Oral Flurbiprofen Metabolic Ratio Assessment Using a Single-Point Dried Blood Spot

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We investigated whether a single blood measurement using the minimally invasive technique of a finger prick to draw a blood sample of 5 µl (to yield a dried blood spot (DBS)) is suitable for the assessment of flurbiprofen (FLB) metabolic ratio (MR). Ten healthy volunteers who had been genotyped for *CYP2C9* were recruited as subjects. They received FLB alone in session 1 and FLB with fluconazole in session 2. In session 3, the subjects were pretreated for 4 days with rifampicin and received FLB with the last dose of rifampicin on day 5. Plasma and DBS samples were obtained between 0 and 8 h after FLB administration, and urine was collected during the 8 h after administration. The pharmacokinetic profiles of the drugs were comparable in DBS and plasma. FLB's apparent clearance values decreased by 35% in plasma and DBS during session 2 and increased by 75% in plasma and by 30% in DBS during session 3. Good correlations were observed between MRs calculated from urine, plasma, and DBS samples.

In the past decade, a novel approach involving the use of dried blood spots (DBSs) has emerged for the quantitative determination of circulating drugs.[1–4] Briefly, whole-blood samples are directly collected on a filter paper card using a small finger prick (or heel prick in pediatric patients). After a short air-drying period, DBS cards are easily shipped to the analytical laboratory and can be stored for long periods at ambient temperatures.[5] Because of the simplicity of this method, the DBS samples can be collected in nonhospital environments by technicians or even by patients themselves after minimal training. Beyond the logistical aspects, this new method offers several advantages over conventional plasma collection, including a less invasive sampling process, a smaller blood volume (<10 µl) without the need for anticoagulants, and improved safety because of reduced pathogen transmission.[6] These combined advantages make DBS a patient-friendly blood-collection procedure for numerous biomedical applications such as neonatal screening and for preclinical, clinical, and epidemiological investigations.

Cytochrome P450 2C9 (CYP2C9) is involved in the metabolism of a wide variety of therapeutic drugs such as warfarin and acenocoumarol, nonsteroidal anti-inflammatory drugs, sulfonylureas, phenytoin, and valproic acid.[7] Owing to the known interindividual variability of CYP2C9 activity caused by genetic (polymorphisms) and environmental factors, there is a real clinical need for a reliable phenotypic measure of CYP2C9 activity in humans. Indeed, phenotyping provides the most clinically relevant information about enzyme activity, because it is a measure of the combined effects of genetic, environmental, and endogenous factors on CYP activity.[8] Several drugs have been proposed as being potentially useful as probes for CYP2C9 phenotyping, including tolbutamide,[9] phenytoin,[10] warfarin,[11] and losartan.[12] However, each substrate is associated with limitations that make these drugs less than ideal as probes for CYP2C9.[13] Recently, flurbiprofen (FLB) was validated as a reliable probe for CYP2C9 phenotyping in urine.[13] In fact, CYP2C9 has been shown to be exclusively involved in the formation of 4′-hydroxyflurbiprofen (OHFLB).[14] The principal drawback of this method is the requirement for overnight or 8-h urine collection after the administration of the probe test. This procedure is very tedious and time consuming for both patients and medical staff. The CYP2C9 phenotype test was also performed in plasma 2 h after the probe administration.[15] However, this procedure is invasive and requires that a blood sample be drawn by a qualified nurse in a clinical environment.

The purpose of this study was to evaluate the usefulness and effectiveness of an innovative and minimally invasive approach (DBS) to determine FLB metabolic ratio (MR), from 5 µl of blood taken from a finger prick. Using this method, the kinetics

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of oral FLB were measured in 10 healthy subjects over a period of 8 h. The subjects were free of known CYP2C9 inducers and inhibitors in session 1, received the strong CYP2C9 inhibitor fluconazole in session 2, and received the strong CYP2C9 inducer rifampicin in session 3.

RESULTS

The subjects reported no significant adverse effects after administration of FLB, whether alone or in combination with either fluconazole or rifampicin. One subject was a heterozygous carrier of *CYP2C9**2 and another was a heterozygous carrier of *CYP2C9**3. Nevertheless, these two subjects were included in the study because their FLB and metabolite pharmacokinetic data were not different from those of the subjects with homozygous *CYP2C9**1 alleles.

Pharmacokinetic data

As shown in **Figure 1** and **Table 1**, the pharmacokinetic profiles of FLB and OHFLB were similar in DBS and plasma in terms of distribution and elimination. No statistically significant differences were observed between plasma and DBS with respect to half-lives or time to maximum concentration T_{max} during each session. Maximum concentration (C_{max}) and area under the concentration–time curve (AUC) were proportionally higher in plasma than in DBS, with an approximate mean ratio of 0.5 corresponding to the dilution factor due to the presence of red blood cells (hematocrit) in the whole blood forming the spot in DBS.

