substrates and their Phase I metabolites in DBS and plasma. P-gp (fexofenadine) and CYP-specific substrates (caffeine for CYP1A2, bupropion for CYP2B6, flurbiprofen for CYP2C9, omeprazole for CYP2C19, dextromethorphan for CYP2D6 and midazolam for CYP3A4) and their metabolites were extracted from DBS (10 µl) using methanol. Analytes were separated on a reversed-phase LC column followed by SRM detection within a 6 min run time. **Results:** The method was fully validated over the expected clinical concentration range for all substances tested, in both DBS and plasma. The method has been successfully applied to a PK study where healthy male volunteers received a low dose cocktail of the here described P-gp and CYP probes. Good correlation was observed between capillary DBS and venous plasma drug concentrations. **Conclusion:** Due to its low-invasiveness, simple sample collection and minimal sample preparation, DBS represents a suitable method to simultaneously monitor *in vivo* activities of P-gp and CYP.

The **cytochrome P450** (CYP) enzymes are the major drug-metabolizing enzyme system in humans. Inter-individual variability affects the activity of these enzymes, and consequently drug clearance. Besides the metabolizing enzymes, another source of PK variability in drug response are the influx and efflux proteins, such as **P-glycoprotein** (P-gp). The PK variability and the modifications in CYP and/or transporter activities (such as P-gp) can cause various pharmacological and toxicological consequences. It is therefore important to precisely and reliably evaluate their *in vivo* activity (**phenotyping**). Phenotyping tests can be either individual (evaluating the activity of a single cytochrome or transporter) or simultaneous (assessing the activity of multiple enzymes/transporters). Simultaneous phenotyping is performed by the *in vivo* administration of a **cocktail** of probe drugs, each of which is metabolized by one specific cytochrome or transported by P-gp, followed by the determination of an appropriate PK parameter of the probe drug or a ratio between the drug and its metabolite (metabolic ratio) [1].

Several probes have been validated and used to assess the activity of the most important CYPs and P-gp. Among them, caffeine (CAF) is most widely used as a probe for CYP1A2; bupropion (BUP) has been proposed by the European Medicines Agency as a probe for 2B6 activity [101]; flurbiprofen (FLB) is used for CYP2C9 phenotyping [2,3]. CYP2C19 activity can be assessed using omeprazole (OPZ); dextromethorphan (DEM) and midazolam (MDZ) are used as probes for CYP2D6 and CYP3A4/5, respectively [4,5], while P-gp transporting activity can be evaluated by the administration of fexofenadine (FEX) [6,7]. When a cocktail approach is used, it is important to overcome the problem of potential drug–drug interactions between the various substrates. The probability of such interactions may be minimized by the use of low probe drug doses [8,9]. The use of low doses also has the advantage of diminishing the risk of adverse effects, but requires the development of sensitive analytical methods.

Most of the currently validated phenotyping procedures require tedious venous blood or 8 h urine sample collection. A novel and promising approach for CYP activity phenotyping is the use of DBS as a sampling procedure. In the past few years DBS has increased in popularity since it offers several advantages over conventional whole blood or plasma sampling. Due to the low blood volume required, obtained from a small finger prick, this method is less invasive and

Marija Bosilkovska[1], Julien Déglon[2], Caroline Samer[1], Bernhard Walder[3], Jules Desmeules[1], Christian Staub[2] & Youssef Daali*[1]

[1]Division of Clinical Pharmacology & Toxicology, Geneva University Hospitals, Rue Gabrielle Perret-Gentil 4, 1211, Geneva, Switzerland
[2]Unit of Toxicology, University Center of Legal Medicine, Geneva, Switzerland
[3]Division of Anesthesiology, Geneva University Hospitals, Geneva, Switzerland
*Author for correspondence:
Tel.: +41 223 795 430
Fax: +41 223 829 945
E-mail: youssef.daali@hcuge.ch

FUTURE SCIENCE part of fsg

Key Terms

Cytochrome P450: Superfamily of enzymes responsible for the Phase I metabolism of a large amount of drugs.

P-glycoprotein: Efflux transporter responsible for the active transport of a wide variety of endogenous and exogenous substances (drugs) out of the cell.

Phenotyping: Measuring of the *in vivo* activity of an enzyme or transporter (using a probe drug metabolized by a specific enzyme isoform or transported by a specific transporter).

Cocktail: Mix of substances (often drugs) each of which is specifically metabolized or transported by one enzyme isoform or transporter. Used for the simultaneous determination of the function of several enzymes and/or transporters.

more patient-friendly [10]. Moreover, DBS sampling does not require the use of anticoagulant or plasma separation and can be easily stored and shipped to analytical laboratories without using refrigerated devices, which also makes it more cost effective in comparison with the conventional sampling methods [11].

The DBS sampling method has been successfully applied for the determination of drug concentrations in numerous TK and PK studies [12–16], as well as for therapeutic drug monitoring [17–20]. Some studies have shown that DBS sampling could also be used for individual cytochrome phenotyping of either CYP2C9 [2,21] or CYP3A [22] activities. The utility of DBS sampling for cytochrome activity assessment has been underlined in a recent study, where five individual analytical methods for the quantification of CYP probe substrates in DBS have been developed [23].

This article describes the development, validation and application of a single method for the quantification of six CYP specific probe substrates (CAF, BUP, FLB, OPZ, DEM and MDZ) and their metabolites, as well as a P-gp substrate (FEX) in DBS and plasma.

Experimental

■ Chemicals & reagents

CAF, paraxanthine (PAR), FLB, OPZ and FEX were purchased from Sigma–Aldrich (Buchs, Switzerland). DEM, dextrorphan (DOR), MDZ and 1-hydroxy-MDZ (OH-MDZ) were kindly donated by Hoffman-La-Roche (Basel, Switzerland). BUP, hydroxybupropion (OH-BUP) and MDZ-d4 were purchased from Cerilliant (TX, USA). 5-hydroxyOPZ (OH-OPZ), 4-hydroxy-FLB (OH-FLB), OPZ-d3 and FLB-d3 were purchased from Toronto Research Chemicals (ON, Canada). Both acetonitrile (ACN) and methanol (MeOH) were of HPLC grade from Merck (Darmstadt, Germany). Fresh human CAF and drug-free blood and plasma with EDTA as anticoagulant were supplied by Geneva University Hospitals (Geneva, Switzerland). Stock solutions of each analyte at a concentration of 1 mg/ml were prepared in MeOH, except for OH-BUP in ACN, and were stored at -20°C.

Working standard solutions were prepared by dilution of the stock solutions in MeOH to reach concentration of 5000 ng/ml for CAF, PAR and FLB; 1000 ng/ml for OH-FLB; 500 ng/ml for DOR; 200 ng/ml for BUP, OH-BUP, OPZ, OH-OPZ, DEM, OH-MDZ and FEX; and 100 ng/ml for MDZ.

■ Preparation of calibration standards & QC samples

Spiked blood and plasma needed for the calibrators and QCs were prepared using fresh EDTA whole blood or plasma, respectively. Appropriate volumes of the working standard solutions were evaporated in Eppendorf tubes and whole blood or plasma was then added into the tubes to reach the desired concentrations.

Plasma and DBS calibration standards and QCs were prepared using separate working solutions.

■ Sample pretreatment

DBS

A 10 µl volume of real or spiked whole blood was spotted onto a filter paper 903 protein saver card from Whatman (MA, USA) using a volumetric (0.1–10 µl) micropipette (Rainin, CA, USA). The DBS collection cards were bent so that the back of the card was not in contact with any surface to prevent loss of blood that soaked through the filter paper. DBS samples were allowed to dry at room temperature for at least 1 h and were then packed in a sealable plastic bag containing desiccant until analysis (no later than 15 days postspotting). They were stored in the dark at ambient temperature, except for the clinical study samples (at -20°C), and stability experiments in which various storing temperatures were tested.

Discs of 6 mm diameter covering the entire DBS were punched out and folded into the bottom of individual LC vials containing a 300 µl inert insert. Using the whole spot in association with a previous volumetric control has the advantage of overcoming the impact that hematocrit can have on the spreading of the applied drop of blood. For the extraction, MeOH (100 µl) containing the IS (MDZ-d4 1 ng/ml, OPZ-d3 5 ng/ml and FLB-d3 50 ng/ml) was added to each vial. The vials were then sealed, vortex-mixed and positioned in the LC rack.

Plasma

Spiked or real plasma samples (50 µl) were put in an eppendorf tube to which 5 µl of ACN containing the IS (100 ng/ml of MDZ-d4, 500 ng/ml of OPZ-d3 and 5000 ng/ml of FLB-d3) was added. A protein precipitation (PP) was performed by adding 195 µl of ACN, followed by vortex-mixing. The samples were then centrifuged for 3 min at 10000 rpm. The supernatant (50 µl) was added to a LC vial and diluted with water (1:1).

LC–MS/MS analysis

Blood (i.e., DBS or plasma) analysis was performed using a LC–MS/MS system consisting of a 5500QTrap® triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer equipped with a TurboIon Spray™ interface (AB Sciex, ON, Canada) and an Ultimate 3000 RS instrument (Dionex, CA, USA) as LC system. Data were acquired and processed using Analyst software (version 1.5.2; AB Sciex, Toronto, Canada).

Before injection, the autosampler needle was filled with 50 µl of water by means of the user defined program function. Then, 5 µl of DBS or plasma extract was injected into the LC–MS/MS system. To avoid any contact with DBS samples folded into the vial, the needle height was set at 6 mm.

The chromatographic separation was conducted on a 50 mm length × 2.1 mm internal diameter, 2.6 µm particle size, Kinetex™ RP C18 XB column (Phenomenex, CA, USA). The mobile phase consisted of a mixture of H_2O and ACN set at a flow rate of 0.6 ml/min. A linear gradient was employed from H_2O/ACN (98/2, v/v) to H_2O/ACN (2/98, v/v) over 2 min. These proportions were maintained for 1 min before re-equilbrating the column with the initial conditions. The method featured a 6 min total run-time per analysis.

The MS TurboIonSpray interface was operated in dual ionization mode with continuous polarity switching. The capillary potential was of +4500 and -4000 V for the positive and negative ESI modes, respectively. Nitrogen was used as the curtain and nebulizer gas, and the source parameters were set to a temperature of 650°C, an entrance potential of 10 V, a collision cell exit potential of 10 V, a curtain gas pressure of 20 psi, a nebulizer gas (GS1) pressure of 30 psi and an auxiliary gas (GS2) pressure of 40 psi.

The MS detection was operated in SRM mode based on collision-induced dissociation occurring in the collision cell (quadrupole two). The dwell-time was set to 4 ms. All other settings were analyte-specific and were determined using Analyst software in compound optimization mode. Quantification transitions with parameters are listed in Table 1.

Method validation

The method was fully validated according to the guidelines of the European Medicines Agency [102].

For each of the 3 nonconsecutive days, calibration standards were prepared in duplicate (n = 2) at seven concentration levels for CAF, PAR, BUP, OH-BUP, FLB, OH-FLB and fexofenadine, at eight concentration levels for OPZ and OH-OPZ, and at nine concentration levels for DEM, DOR, MDZ and OH-MDZ, which have the largest calibration range (Table 1). QC samples were prepared in whole blood or plasma in quadruplicate (n = 4) at four concentration levels representing

Table 1. Calibration range and LC–MS/MS settings for the analytes and their respective IS.

Analyte	IS	Retention time (min)	Calibration range (ng/ml)	SRM transition (*m/z*)	Polarity	CE (V)	DP (V)	CXP (V)	Estimated LOD (ng/ml)
Caffeine	mida-d4	1.87	25–5000	195 → 138	+	37	180	10	5
Paraxanthine	mida-d4	1.68	25–5000	181 → 124	+	27	120	10	1
Bupropion	mida-d4	2.55	1–200	240 → 131	+	30	80	10	0.1
OH-bupropion	mida-d4	2.40	1–200	256 → 139	+	43	100	14	0.3
flurbi	flurbi-d3	2.74	25–5000	243 → 199	-	-18	-50	-15	1
OH-flurbi	flurbi-d3	2.40	5–1000	259 → 215	-	-12	-25	-15	1
omep	omep-d3	2.25	0.4–200	346 → 198	+	19	66	10	0.1
OH-omep	omep-d3	2.10	0.4–200	362 → 214	+	17	66	12	0.1
Dextromethorphan	mida-d4	2.65	0.2–200	272 → 128	+	85	150	8	0.05
Dextrorphan	mida-d4	2.36	0.5–500	258 → 157	+	50	160	18	0.1
mida	mida-d4	2.57	0.1–100	326 → 291	+	37	166	18	0.03
OH-mida	mida-d4	2.40	0.2–200	342 → 324	+	31	180	16	0.05
Fexofenadine	mida-d4	2.68	1–200	502 → 466	+	45	160	10	0.3
IS									
mida-d4	NA	2.57	NA	330 → 295	+	37	166	10	NA
omep-d3	NA	2.25	NA	349 → 198	+	35	66	10	NA
flurbi-d3	NA	2.74	NA	246 → 202	-	-18	-50	-15	NA

LODs were set at three-times the S/N.
CE: Collision energy; CXP: Collision cell exit potential; DP: Declustering potential; flurbi: Flurbiprofen; mida: Midazolam; omep: Omeprazole.

the entire range of concentrations tested. The individual QC concentrations for each analyte are listed in Tables 2 & 3. The validation process allowed the determination of specific criteria such as accuracy, precision, linearity, LLOQ and LOD.

The linearity of the method was evaluated by back-calculating QC samples using the calibration curve. LLOQ was determined as the lowest QC with accuracy and precision under 20%, while LOD was set at three-times the S/N ratio.

Selectivity was tested by analyzing six human blank DBS and plasma samples from different sources. Carryover was investigated by injecting a blank sample after the analysis of the highest

Table 2. DBS validation data.