Effect of the CYP2C9 inhibition

As expected, fluconazole significantly decreased OHFLB C_{max} (36% in plasma ($P < 0.0001$) and 50% in DBS ($P < 0.01$)) and AUC (45% in plasma ($P < 0.001$) and 33% in DBS ($P < 0.05$)), whereas no significant change was observed in the time to maximum plasma concentration. The half-life increased significantly (45% in plasma ($P = 0.02$) and 40% in DBS ($P = 0.05$)). In contrast, FLB C_{max} and AUC increased by 28 and 37% ($P < 0.01$) in DBS and 11 and 28% ($P < 0.05$) in plasma, respectively. Mean FLB apparent clearances decreased by 35% in both plasma and DBS ($P < 0.01$).

Effect of CYPC9 induction

Pretreatment with rifampicin induced a significant decrease in FLB AUC of 35% in plasma ($P < 0.01$) and 15% in DBS ($P < 0.05$), whereas FLB CL/F increased by 75% in plasma ($P = 0.002$) and 30% in DBS ($P = 0.01$). Plasma FLB C_{max} decreased by 30% ($P = 0.001$), whereas no statistically significant change was observed in DBS ($P = 0.89$). No significant changes were observed in OHFLB pharmacokinetic parameters between sessions 1 and 3 in either plasma or DBS.

Hydrolysis of glucuroconjugates

As shown in **Figure 2** and **Table 1**, no significant changes in the FLB pharmacokinetic parameters were observed after hydrolysis of plasma samples, whereas OHFLB C_{max} and AUC significantly increased after chemical hydrolysis. Indeed, AUC of OHFLB increased by 21% in session 1 ($P < 0.0001$), 28% in session 2 ($P = 0.014$), and 46% in session 3 ($P = 0.007$). The value of C_{max} increased by 14% in session 1 ($P = 0.017$), 24% in session 2 ($P = 0.015$), and 46% in session 3 ($P = 0.011$).

MRs in urine

Total (unconjugated + conjugated) FLB and OHFLB were determined after the 8-h urine collection. The distributions of the MRs OHFLB/FLB after FLB alone and after coadministration with fluconazole or rifampicin are presented in **Figure 3**.

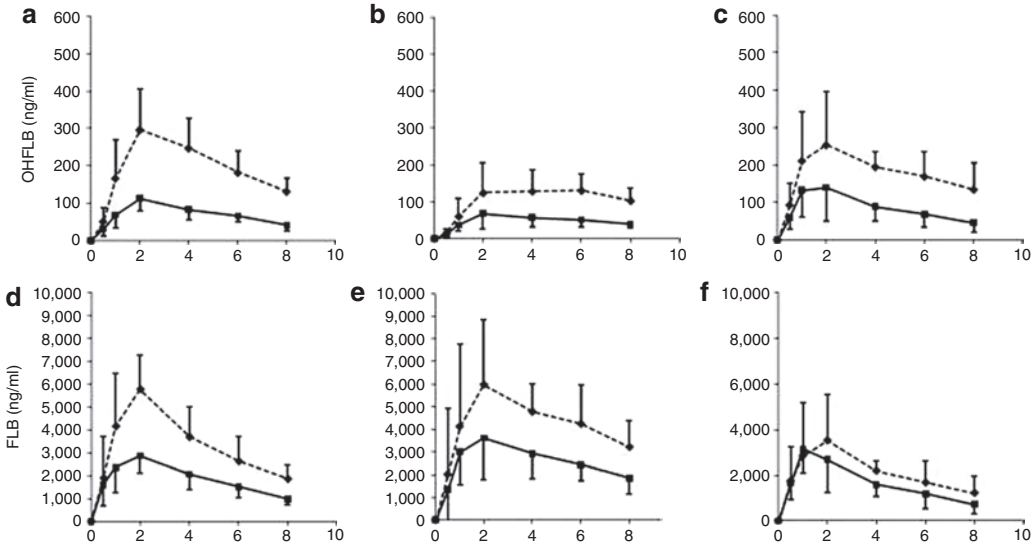

Figure 1 Mean (SD) plasma concentration–time profile of 4′-hydroxyflurbiprofen (OHFLB) and flurbiprofen (FLB) in plasma and OHFLB and FLB in dried blood spots (DBSs) in 10 healthy subjects after a single 50 mg oral dose of FLB. (**a**) OHFLB session 1, (**b**) OHFLB session 2 (treatment with 400 mg of fluconazole), (**c**) OHFLB session 3 (5-day treatment with 600 mg of rifampicin), (**d**) FLB session 1,a (**e**) FLB session 2 (treatment with 400 mg of fluconazole), and (**f**) FLB session 3 (5-day treatment with 600 mg of rifampicin).