Compound	Nominal concentration (ng/ml)	Determined concentration (ng/ml)	Accuracy (%)	Intra-day precision (%RSD)	Inter-day precision (%RSD)
Caffeine	25	24.7	98.9	7.3	8.8
	125	129	102.8	5.8	5.8
	1250	1245	99.6	4.2	9.1
	5000	5143	102.9	4.9	7.3
Paraxanthine	25	25.8	103.2	4.7	7.2
	125	119	95.5	4.7	4.7
	1250	1152	92.2	3.9	6.2
	5000	4970	99.4	5.1	8.5
Bupropion	1	1.04	104.0	2.3	5.3
	5	5.07	101.4	3.9	3.9
	50	48.6	97.3	2.2	4.0
	200	204	101.8	3.3	5.3
OH-bupropion	1	1.05	104.5	4.0	4.8
	5	4.94	98.8	3.3	4.3
	50	47.3	94.6	3.4	3.5
	200	200	100.1	4.1	5.2
Flurbiprifen	25	24.3	97.4	4.0	4.0
	125	121	96.8	4.4	4.6
	1250	1312	104.9	1.7	3.6
	5000	4705	94.1	2.4	2.4
OH-flurbiprifen	5	4.93	98.6	3.4	3.4
	25	24.3	97.1	1.6	2.3
	250	250	99.8	3.5	3.8
	1000	1022	102.2	5.3	5.4
Omeprazole	0.4	0.445	111.1	2.8	2.8
	5	5.03	100.7	2.2	5.1
	50	51.1	102.3	4.1	4.3
	200	194	97.2	4.3	4.5
OH-omeprazole	0.4	0.423	105.8	3.6	4.1
	5	4.88	97.6	3.3	8.4
	50	49.6	99.3	3.2	5.9
	200	206	102.8	4.7	6.4
Dextromethorphan	0.2	0.215	107.7	3.9	4.5
	2	2.09	104.7	2.7	3.6
	50	48.1	96.1	2.6	2.6
	200	201	100.5	3.8	4.7
Dextrorphan	0.5	0.523	104.6	4.3	5.6
	5	5.11	102.1	5.0	7.5
	125	124	99.0	3.9	6.5
	500	494	98.8	3.7	7.1

Analyses were performed in quadruplicate (n = 4) over 3 nonconsecutive days.

Table 2. DBS validation data (cont.).

Compound	Nominal concentration (ng/ml)	Determined concentration (ng/ml)	Accuracy (%)	Intra-day precision (%RSD)	Inter-day precision (%RSD)
Midazolam	0.1	0.109	108.7	5.2	5.2
	1	1.01	101.2	3.8	7.9
	25	25.4	101.7	2.3	3.8
	100	103	102.6	4.1	5.0
OH-midazolam	0.2	0.206	102.7	3.0	4.5
	2	2.08	104.0	3.8	4.6
	50	50.8	101.6	4.6	5.6
	200	195	97.3	4.7	6.4
Fexofenadine	1	0.995	99.5	4.3	10.9
	5	5.30	106.0	4.8	5.7
	50	53.9	107.8	3.6	3.6
	200	198	99.0	5.1	5.1

Analyses were performed in quadruplicate (n = 4) over 3 nonconsecutive days.

QC. Recovery and matrix effect were measured in quadruplicate at two different concentration levels (low and high) as shown in Table 4. Total analyte recoveries were calculated by comparing the absolute analyte peak areas from spiked DBS (or spiked extracted plasma) to that obtained from the corresponding methanolic solutions added into an empty vial (plasma recovery) or into a vial containing a blank filter paper (DBS recovery). The quantitative evaluation of matrix effect was carried out by comparing the absolute analyte peak area of spiked methanolic solutions added into the vial containing a blank DBS with that obtained from spiked methanolic solutions added in a vial containing a blank filter paper. Similarly, matrix effect for plasma analysis was carried out by comparing the absolute analyte peak area of spiked methanolic solutions added into the vial containing blank extracted plasma to peak area of neat spiked methanolic solutions. Additionally, the matrix effect was evaluated qualitatively by postcolumn infusion of the reference standard solutions (c = 10 ng/ml for all substances except CAF, PAR and OH-BUP for which c = 100 ng/ml, speed = 10 µl/min), while injecting six blank DBS or plasma samples from different sources [24].

To verify the short-term stability of the analytes on filter paper, DBS containing all the analytes at two concentration levels were prepared and stored at three different temperatures (room temperature, +4°C and -20°C). Stability was evaluated after 15 and 30 days by comparing the concentrations obtained after analysis at day 15, that is, day 30 with those obtained at day 0. Each measurement was performed in triplicate.

PK study

The clinical applicability of the method was evaluated in a PK study in which 10 healthy male volunteers received an oral 'cocktail' capsule containing low dose BUP (25 mg), FLB (25 mg), DEM (10 mg), OPZ (5 mg), MDZ (1 mg) and FEX (25 mg) together with a cup of coffee or coke. Capillary and venous blood samples were collected simultaneously before drug administration as well as 0.5, 1, 2, 3, 4, 6 and 8 h after intake. Capillary whole blood (10 µl) was collected on filter paper after a small finger prick (BD Microtainer, Contact-Activated Lancet, Plymouth, UK), whereas venous blood was collected into EDTA tubes (BD Vacutainer, Plymouth, UK). Plasma was obtained after centrifugation at 2500 rpm for 10 min and stored together with DBS cards at -20°C. Both DBS and plasma samples were analyzed (in duplicate) with the method described above. The clinical study was composed of four sessions in which the volunteers received the 'cocktail' capsule alone or in presence of CYP and P-gp inhibitors or inducer.

The study was conducted according to the standards of Good Clinical Practice, the Declaration of Helsinki and the Swiss regulatory requirements. The protocol was approved by the Ethics Committee of Geneva University Hospitals (ID: 12–085) and the trial was registered at ClinicalTrials.gov (ID: NCT01731067). Written informed consent was obtained from each subject prior to inclusion.

Results & discussion

Modifications and variability in drug disposition as a result of drug–drug interactions, genetic or

Table 3. Plasma validation data.

Compound	Nominal concentration (ng/ml)	Determined concentration (ng/ml)	Accuracy (%)	Intra-day precision (%RSD)	Inter-day precision (%RSD)
Caffeine	25	27.3	109.2	8.7	9.1
	125	122	97.9	6.7	6.8
	1250	1301	104.1	4.5	5.3
	5000	4687	93.7	6.1	6.1
Paraxanthine	25	26.9	107.6	9.4	9.8
	125	131	105.1	6.3	6.4
	1250	1232	98.6	7.2	7.2
	5000	4852	97.0	4.2	5.1
Bupropion	1	1.10	109.5	7.1	7.8
	5	4.79	95.8	8.8	11.2
	50	50.2	100.5	3.9	7.3
	200	200	100	10.9	10.9
OH-bupropion	1	1.08	107.7	6.9	8
	5	4.81	96.2	7.5	8.8
	50	50.3	100.6	2.5	4.5
	200	207	103.5	6.7	6.7
Flurbiprifen	25	26.2	104.8	4.8	6.3
	125	124	99.1	6.1	6.1
	1250	1293	103.4	4.7	5.2
	5000	4602	92	7.9	8.9
OH-flurbiprifen	5	5.20	103.9	5.4	6
	25	24.7	98.8	3.4	5.4
	250	252	100.9	6.2	6.9
	1000	921	92.1	6.5	7.2
Omeprazole	0.4	0.407	101.8	3.8	8.2
	5	4.96	99.1	4.7	4.7
	50	52.2	104.3	3.1	3.2
	200	207	103.5	5.3	5.3
OH-omeprazole	0.4	0.462	115.5	7.4	9.6
	5	4.56	91.3	6.2	6.2
	50	49.9	99.9	3.2	5.2
	200	203	101.6	4.3	4.4
Dextromethorphan	0.2	0.421	105.2	7.7	14.6
	2	5.05	101.1	7.2	7.6
	50	52.9	105.8	3.9	6.1
	200	199	99.3	6.8	8.4
Dextrorphan	0.5	0.512	102.5	6.9	11.6
	5	4.92	98.3	7	7
	125	131	104.9	2.7	5.8
	500	504	100.8	4.2	6.2
Midazolam	0.1	0.107	106.8	5.4	12
	1	1.00	99.7	4	4.9
	25	26.4	105.5	3.4	5.2
	100	90.2	90.2	4.6	10.1
OH-midazolam	0.2	0.208	103.9	4.8	10.9
	2	2.02	101.2	4.2	4.5
	50	50.7	101.5	2.9	5.2
	200	197	98.6	2.7	7

Analyses were performed in quadruplicate (n = 4) over 3 nonconsecutive days.

Table 3. Plasma validation data (cont.).

Compound	Nominal concentration (ng/ml)	Determined concentration (ng/ml)	Accuracy (%)	Intra-day precision (%RSD)	Inter-day precision (%RSD)
Fexofenadine	1	1.03	103.1	6.5	10.6
	5	4.96	99.2	4.7	5.6
	50	51.0	102	3.9	3.9
	200	195	97.4	4.9	4.9

Analyses were performed in quadruplicate (n = 4) over 3 nonconsecutive days.

environmental factors can lead to adverse effects or therapeutic inefficacy. Since CYP are considered a major source of inter-individual variability in drug response and of potential drug–drug interactions, we decided to assess a low dose cocktail approach to phenotype these enzymes in association with P-gp using DBS with subsequent LC–MS/MS analysis. The objective was to optimize the entire analytical workflow (including sample collection, extraction and LC–MS analysis) in order to improve the throughput of the method and therefore the clinical output.

It is now well-established that filter paper offers an attractive alternative to conventional venipuncture as it provides a minimally invasive technique and an easy way to collect blood. However, filter paper is not only a sampling support; it is also a promising technique to simplify sample preparation of complex matrices before LC–MS analysis [24]. Indeed, the use of an appropriate organic solvent (for example MeOH

Table 4. Recovery and matrix effects in DBS and plasma for all analytes at two concentration levels.

Compound	Concentration (ng/ml)	DBS (%)		Plasma (%)	
		Recovery	Matrix effect	Recovery	Matrix effect
Caffeine	100	78.6	+6.0	92.4	-3.8
	5000	101.4	+1.4	83.9	-8.9
Paraxanthine	100	107.4	+14.3	98.1	+9.0
	5000	100.2	+1.7	86.5	-4.5
Bupropion	2	74.8	-1.8	92.0	+5.6
	200	81.3	-2.4	94.3	+1.5
OH-bupropion	2	86.8	+1.0	86.5	+5.1
	200	67.0	-3.2	88.4	-1.9
Flurbiprofen	100	92.3	+2.1	96.9	+3.7
	2500	88.2	+6.2	89.1	+1.7
OH-flurbiprofen	25	73.0	-3.0	86.2	-4.3
	1000	79.6	-3.9	81.4	-6.6
Omeprazole	1	98.0	+3.5	96.3	+7.6
	200	114.8	+5.1	90.3	+2.9
OH-omeprazole	1	83.2	-5.6	72.2	-11.3
	200	95.2	-4.7	67.0	-5.0
Dextromethorphane	1	56.6	+0.4	102.4	+0.9
	200	81.5	-8.5	95.9	+3.1
Dextrorphane	2.5	73.1	-9.8	90.5	+5.2
	500	88.0	+3.5	94.6	+7.0
Midazolam	0.5	95.2	+1.3	92.5	+2.3
	100	103.2	+1.7	90.1	-0.1
OH-midazolam	1	90.7	+7.9	89.8	+13.7
	200	91.4	-4.5	91.1	+2.3
Fexofenadine	2	86.3	+16.4	74.8	-9.0
	200	87.7	+7.5	80.3	-5.4

Analyses were performed in quadruplicate (n = 4).

or ACN) allows the fixation of proteins and cell fragments onto the filter, while permitting the extraction of the analytes into a clear extract. Taking advantage of this, the DBS extraction can be coupled directly with LC system in an on-line mode. Recent papers have demonstrated the potential of this approach for drug analysis using dedicated DBS autosamplers [25,26]. For the present development, we decided to use a simplified offline extraction previously described [17,27]. Even if this setup features additional manipulations compared with online methods, it allows the DBS extraction to be achieved using conventional LC autosampler and simplifies the LC method avoiding column switching setup.

The main challenge of this work was to develop a versatile LC–MS/MS platform enabling the simultaneous quantification of six CYP probes and their respective phase I metabolites, as well as a P-gp substrate. Regarding the extraction, DBS pretreatment by means of soaking filter paper with MeOH into the vial allowed for a reliable and unbiased extraction of all tested substances. Moreover, the MeOH extraction provided sufficiently clean DBS extracts to avoid any additional clean-up step. For plasma extraction, the same strategy was applied. A generic PP was performed using ACN in order to have a nondiscriminating extraction. Despite the simplicity of both DBS and plasma extraction protocols, the selectivity of the method was confirmed since no interfering peaks were observed in blank DBS or in drug-free plasma at the retention times of the target analytes.

Within the same analytical run, generic LC conditions enabled the separation of different molecules with a wide range of polarity. The use of H_2O without buffer or formic acid associated to a low percentage of organic solvent (i.e., 2% of ACN) during the initial step of the gradient allowed for the good focalization and separation of polar compounds (such as PAR or CAF with a LogP of -0.9 and -0.6, respectively), as well as more hydrophobic compounds on the same RP C18 column. Furthermore, the combination of a short fused-core LC column (Kinetex™, Phenomenex Inc., CA, USA) with a rapid ACN gradient delivered at a flow rate of 0.6 ml/min allowed for the rapid and efficient chromatographic separation of the analytes in less than 3 min, as illustrated in Figure 1.

Due to the polarity switching of the TurboIonSpray interface, both positive and negative ions could be acquired simultaneously. In this way, acid compounds such as FLB and OH-FLB could be detected in negative mode and basic compounds in positive mode within the same SRM method (Table 1).

■ Method performance

Response function

For both plasma and DBS, the response function was linear for each analyte over the concentration ranges indicated in Table 1. These concentration ranges were chosen based on expected plasma and/or blood concentrations for each substance after oral administration of the drug cocktail described above. The best-fit line of the calibration curve for each analyte was obtained by using a weighting factor of 1/x.