Table 1 Pharmacokinetic parameter estimate of flurbiprofen and 4′-hydroxyflurbiprofen after 50 mg oral administration with and without fluconazole and rifampicin

Variable	Units	DBS			Plasma (unconjugated)			Plasma (conjugated + unconjugated)		
		Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
Flurbiprofen										
$t_{1/2}$	h	3.9 (0.7)	4.5 (1.1)	2.6 (0.5)	4.1 (1.2)	5.2 (1.7)	3.0 (0.6)	4.0 (1.1)	5.7 (1.7)	3.2 (0.8)
		—	n.c.	n.c.	—	n.c.	n.c.	—	n.c.	n.c.
C_{max}	ng/ml	3,237 (434)	4148.0 (1,110)	3,255 (1,103)	6,370 (1,012)	7,076 (1,812)	4,410 (1,206)	5,973 (1,294)	6,601 (1,766)	4,695 (1,831)
Ratio (90% CI)		—	1.32 (0.76–1.68)	0.92 (0.65–1.22)	—	1.14 (0.81–1.52)	0.71 (0.58–0.86)	—	1.08 (0.84–1.47)	0.76 (0.54–1.01)
T_{max}	h	2.0 (0.8)	2.8 (1.7)	2.8 (1.9)	1.9 (0.9)	2.9 (1.8)	2.0 (1.9)	2.0 (0.8)	2.9 (2.4)	2.5 (2.0)
		—	n.c.	n.c.	—	n.c.	n.c.	—	n.c.	n.c.
AUC_{∞}	h·ng/ml	14,991 (2,344)	20,507 (3,524)	12,822 (3,458)	26,789 (5,366)	34,327 (10,828)	17,315 (4,508)	25,437 (3,673)	33,833 (9,235)	18,946 (6,000)
Ratio (90% CI)		—	1.47 (1.11–2.01)	0.88 (0.62–0.98)	—	1.21 (0.94–1.65)	0.56 (0.53–0.83)	—	1.27 (1.18–1.59)	0.71 (0.49–0.82)
Vz/F	ml	13,775 (2,130)	9,922 (2,507)	12,254 (4,464)	7,802 (2,396)	6,227 (743)	10,269 (3,587)	7,877 (1,738)	6,499 (829)	10,373 (4,234)
		—	n.c.	n.c.	—	n.c.	n.c.	—	n.c.	n.c.
Cl/F	ml/h	2,511 (496)	1,607 (513)	3,254 (815)	1,358 (286)	891 (253)	2,374 (689)	1,383 (292)	860 (281)	2,244 (796)
Ratio (90% CI)		—	0.64 (0.42–0.72)	1.33 (1.07–1.87)	—	0.67 (0.51–0.99)	1.85 (1.26–2.60)	—	0.61 (0.45–0.85)	1.71 (1.26–1.83)
4′-hydroxyflurbiprofen										
$t_{1/2}$	h	4.1 (1.1)	5.8 (1.1)	2.9 (0.5)	5.6 (2.9)	8.1 (1.5)	3.9 (0.9)	5.6 (1.4)	6.7 (2.3)	5.1 (3.0)
		—	n.c.	n.c.	—	n.c.	n.c.	—	n.c.	n.c.
C_{max}	ng/ml	122 (26)	77 (29)	166 (65)	313 (90)	154 (57)	309 (69)	356 (102)	192 (69)	452 (157)
Ratio (90% CI)		—	0.64 (0.39–0.88)	1.25 (1.05–1.67)	—	0.43 (0.36–0.66)	1.10 (0.79–1.4)	—	0.52 (0.39–0.69)	1.26 (0.84–1.46)
T_{max}	h	2.2 (0.6)	2.8 (1.7)	2.8 (1.9)	2.2 (0.6)	4.0 (2.1)	3.1 (2.3)	2.4 (0.8)	4.4 (1.8)	2.9 (1.8)
		—	n.c.	n.c.	—	n.c.	n.c.	—	n.c.	n.c.
AUC_{∞}	h·ng/ml	578 (114)	389 (159)	679 (260)	1,551 (463)	856 (371)	1,408 (336)	1,882 (512)	1,099 (478)	2,063 (706)
Ratio (90% CI)		—	0.75 (0.33–0.84)	1.11 (0.86–1.42)	—	0.51 (0.38–0.81)	0.97 (0.83–1.19)	—	0.58 (0.38–0.68)	1.08 (0.73–1.57)
Vz/F	ml	373,067 (112,708)	578,028 (210,178)	265,958 (128,443)	146,299 (42,212)	319,592 (146,494)	142,352 (41,504)	129,971 (35,383)	184,212 (42,074)	115,226 (52,122)
		—	n.c.	n.c.	—	n.c.	n.c.	—	n.c.	n.c.
Cl/F	ml/h	63,850	69,225 (21,750)	64,462 (26,427)	20,263 (7,331)	27,191 (11,657)	25,585 (7,380)	14,199 (7,453)	23,272 (7,570)	17,000 (7,561)
Ratio (90% CI)		(16,403)	1.05 (0.92–1.35)	1.13 (0.71–1.29)	—	1.27 (1.05–1.58)	1.23 (0.86–2.20)	—	1.23 (0.44–1.84)	1.26 (0.51–1.32)

For C_{max}, AUC, and CL/F, data are mean values (±SD) and median ratios (session 2 vs. session 1 and session 3 vs. session 1) with 90% confidence interval (90% CI). Other parameters (T_{max}, $t_{1/2}$, and Vz/F) are mean values (±SD).