Accuracy, precision & linearity

Accuracy and precision were determined by injecting independent QC samples at four different concentration levels. Accuracy was determined as a percentage of the ratio between experimental and theoretical concentrations. Precision values were assessed by calculating the relative standard deviations for the intra-day repeatability and the inter-day variability. As shown in Table 2 (DBS validation), for each compound and concentration tested the accuracy was included in the interval 92.2–111.1%, whereas both intra-day and inter-day precision were below 11%. Plasma validation data in Table 3 show that the accuracy values for every substance and concentration were in the interval 90.2–109.5%, except for the lowest OH-OPZ QC, which was slightly overestimated (115.5%) but still within the acceptable limit criteria. Precision values for all of the substances in plasma samples were lower than 15%.

For all analytes, the method was linear within the chosen concentration range with slope values between 0.922 and 1.037 for plasma, and between 0.937 and 1.030 for DBS. The coefficients of determination (R^2) were above 0.995 and 0.999 for plasma and DBS, respectively (data not shown).

LOD & LLOQ

LODs and LLOQs were identical for plasma and DBS since the same dilution factor was applied during the respective extraction procedures. Despite the use of a simple extraction procedure without preconcentration step for both DBS and plasma samples, the method provided sufficient sensitivity to encompass

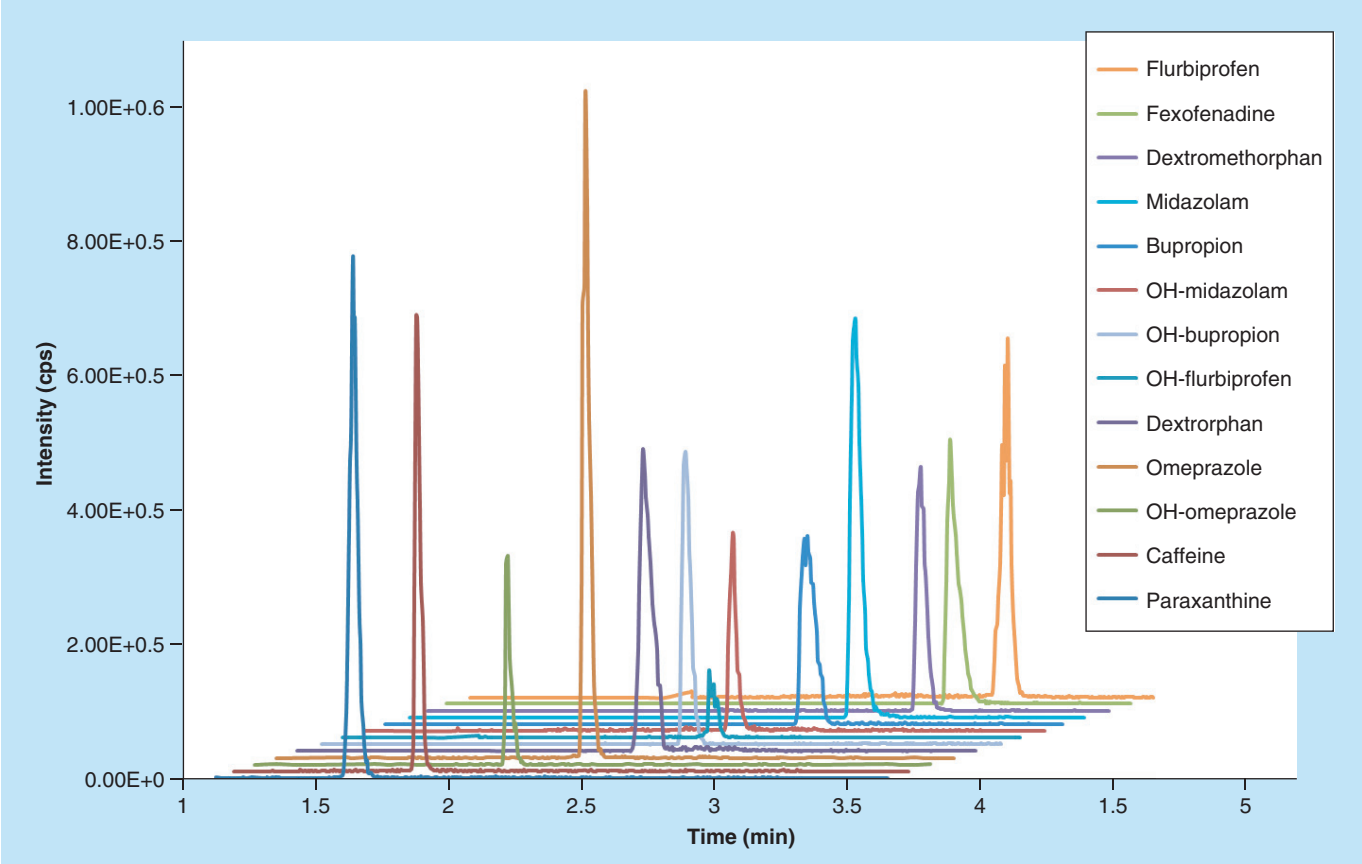

Figure 1. SRM chromatograms of a 10 µl DBS sample containing P-glycoprotein and cytochrome P450 probe substrates and their metabolites at the following concentrations. Caffeine 125 ng/ml; paraxanthine 125 ng/ml; OH-omeprazole 5 ng/ml; omeprazole 5 ng/ml; dextrorphan 5 ng/ml; OH-flurbiprofen 25 ng/ml; OH-bupropion 5 ng/ml; OH-midazolam 2 ng/ml; bupropion 5 ng/ml; midazolam 1 ng/ml; dextromethorphan 2 ng/ml; fexofenadine 5 ng/ml and flurbiprofen 125 ng/ml.

the expected concentration domain of each substance. LLOQs corresponding to the lowest QC concentrations are summarized in Tables 2 & 3, whereas estimated LODs are listed in Table 1.

Recovery & matrix effect

Recovery and matrix effect results for both plasma and DBS are summarized in Table 4. As shown, total recoveries for all substances at the two concentration levels were in the interval 56.6–114.8% for DBS and 67.0–102.4% for plasma. Recoveries for all substances were reproducible, which is in agreement with the good precision observed.

An issue of concern when DBS samples are used, especially when sample preparation is minimal, is the impact that hematocrit may have on the extraction efficiency of drugs. This issue was investigated in a study using similar experimental design to the present [28]. The biases observed at different hematocrit levels (from 25 to 75%) were found to be very low (between +6.3 and -5.4). These biases are within the accepted validation criteria, showing that differences in hematocrit levels would not influence the extraction efficiency of drugs.

Ion suppression or enhancement was lower than 20% for all of the substances in both DBS and plasma, indicating that there was no significant matrix effect. These results were confirmed with the postcolumn infusion test, since no variation in the MS response was observed in the elution windows of the analytes (data not shown).

Carryover

Analyte concentrations in the blank sample injected after the highest QC were lower than 20% of the LLOQ for all substances except for MDZ and OH-MDZ. For these two compounds, a carryover was observed in the blank filter injected after the highest QC sample as the signal was equal to approximately 50%

of the signal of the LLOQ. However, no carryover was observed after the injection of the QC 3 for both MDZ and OH-MDZ. Therefore, a blank filter paper is recommended after the injection of DBS samples containing MDZ and OH-MDZ concentrations higher than 25 and 50 ng/ml, respectively. During the study, plasma and DBS concentrations in the clinical samples were found to be lower than 20 ng/ml and 5 ng/ml for MDZ and OH-MDZ, respectively.

Stability

To verify if DBS samples collected during the PK study could be shipped and stored at ambient temperature, analyte stability in DBS was investigated after storing the samples for 15 and 30 days at room temperature, +4 and -20°C. When stored for 15 days, all of the substances were stable at all temperatures (concentrations at day 15 were between 85 and 115% of the corresponding day 0 DBS samples). All substances were stable after 30-day storing at all temperatures, except for bupropion whose concentrations were 53, 71 and 81% of the corresponding day 0 sample at room temperature, +4 and -20°C, respectively. No differences in stability were observed between the high- and the low-concentrated QC samples.

The observed results indicate that DBS cards could be collected, shipped and stored at ambient temperature as long as they were analyzed in the 15 days following sampling. For longer storage periods (up to 30 days), DBS cards containing bupropion should be kept at -20°C.

■ PK study

The developed method was used to determine the concentrations of the probe substrates and their specific metabolites in a PK study in which ten healthy volunteers received a cocktail of the probe substances. These probes have been used and validated in different combinations in several previously published cocktail approaches at therapeutic [4,29,30] or subtherapeutic doses [7,12]. The doses given to the volunteers in this study were five- to ten-times lower than usual therapeutic doses, therefore no interactions or side effects are expected to occur.

Figure 2 illustrates representative concentration–time profiles for all substances and their metabolites, in both plasma and DBS, after the administration of cocktail drugs. Despite the low doses administered and the minimal blood volume used, the sensitivity of the method allowed the quantification of all substances over the entire sampling period (8 h) except for MDZ and OH-MDZ, which are rapidly eliminated from the organism and could only be quantified over 6 h. As it can be deduced from Figure 2, good correlation was observed between DBS and plasma concentrations. This correlation is visually represented in Figure 3 and confirmed by the high values (0.843–0.985) of the coefficients of determination (R^2).

Blood to plasma concentration ratios (R_{BP}) range between 0.62–1.67 for the compounds assessed with ten of the 13 compounds partitioning predominantly into plasma compared with red blood cells [31]. Some molecules such as CAF, PAR and FEX might enter the erythrocytes, but do not bind (or bind only slightly) to proteins there, yielding R_{BP} values close to 1. Drugs such as DEM and its metabolite, which have high R_{BP}, enter and probably bind to blood cells, although there are no previous studies that evaluate this question.

Detailed results of the PK study and the interpretation of the metabolic ratios for the assessment of CYP and P-gp activity will be published elsewhere.

Conclusion

This article describes simple extraction procedures and LC–MS/MS method for the simultaneous quantification of P-gp and CYP probe substrates, as well as their metabolites, in plasma and 10 µl DBS samples. Due to its simplicity, the present method can be easily implemented in conventional biomedical laboratories and it fulfills the expectations in terms of throughput, since all probes could be quantified within a single 6 min analytical run. The method satisfied the required international validation criteria for all of the tested compounds. Despite the simple extraction procedure (plasma PP and DBS soaking, respectively) good recoveries and no matrix effect were observed.

The validated method was successfully applied to a PK study in which healthy male volunteers received a low dose (five- to ten-times lower doses than conventional therapeutic doses) cocktail of P-gp and CYP probe substrates. Good DBS–plasma concentration correlation was observed for all of the analytes, indicating that DBS may provide an alternative sampling technique to classic venous plasma collection. DBS procedure presents several advantages in terms of sample storage and shipment (analyte stability at room temperature for at least 15 days), as well as ease of sampling and patient-friendliness, and

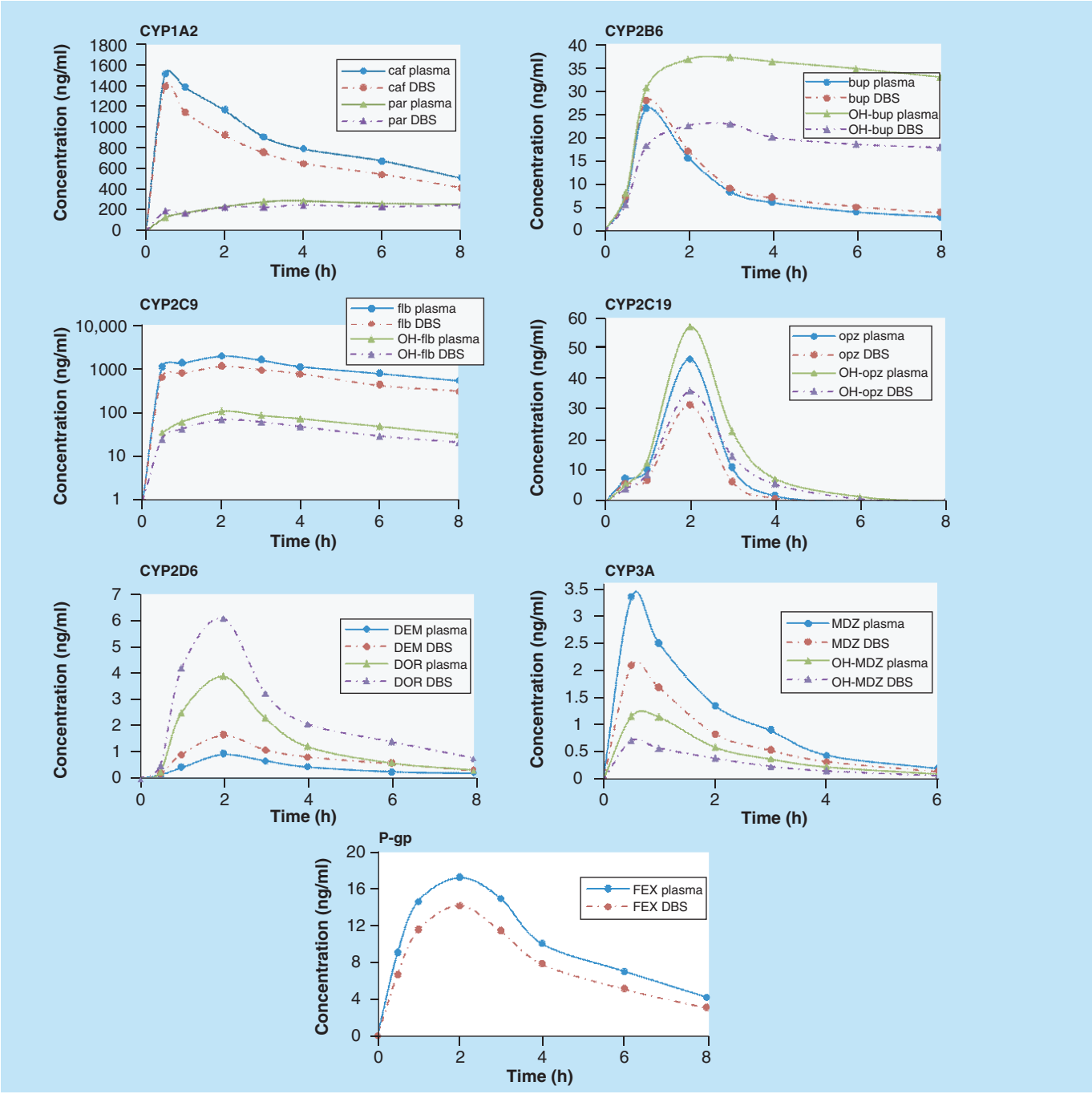

Figure 2. Representative concentration–time profiles for P-glycoprotein and cytochrome P450 probe substrates (circles) and their metabolites (triangles) obtained in 10 µl capillary DBS (dashed lines) and venous plasma samples (continuous lines) from a single volunteer after oral administration of cocktail drugs.
bup: Bupropion; Caf: Caffeine; CYP: Cytochrome P450; DEM: Dextromethorphan; DOR: Dextrorphan; FEX: P-glycoprotein substrate; flb: Flurbiprofen; MDZ: Midazolam; opz: Omeprazole; P-gp: P-glycoprotein; par: Paraxanthine.

therefore can be preferred over plasma for CYP and P-gp phenotyping.