AUC, area under the plasma–concentration time curve; Cl/F, apparent clearance; CI, confidence interval; C_{max}, maximum plasma concentration; DBS, dried blood spot; n.c., not calculated; $t_{1/2}$, half life; T_{max}, time to maximum plasma concentration ; Vz/F, volume of distribution.

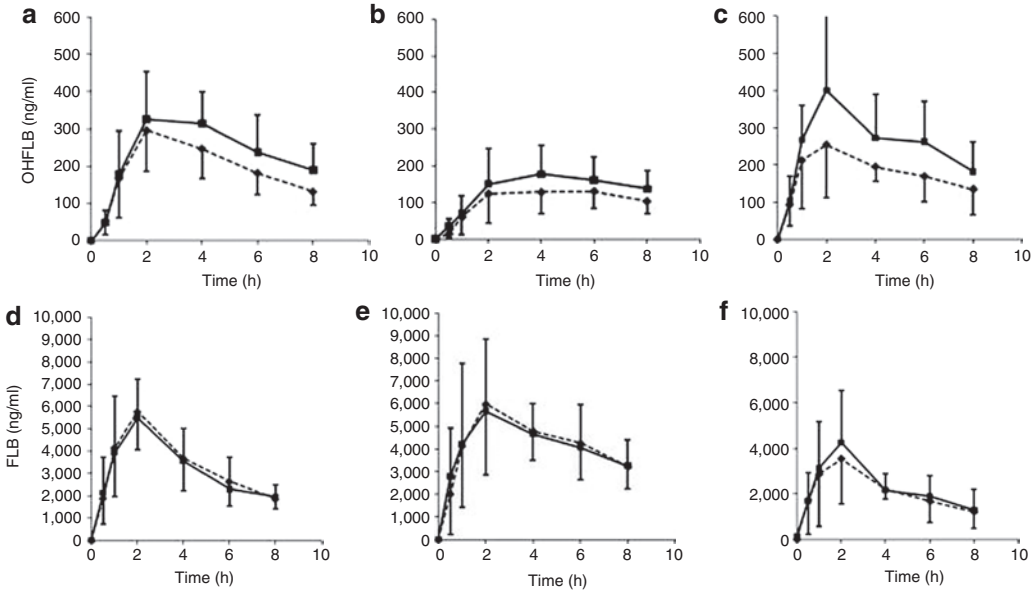

Figure 2 Mean (SD) plasma concentration–time profile of unconjugated 4′-hydroxyflurbiprofen (OHFLB) and flurbiprofen (FLB), and (unconjugated + conjugated) OHFLB and FLB in 10 healthy subjects after a single 50-mg oral dose of FLB. (**a**) OHFLB session 1, (**b**) OHFLB session 2 (treatment with 400 mg of fluconazole), (**c**) OHFLB session 3 (5-day treatment with 600 mg of rifampicin), (**d**) FLB session 1, (**e**) FLB session 2 (treatment with 400 mg of fluconazole), and (**f**) FLB session 3 (5-day treatment with 600 mg of rifampicin).

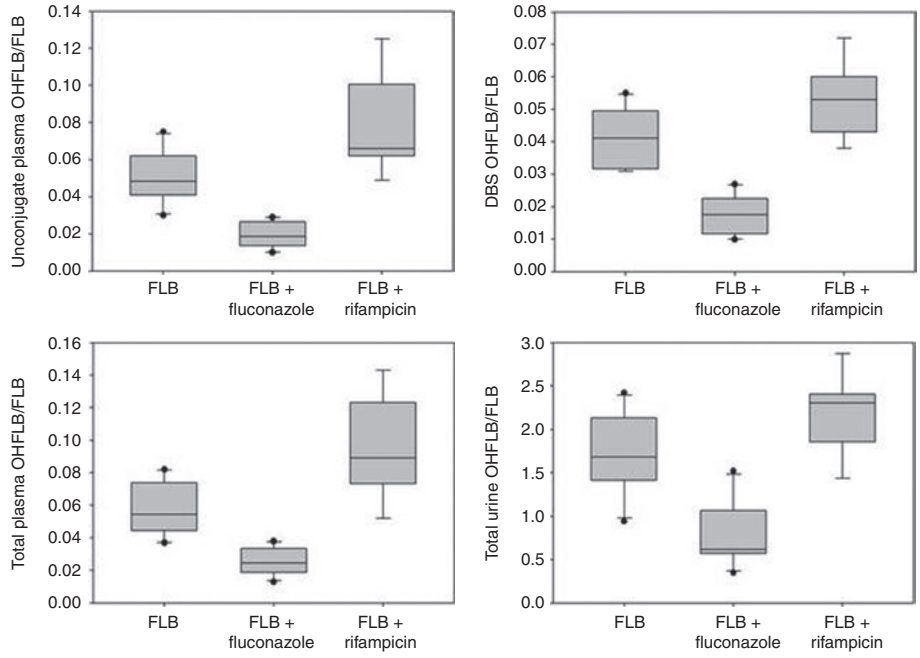

Figure 3 Box plots of the distribution of OHFLB/FLB metabolic ratios in unconjugated plasma, dried blood spots, and total plasma at 2 h after oral administration of 50 mg flurbiprofen in 10 healthy subjects, and in 8-h urine collection. DBS, dried blood spot; FLB, flurbiprofen; OHFLB, 4′-hydroxyflurbiprofen.

Statistically significant differences were observed among the three sessions ($P < 0.001$). The mean (SD) OHFLB/FLB MRs were 1.73 (0.45), 0.79 (0.36), and 2.19 (0.44) in sessions 1, 2, and 3, respectively.

MRs in plasma and DBS

Mean (SD) MRs over time in plasma (unconjugated), plasma (conjugated + unconjugated), and DBS after oral administration of 50 mg of FLB, alone and with pretreatment, are shown in Figure 4. MRs varied only moderately over 0.5–8 h after drug administration regardless of pretreatment condition. Statistically significant differences were observed in plasma (total and unconjugated) and DBS among the three sessions at 2, 4, 6, and 8 h after FLB administration. However, MRs at 2 h after FLB administration in plasma and DBS showed the best statistical data ($P < 0.001$ for all sessions in hydrolyzed plasma and for

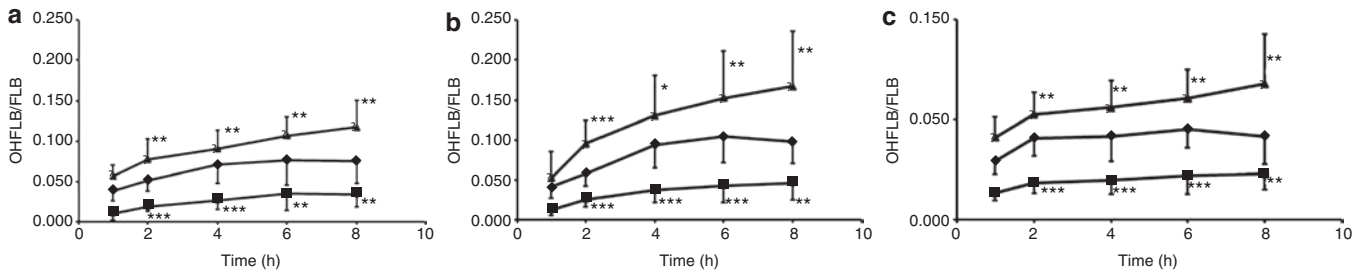

Figure 4 Metabolic ratio profiles after oral administration of 50 mg flurbiprofen in 10 healthy subjects without pretreatment (diamonds), after fluconazole (squares), and after rifampicin (triangles) in (**a**) plasma (unconjugated), (**b**) in plasma (unconjugated + conjugated), and (**c**) in dried blood spots. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. OHFLB, 4´-hydroxyflurbiprofen.

session 2 in plasma and DBS, $P < 0.01$ for session 3 in plasma and DBS).

The Spearman rank correlation coefficients (ρ_s) between OHFLB/FLB MRs in plasma (unconjugated), plasma (unconjugated + conjugated), and DBS are shown in **Table 2**. Good correlations were observed at 2, 4, 6, and 8 h after FLB administration during the three sessions, except for MRs in plasma (unconjugated + conjugated) and DBS at session 3, at 6 h after FLB administration ($\rho_s = 0.467$, $P = 0.2$). Moreover, good correlations were also observed between MRs in urine and OHFLB/FLB MRs in plasma and DBS at 2, 4, 6, and 8 h after FLB administration (**Table 3**). Similarly, good correlations were seen between FLB clearances and OHFLB/FLB MRs in plasma and DBS (**Table 3**).

Table 2 Spearman rank correlation (ρ_s) between the metabolic ratios in DBS, plasma (unconjugated) and plasma (unconjugated + conjugated) at various time points of the three sessions

	OHFLB/FLB in DBS			
	2 h	4 h	6 h	8 h
Session 1				
OHFLB/FLB in plasma (unconjugated)				
Spearman correlation	0.753	0.915	0.740	0.786
P value	0.019	<0.001	0.023	0.021
OHFLB/FLB in plasma (unconjugated + conjugated)				
Spearman correlation	0.904	0.842	0.824	0.714
P value	0.001	0.002	0.006	0.047
Session 2				
OHFLB/FLB in plasma (unconjugated)				
Spearman correlation	0.950	0.842	0.960	0.874
P value	<0.001	0.002	<0.001	0.001
OHFLB/FLB in plasma (unconjugated + conjugated)				
Spearman correlation	0.885	0.964	0.973	0.847
P value	0.002	<0.001	<0.001	0.002
Session 3				
OHFLB/FLB in plasma (unconjugated)				
Spearman correlation	0.667	0.743	0.862	0.833
P value	NS (0.071)	0.035	0.006	0.005
OHFLB/FLB in plasma (unconjugated + conjugated)				
Spearman correlation	0.753	0.970	0.467	0.817
P value	0.019	<0.001	NS	0.007

DBS, dried blood spot; FLB, flurbiprofen; NS, not significant; OHFLB, 4′-hydroxyflurbiprofen.

DISCUSSION

DBS has recently received great interest as an alternative matrix to plasma samples in pharmacokinetic and therapeutic drug monitoring studies.[4,16] Several technical advantages have been demonstrated for the use of DBS in place of plasma, including small volume collection, low storage and transport costs, enzyme and pathogen inactivation, and higher drug and metabolite stability.[5] A considerable number of papers have recently been published on the development of highly selective and sensitive bioanalytical methods. However, less attention has been given to DBS-related pharmacokinetic and pharmacodynamic issues.[17] The aim of our study was to determine whether a single-point method involving the use of DBS can be effective in assessing CYP2C9 activity while using FLB as a probe.

In our study, pharmacokinetic profiles were similar in plasma and DBS. The drug concentrations in plasma were higher than in DBS because the latter contains blood cells. The hematocrit content is an important issue in drug determination involving the use of DBS, because it affects blood viscosity and the dilution factor, and should be taken into account as a variability factor. Normally, it is relatively stable at ~0.45. Nevertheless, it can decrease to 0.2 in conditions such as anemia.[17] It has to be noted that the most frequent phenotyping procedure is the measurement of the MR between a metabolite produced by the specific cytochrome of interest and the probe drug.[18] Hence, the hematocrit content will have no effect on the MR, given that its effect relates to both the metabolite and the probe drug. Moreover, the degree of precision of the sampling volume and the quality of the filter paper used during sample collection may affect the drug-level measurements; this issue is still a matter of debate and requires further investigation. Here again, the MR may not be significantly affected by these parameters. Another important factor to be kept in mind while using DBS for drug determination is the pattern of drug distribution in erythrocytes. Differential distribution of the drug and its metabolite in blood cells could have an effect on the evaluation of the MR. However, acidic molecules are known to have a weak affinity for erythrocytes.[19] Hence, the measurements of FLB and OHFLB may not be materially affected by this factor.

Table 3 Spearman rank correlation (ρ_s) between flurbiprofen clearance, metabolic ratios in urine, and the metabolic ratios in plasma (unconjugated), plasma (unconjugated + conjugated) and DBS at various time points of the three sessions

	FLB clearance				Urine metabolic ratio			
	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
Session 1								
OHFLB/FLB in plasma (unconjugated)								
Spearman correlation	0.79	0.71	0.60	0.76	0.85	0.65	0.83	0.64
P value	0.02	0.04	0.09	0.03	<0.01	0.04	0.01	0.08
OHFLB/FLB in plasma (unconjugated + conjugated)								
Spearman correlation	0.70	0.60	0.77	0.84	0.81	0.66	0.57	0.8
P value	0.08	0.01	0.07	<0.01	<0.01	0.08	0.11	0.01
OHFLB/FLB in DBS								
Spearman correlation	0.83	0.77	0.51	0.47	0.91	0.61	0.78	0.60
P value	0.04	0.05	0.10	NS	<0.01	0.05	0.012	0.09
Session 2								
OHFLB/FLB in plasma (unconjugated)								
Spearman correlation	0.77	0.70	0.71	0.77	0.88	0.93	0.88	0.83
P value	0.07	0.10	0.11	0.07	0.002	<0.01	<0.01	0.01
OHFLB/FLB in plasma (unconjugated + conjugated)								
Spearman correlation	0.89	0.83	0.86	0.82	0.76	0.85	0.95	0.95
P value	0.007	0.04	<0.01	0.04	0.02	<0.01	<0.01	<0.01
OHFLB/FLB in DBS								
Spearman correlation	0.94	0.65	0.66	0.83	0.90	0.85	0.85	0.81
P value	0.005	0.10	0.11	0.04	0.002	<0.01	<0.01	0.02
Session 3								
OHFLB/FLB in plasma (unconjugated)								
Spearman correlation	0.77	0.71	0.8	0.61	0.79	0.46	0.81	0.83
P value	0.07	0.11	0.10	0.09	0.03	NS	0.015	0.04
OHFLB/FLB in plasma (unconjugated + conjugated)								
Spearman correlation	0.66	0.76	0.65	0.48	0.51	0.61	0.56	0.6
P value	0.10	0.14	0.09	NS	NS	0.11	0.09	0.09
OHFLB/FLB in DBS								
Spearman correlation	0.75	0.72	0.5	0.64	0.82	0.66	0.67	0.88
P value	0.08	0.09	0.11	0.11	0.02	0.10	0.06	<0.01

DBS, dried blood spot; FLB, flurbiprofen; NS, not significant; OHFLB, 4′-hydroxyflurbiprofen.

The pharmacokinetics of FLB did not show significant changes in any of the three sessions after chemical hydrolysis of plasma samples, whereas the AUC and C_{max} of OHFLB showed increases. Previous *in vivo* and *in vitro* reports found that 15% of the total FLB is conjugated by UGT2B7 to produce the acyl-glucuronide that is eliminated in the urine.[20,21] However, it was shown that the FLB glucuronidation fraction is difficult to determine because of the instability of acyl-glucuronide *in vivo*.[22]

One subject was a heterozygous carrier of *CYP2C9**2, and another was a heterozygous carrier of *CYP29C9**3. However, no differences were observed in the pharmacokinetic profiles of FLB and OHFLB in these subjects as compared with other subjects with homozygous *CYP2C9**1 alleles. In an earlier study, Lee *et al*. demonstrated that FLB clearance was lower in individuals with the *CYP2C9**1/*3 genotype whereas no differences were observed in those with the *CYP2C9**1/*2 genotype.[23] In our study, only one subject was a heterozygous carrier of *CYP2C9**3, and therefore statistical interpretation is difficult.

As expected, fluconazole was found to be a potent inhibitor of CYP2C9 *in vivo*. The changes seen in pharmacokinetic parameters are in accordance with those of earlier reports.[13,24] Zgheib *et al*. observed greater CYP2C9 inhibition after 7-day vs. single-dose fluconazole.[13] To address ethical concerns and to reduce the potential side effects caused by long-term use, we used only one dose of fluconazole in our study. Slight increases in half-life and AUC were observed in our study relative to those reported in an earlier study by Zgheib *et al*.;[13] that is, our study data indicated weaker inhibition by CYP2C9. However, the MR values in

urine in session 2 (0.79 (0.36)) in our study were in accordance with their data.[13] In fact, MRs (SD) in urine were 0.91 (0.68) and 0.74 (0.48) after dose 1 and dose 7, respectively.

Rifampicin is one of the most potent inducers of phase I and phase II drug-metabolizing enzymes.[25] Rifampicin pretreatment had a major influence on FLB clearance, whereas no significant change was observed for OHFLB. This could be explained by UGT2B7 induction by rifampicin, which increases OHFLB clearance generated by induced CYP2C9. An approximate twofold increase in FLB clearance was observed in plasma, which is in good agreement with earlier findings when subjects were exposed to moderate doses of rifampicin for several days.[26] The increases in MR values in urine in session 3 were weak in comparison with those in session 1, yielding a notable overlap of the MR values in sessions 1 and 3. In our study, rifampicin was administered for 5 days. In an earlier study on the effect of rifampicin on prednisolone pharmacokinetics, only half of the maximal induction effect was reached within 5 days.[27] Another study showed that the effect of rifampicin as measured by the urinary 6β-hydroxycortisol-to-cortisol ratio reached a plateau on day 6 of rifampicin administration.[28] Consequently, more pronounced induction could possibly have been observed if rifampicin had been administered beyond 5 days.

The subjects were given breakfast 1 h after drug ingestion and another meal 4 h after drug ingestion. A delay in the absorption phase has sometimes been observed when food and a drug are ingested simultaneously. In our study, pharmacokinetic parameters were in accordance with those of earlier reports,[13,24] indicating that the two meals given at these time points had no remarkable influence on FLB and OHFLB pharmacokinetics.

Complete agreement was observed between the MR values determined using DBS and those using plasma, as demonstrated by the excellent Spearman correlation coefficients. Good correlations were also observed between MR and FLB clearance. However, further studies (such as intraindividual comparisons of *in vitro* enzyme activities to phenotyping metrics *in vivo*)[29] are needed to further validate FLB as a probe drug for CYP2C9 activity assessment.

In summary, this study demonstrated that the single-point method using a DBS sample drawn 2 h after FLB administration provides a simple, minimally invasive alternative to plasma collection for assessing metabolic enzyme activity. Perfect correlations were observed between the MRs determined in plasma, DBS, and urine.

METHODS

This study was approved by the institutional ethics committee of the Anaesthesiology, Pharmacology and Intensive Care Department, Geneva University Hospitals; registered with the Swiss Agency for Therapeutic Products (Swissmedic); and performed in accordance with good clinical practices.

After giving informed consent, 10 healthy male subjects, all of them volunteers, were included in an open-label study.

The mean age was 27 years (range, 23–39 years). One subject was originally from Africa and all the others were Caucasians. They had normal results on physical examination, ECG, and liver- and kidney-function tests and were not taking any medications. The subjects did not ingest alcohol or caffeine-containing products for 48 h before each study day and abstained from grapefruit juice for at least 1 week before the study. The open study was performed in three sessions. During each session, at ~0800 h (after an overnight fast), an intravenous catheter was inserted into the left forearm vein, and a blood sample was drawn into an ethylenediamine-tetraacetic acid tube. For session 1, each subject was requested to empty the bladder before receiving 50 mg of FLB alone (Froben 50 mg). During session 2, after at least a 1-week washout period, each subject was given 400 mg of fluconazole followed by 50 mg of FLB 2 h later. Four days before the third session, after at least a 2-week washout period, the subjects were given four capsules of rifampicin (600 mg) and asked to take one a day. They were specifically asked about their compliance with the rifampicin regimen. On day 5, they received the last dose of rifampicin along with 50 mg of FLB. The subjects were instructed to collect their urine for 8 h after drug administration. The exact duration of urine collection and total urine volume were recorded for each subject, and aliquots (10 ml) were stored at −20 °C until analysis. Venous blood samples (total of 21 ml/session) were taken at 0, 0.5, 1, 2, 4, 6, and 8 h after FLB administration. Plasma was obtained by centrifugation at 3,350*g* for 10 min at 6 °C and frozen at −20 °C until analysis. Along with venous blood sampling, capillary whole blood (i.e., 5 µl) was collected on a Whatman 903 filter paper card (Dassel, Germany) from a finger prick (BD Microtainer: Contact-Activated Lancet, Plymouth, UK) using a volumetric micropipette (Eppendorf, Hamburg, Germany). DBS samples were allowed to dry at room temperature, packed in sealable plastic bags containing a desiccant, and stored in the dark at an ambient temperature until analysis. The subjects were given breakfast 1 h after drug administration and another meal 4 h after drug administration.

Genotyping. Genomic DNA was extracted from whole blood (200 µl) using the QIAamp DNA blood Mini Kit (Qiagen, Hombrechtikon, Switzerland). *CYP2C9**2 and *CYP2C9**3 genotyping was performed by means of multiplex PCR with fluorescent probes (LightMix 40-0298-16, TibMolbiol, Berlin, Germany) and melting curve analysis on a LightCycler (Roche, Basel, Switzerland).

Analytical methods

FLB and 4′-hydroxyflurbiprofen determination in plasma. Unconjugated and total (unconjugated + conjugated) FLB and 4-OHFLB concentrations in plasma were determined using a validated high-performance liquid chromatography (HPLC) coupled to a fluorescence detector. Total FLB and 4-OHFLB were obtained from 50 µl of plasma after chemical hydrolysis using hydrochloric acid, as described previously.[29] For sample preparation, the method involved liquid–liquid extraction. Analytes were separated on a reverse-phase column in gradient mode.

FLB and 4′-hydroxyflurbiprofen determination in DBS. Unconjugated FLB and 4-OHFLB in capillary DBS were quantified using reverse-phase-HPLC coupled to a tandem mass spectrometer (MS/MS) operating in negative electrospray ionization mode. The extraction of DBS samples was performed by means of an automated system, allowing for the online desorption of DBS directly into the HPLC-MS/MS device without sample pretreatment.[30,31] The method was fully validated on the basis of international criteria.

FLB and 4′-hydroxyflurbiprofen determination in urine. Total (unconjugated + conjugated) FLB and OHFLB were quantified using an HPLC assay as previously described, with slight modifications.[13] Briefly, 350 µl of purified water and 100 µl of 10N hydrochloric acid were added to 50 µl of urine, and the vials were screw-capped. Chemical hydrolysis was performed at 90 °C for 30 min. Once the hydrolysis was completed, 50 µl of internal standard (I.S.) (naproxen: 1 µg/ml) was added, and the samples were extracted using 5 ml of hexane-ethyl acetate (50–50) for 15 min. The extracted solution was subjected to centrifugation at 5,000*g* and the upper organic phase was transferred to a second tube and evaporated to dryness. Residues were reconstituted in 250 µl of mobile phase, and 20 µl were injected onto the HPLC system. The separation method used was the same as the one described for the plasma samples.

Data analysis. Pharmacokinetic parameters were estimated using a noncompartmental method using WinNonlin version 5.2 (Pharsight, Mountainview, CA, USA). The results are presented as mean values (±SD) and median ratios with 90% confidence intervals. All statistical analyses were performed using SPSS software version 17 (Chicago, IL, USA). All tests were two-tailed, and a probability of $P < 0.05$ was considered significant.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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