Future perspective

As MS coupled with LC separation continuously gain in both sensitivity and selectivity, cytochrome phenotyping could be performed with even lower probe doses and very small amounts of blood material (lower than 10 µl). The low invasiveness of the DBS sampling method represents a great advantage, which can be further used for phenotyping or PK studies in

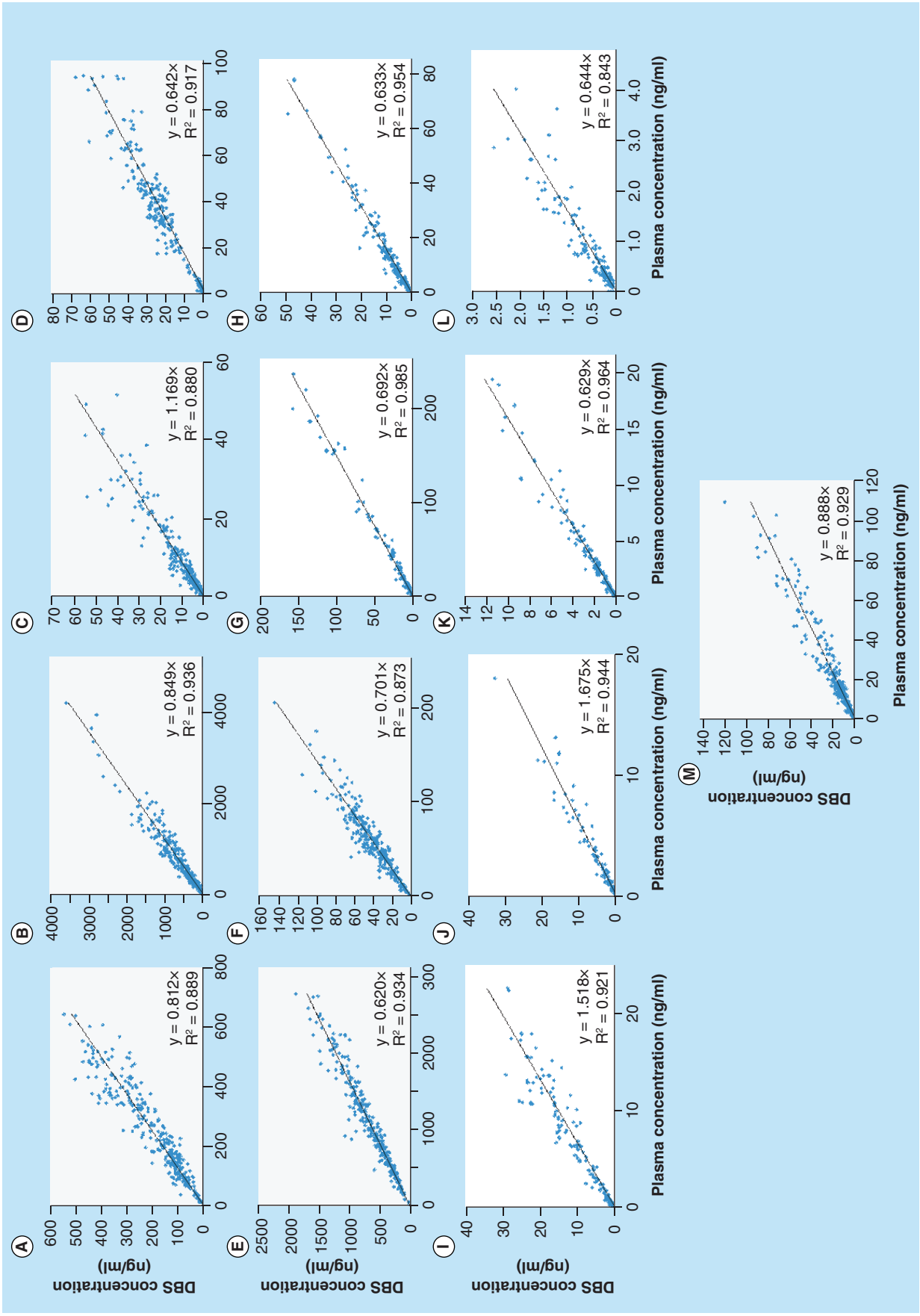

Figure 3. Comparison between capillary DBS (y-axis) and venous plasma (x-axis) concentrations. (A) Caffeine; **(B)** paraxanthine; **(C)** bupropion; **(D)** 4-hydroxybupropion; **(E)** flurbiprofen; **(F)** 4-hydroxyflurbiprofen; **(G)** omeprazole; **(H)** 5-hydroxyomeprazole; **(I)** dextromethorphan; **(J)** dextrorphan; **(K)** midazolam; **(L)** 1-hydroxymidazolam; and **(M)** fexofenadine. Concentration values were obtained from the PK study performed on ten volunteers at four study sessions.

more vulnerable patients, such as the pediatric or elderly population.

No writing assistance was utilized in the production of this manuscript.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Experimental (LC–MS/MS analysis)

- A single, fast (6 min) and sensitive method for the quantification of P-glycoprotein and cytochrome P450 probe substrates as well as their metabolites in DBS and/or plasma has been developed.

Results (method performance)

- The LC–MS/MS method fulfilled all of the required validation criteria and was successfully applied for cytochrome and P-glycoprotein phenotyping in healthy volunteers.

Results (PK study)

- The good correlation observed between plasma and DBS analyte concentrations indicates that the use of capillary DBS could be a suitable alternative to classical venous plasma analysis.

Conclusion

- Due to the facility of sample collection and the simple extraction procedure, DBS sampling can be easily used in clinical setting for the evaluation of cytochromes and P-glycoprotein activities.

References

Papers of special note have been highlighted as:
▪ of interest

1 Fuhr U, Jetter A, Kirchheiner J. Appropriate phenotyping procedures for drug metabolizing enzymes and transporters in humans and their simultaneous use in the 'cocktail' approach. *Clin. Pharmacol. Ther.* 81(2), 270–283 (2007).

▪ **Explains phenotyping methods and requirements.**

2 Daali Y, Samer C, Deglon J *et al.* Oral flurbiprofen metabolic ratio assessment using a single-point dried blood spot. *Clin. Pharmacol. Ther.* 91(3), 489–496 (2012).

▪ **Use of DBS for cytochrome P450 2C9 phenotyping.**

3 Zgheib NK, Frye RF, Tracy TS, Romkes M, Branch RA. Evaluation of flurbiprofen urinary ratios as *in vivo* indices for CYP2C9 activity. *Br. J. Clin. Pharmacol.* 63(4), 477–487 (2007).

4 Ryu JY, Song IS, Sunwoo YE *et al.* Development of the "Inje cocktail" for high-throughput evaluation of five human cytochrome P450 isoforms *in vivo*. *Clin. Pharmacol. Ther.* 82(5), 531–540 (2007).

5 Videau O, Delaforge M, Levi M *et al.* Biochemical and analytical development of the CIME cocktail for drug fate assessment in humans. *Rapid Commun. Mass Spectrom.* 24(16), 2407–2419 (2010).

6 Kim KA, Park PW, Park JY. Short-term effect of quercetin on the pharmacokinetics of fexofenadine, a substrate of P-glycoprotein, in healthy volunteers. *Eur. J. Clin. Pharmacol.* 65(6), 609–614 (2009).

7 Croft M, Keely B, Morris I, Tann L, Lappin G. Predicting drug candidate victims of drug-drug interactions, using microdosing. *Clin. Pharmacokinet.* 51(4), 237–246 (2012).

8 Frye RF, Matzke GR, Adedoyin A, Porter JA, Branch RA. Validation of the five-drug "Pittsburgh cocktail" approach for assessment of selective regulation of drug-metabolizing enzymes. *Clin. Pharmacol. Ther.* 62(4), 365–376 (1997).

9 Tomalik-Scharte D, Jetter A, Kinzig-Schippers M *et al.* Effect of propiverine on cytochrome P450 enzymes: a cocktail interaction study in healthy volunteers. *Drug Metab. Dispos.* 33(12), 1859–1866 (2005).

10 Mcdade TW, Williams S, Snodgrass JJ. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography* 44(4), 899–925 (2007).

11 Deglon J, Thomas A, Mangin P, Staub C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal. Bioanal. Chem.* 402(8), 2485–2498 (2012).

12 Ancrenaz V, Deglon J, Samer C *et al.* Pharmacokinetic interaction between prasugrel and ritonavir in healthy volunteers. *Basic Clin. Pharmacol. Toxicol.* 112(2), 132–137 (2013).

13 Li Y, Henion J, Abbott R, Wang P. Dried blood spots as a sampling technique for the quantitative determination of guanfacine in clinical studies. *Bioanalysis* 3(22), 2501–2514 (2011).

14 Filippi L, La Marca G, Cavallaro G *et al.* Phenobarbital for neonatal seizures in hypoxic ischemic encephalopathy: a pharmacokinetic study during whole body hypothermia. *Epilepsia* 52(4), 794–801 (2011).

15 Deglon J, Lauer E, Thomas A, Mangin P, Staub C. Use of the dried blood spot sampling process coupled with fast gas chromatography and negative-ion chemical ionization tandem mass spectrometry: application to fluoxetine, norfluoxetine, reboxetine, and paroxetine analysis. *Anal. Bioanal. Chem.* 396(7), 2523–2532 (2010).

16 Barfield M, Spooner N, Lad R, Parry S, Fowles S. Application of dried blood spots combined with HPLC–MS/MS for the quantification of acetaminophen in toxicokinetic studies. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 870(1), 32–37 (2008).

17 Ansari M, Uppugunduri CR, Deglon J *et al.* A simplified method for busulfan monitoring using dried blood spot in combination with liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 26(12), 1437–1446 (2012).

18 Kromdijk W, Mulder JW, Smit PM, Ter Heine R, Beijnen JH, Huitema AD. Therapeutic drug monitoring of antiretroviral drugs at home using dried blood spots: a proof of concept study. *Antivir. Ther.* 18(6), 821–825 (2013).

19 Vu DH, Bolhuis MS, Koster RA *et al.* Dried blood spot analysis for therapeutic drug monitoring of linezolid in patients with multidrug-resistant tuberculosis. *Antimicrob. Agents Chemother.* 56(11), 5758–5763 (2012).

20 Wilhelm AJ, Klijn A, Den Burger JC *et al.* Clinical validation of dried blood spot sampling in therapeutic drug monitoring of ciclosporin A in allogeneic stem cell transplant recipients: direct comparison between capillary and venous sampling. *Ther. Drug Monit.* 35(1), 92–95 (2013).

21 Deglon J, Thomas A, Daali Y *et al.* Automated system for on-line desorption of dried blood spots applied to LC–MS/MS pharmacokinetic study of flurbiprofen and its metabolite. *J. Pharm. Biomed. Anal.* 54(2), 359–367 (2011).

22 De Boer T, Wieling J, Meulman E *et al.* Application of dried blood spot sampling combined with LC–MS/MS for genotyping and phenotyping of CYP450 enzymes in healthy volunteers. *Biomed. Chromatogr.* 25(10), 1112–1123 (2011).

23 Lad R. Validation of individual quantitative methods for determination of cytochrome P450 probe substrates in human dried blood spots with HPLC–MS/MS. *Bioanalysis* 2(11), 1849–1861 (2010).

24 Lauer E, Widmer C, Versace F *et al.* Body fluid and tissue analysis using filter paper sampling support prior to LC–MS/MS: application to fatal overdose with colchicine. *Drug Test. Anal.* (2013).

25 Heinig K, Wirz T, Bucheli F, Gajate-Perez A. Determination of oseltamivir (Tamiflu(R)) and oseltamivir carboxylate in dried blood spots using offline or online extraction. *Bioanalysis* 3(4), 421–437 (2011).

26 Ooms JA, Knegt L, Koster EH. Exploration of a new concept for automated dried blood spot analysis using flow-through desorption and online SPE-MS/MS. *Bioanalysis* 3(20), 2311–2320 (2011).

27 Deglon J, Versace F, Lauer E *et al.* Rapid LC–MS/MS quantification of the major benzodiazepines and their metabolites on dried blood spots using a simple and cost-effective sample pretreatment. *Bioanalysis* 4(11), 1337–1350 (2012).

28 Youhnovski N, Bergeron A, Furtado M, Garofolo F. Pre-cut dried blood spot (PCDBS): an alternative to dried blood spot (DBS) technique to overcome hematocrit impact. *Rapid Commun. Mass Spectrom.* 25(19), 2951–2958 (2011).

29 Chainuvati S, Nafziger AN, Leeder JS *et al.* Combined phenotypic assessment of cytochrome p450 1A2, 2C9, 2C19, 2D6, and 3A, N-acetyltransferase-2, and xanthine oxidase activities with the "Cooperstown 5+1 cocktail". *Clin. Pharmacol. Ther.* 74(5), 437–447 (2003).

30 Zgheib NK, Frye RF, Tracy TS, Romkes M, Branch RA. Validation of incorporating flurbiprofen into the Pittsburgh cocktail. *Clin. Pharmacol. Ther.* 80(3), 257–263 (2006).

31 Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. *Bioanalysis* 2(11), 1791–1796 (2010).

▪ **Indicates physiological parameters and calculations to be considered when using DBS.**

Websites

101 European Medicines Agency. Guideline on the Investigation of Drug Interactions. www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/05/WC500090112.pdf

102 European Medicines Agency. Guideline on bioanalytical method validation. www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf