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Authors: De Kesel P., Lambert W., Stove C.

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Alternative sampling strategies for cytochrome P450 phenotyping

Pieter M.M. De Kesel, Willy E. Lambert and Christophe P. Stove*

Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, B-9000 Ghent, Belgium

*Corresponding author:

Christophe P. Stove

Laboratory of Toxicology

Department of Bioanalysis

Faculty of Pharmaceutical Sciences

Ghent University

Ottergemsesteenweg 460

B-9000 Ghent

Belgium

E-mail: Christophe.Stove@UGent.be

Tel.: +32 9 264 81 21

Fax: +32 9 264 81 83

Abstract

Interindividual variability in the expression and function of drug metabolizing cytochrome P (CYP) 450 enzymes, determined by a combination of genetic, non-genetic and environmental parameters, is a major source of variable drug response. Phenotyping by administration of a selective enzyme substrate, followed by the determination of a specific phenotyping metric, is an appropriate approach to assess the *in vivo* activity of CYP450 enzymes, as it takes into account all influencing factors. A phenotyping protocol should be as simple and convenient as possible. Typically, phenotyping metrics are determined in traditional matrices, such as blood, plasma or urine. Several sampling strategies have been proposed as an alternative for these traditional sampling techniques. In this review, we provide a comprehensive overview of available methods using dried blood spots, hair, oral fluid, exhaled breath and sweat for *in vivo* CYP450 phenotyping. We discuss the relation between phenotyping metrics measured in these samples and those in conventional matrices, along with the advantages and limitations of the alternative sampling techniques. Reliable phenotyping procedures for several clinically relevant CYP450 enzymes, including CYP1A2, CYP2C19 and CYP2D6, are currently available for oral fluid, breath or dried blood spots, while additional studies are needed for other CYP450 isoforms, such as CYP3A4. The role of hair analysis for this purpose remains to be established. Being non- or minimally invasive, these sampling strategies provide convenient and patient-friendly alternatives for classical phenotyping procedures, which may contribute to the implementation of CYP450 phenotyping in clinical practice.

Key Points

- Phenotyping by administration of a selective probe drug, followed by determination of a specific phenotyping metric, allows to assess the *in vivo* enzyme activity of CYP450 enzymes, taking into account genetic, non-genetic and environmental influencing factors.
- In addition to classical sampling techniques, such as blood or urine collection, reliable phenotyping procedures for several clinically relevant CYP450 enzymes are currently available for oral fluid, breath and dried blood spots.
- Alternative sampling strategies may aid to implement CYP450 phenotyping in clinical practice or at home.

1 Introduction

Enzymatic biotransformation is one of the main routes of elimination of drugs and other xenobiotics from the human body. These enzymatic processes can be divided into two phases: compounds are modified by oxidation, reduction or hydrolysis in phase I, while conjugation reactions in phase II typically increase the hydrophilic character of substrates by adding endogenous molecules, such as glucuronic acid or gluthathion. Phase I metabolism, being the rate-limiting step of enzymatic biotransformation, is mainly catalyzed by cytochrome P (CYP) 450 enzymes [1]. CYP450 is a superfamily of heme-containing enzymes located primarily in the endoplasmatic reticulum of liver cells and enterocytes in the epithelium of the small intestine. On the basis of increasing amino acid similarity, this group of enzymes is divided into families and subfamilies, denoted by numbers and letters, respectively. Individual enzymes within a subfamily are given an additional number. While 57 functional human *CYP450* genes have been identified, only a limited number of the resulting proteins plays a role in the metabolism of drugs. The latter enzymes mainly belong to the CYP1, CYP2 and CYP3 families [2].

Many CYP450 enzymes display a considerable interindividual variability in their expression and function, which is determined by a combination of genetic, epigenetic and non-genetic host factors (e.g. sex, age, pathophysiological conditions) as well as environmental influences (e.g. diet, tobacco smoke, drug intake) [3]. Consequently, each individual has its own CYP450 metabolic profile. As it is estimated that the major CYP450 enzymes are involved in 70-80 % of phase I reactions metabolizing clinically used drugs [4], variability in enzyme activity may result in variability in bioavailable drug levels and, consequently, variable response to drug therapy. When the parent drug is pharmacologically active, impaired enzyme function may increase the risk of developing adverse drug reactions (ADRs). For example, Phillips *et al.* found that 59 % of drugs cited in ADR studies are, at least partially, metabolized by enzymes with reduced function [5]. Likewise, when metabolization results in inactivation of a parent drug, higher than normal enzyme activity may cause standard drug therapy to fail. In these cases, increasing the drug dose is needed to obtain the same level of efficacy [3,6].

Therefore, estimating the metabolic status of an individual for a given pathway can be a useful tool to explain unexpected drug plasma concentrations, identify causes of ADRs, guide therapy or even predict the appropriate drug dose prior to the start of a therapy. Some CYP450 enzymes, in particular CYP2D6, CYP2C19 and CYP2C9, are encoded by highly polymorphic genes and distinct subpopulations

can be identified based on the genetic signature [4,7,8]. For many drugs metabolized by these enzymes, clinical outcome could be correlated with a certain genotype and genotyping a patient has proven to be a reliable strategy to assess the metabolic phenotype [3,6,8]. This approach holds the advantages that is has to be performed only once for each individual and that intake of test drugs is not required. Also for other important CYP450 enzymes, such as CYP1A2 and CYP3A4, a wide interindividual variability in enzyme activity has been described. Although multiple polymorphisms of the respective genes have been identified, variant alleles giving rise to altered enzyme function are very rare and genetic factors alone are unable to fully explain the variation in enzyme function [2,9]. These enzymes are highly influenced by environmental factors, such as inhibitor or inducer drugs, diet components and tobacco smoke [3]. Therefore, phenotyping by administration of a selective substrate is the preferred approach to assess the *in vivo* activity of these enzymes, as it takes into account genetic, non-genetic and environmental influences. In addition, even for those enzymes whose function is mainly genetically controlled, individuals that were genotyped as extensive metabolizers may display a (transient) poor metabolizer phenotype, due to intake of enzyme-inhibiting drugs or certain comorbidities influencing enzyme activity, such as HIV, liver disease and cancer. This phenomenon, called phenoconversion, has been observed mainly for CYP2D6 and CYP2C19 [10]. Also here, phenotyping can be applied in conjunction with genotyping to obtain an integrated image of enzyme function.

In general, phenotyping for a drug metabolizing enzyme is performed by the administration of a selective substrate of the enzyme, followed by the determination of a specific phenotyping metric. The latter may comprise various pharmacokinetic parameters, such as the systemic clearance of the probe drug, single point concentrations or metabolite/parent drug concentration ratios (or parent drug/metabolite ratios), typically obtained from plasma or urine samples. Multiple probe drugs and phenotyping protocols have been described for different CYP450 enzymes, amongst which several have been validated to provide a reliable estimate of the actual enzyme activity. We refer to the work of Streetman *et al.* [11] and Fuhr *et al.* [12] for an overview of available phenotyping procedures. Fuhr *et al.* also discussed the requirements for appropriate phenotyping procedures. Apart from the use of selective substrates and metrics and the need of probe drugs with an acceptable safety profile, reliable analytics and low intraindividual variability of the metric, the authors stated that phenotyping procedures ideally should be simple and convenient. The use of alternative samples strategies instead of traditional blood/plasma or urine sampling may fulfill this requirement. The major drawback of venous blood sampling in the context of phenotyping is probably the inherent invasiveness, which may specifically pose a problem when repeated sampling is needed to construct a full concentration-time profile. The latter is, for example, required in drug-

drug interaction studies as this approach takes into account the influence of various factors on the kinetics of the probe drug, including processes other than enzyme activity. In addition, venipuncture requires specialized staff. Concerning urine sampling, the susceptibility to non-specific noise caused by urinary flow or pH is a matter of concern. For example, urinary metabolic ratios of the probe drugs caffeine and dextromethorphan for phenotyping of CYP1A2 and CYP2D6, respectively, showed high intraindividual variability caused by these variables [13,14]. Furthermore, phenotyping protocols often require that urine is collected over a period of several hours. Apart from being inconvenient for the patient, this impedes efficient transport of samples.

This review aims at providing an overview of alternative sampling strategies for *in vivo* CYP450 phenotyping. More specifically, the use of dried blood spots, hair, oral fluid, exhaled breath and sweat for this purpose will be discussed. Special emphasis was placed on how phenotyping metrics measured in these alternative samples relate to those in conventional matrices, as we believe that this is a key point to evaluate the validity and usefulness of alternative sampling strategies for CYP450 phenotyping. A discussion on the actual therapeutic usefulness of phenotyping for individual enzymes was out of the scope of this work. A structured literature search was performed using PubMed (U.S National Library of Medicine) and ISI Web of Science™ databases. Keywords were cytochrome P (CYP) 450, all major CYP450 isoforms, phenotyping, enzyme activity and the above mentioned matrices. Only articles in English were selected and references of relevant articles were screened. Although similar sampling procedures as discussed here can also be used for phenotyping other drug metabolizing enzymes, such as conjugative phase II enzymes, these are not covered by this review.

2 Dried blood spots

Since Guthrie and Susi proposed to use newborn dried blood spots (DBS) to determine phenylketonuria [15], DBS sampling and analysis has been extensively used in newborn screening programs. The availability of highly sensitive analytical techniques, mainly liquid chromatography – tandem mass spectrometry (LC-MS/MS), resulted in an exponential increase in the interest in DBS for applications in other fields, including therapeutic drug monitoring [16,17], toxicology [18,19] and toxico- and pharmacokinetic studies in (pre-)clinical phases of drug development [20-22]. In general, DBS are prepared by applying a drop of capillary blood, obtained by a finger or heel prick, on a dedicated filter paper card. This can either be performed in a volumetric way, by spotting a defined volume of blood using a microcapillary, or non-volumetrically, by direct application of a drop of blood from the fingertip. Upon application to the filter paper, the resulting spots are dried to the air. This sampling technique offers several advantages over traditional blood sampling by venipuncture. DBS

sampling by fingerprick is only minimally invasive and it provides a relatively easy and inexpensive way of taking a representative sample. Sample collection can be performed by an informed patient himself, eliminating the need for a trained phlebotomist and enabling sampling in a non-specialized environment (e.g. at home) [23]. Furthermore, as several compounds showed enhanced stability in DBS [24-27], samples can be transferred via regular mail and stored at room temperature in the laboratory. The reduced risk of infection, due the fact that pathogens are deactivated upon drying [28], further contributes to the convenient and cost-effective transport and storage. From an analytical point of view, compounds can often be extracted from DBS cards using straightforward procedures, making this technique particularly suitable for automated processing [29-31].

Despite the many advantages, several issues still surround DBS sampling and analysis. As high quality data can only be derived from high quality samples, good sampling practices are of key importance, especially when non-experienced individuals are involved. Being a microsampling technique, in which small volumes of blood are collected and processed, analytical instruments involved in DBS analysis should provide sufficient sensitivity. Furthermore, when capillary DBS are analyzed, knowledge of the correlation between capillary and venous concentrations of the analyte of interest is needed [32,33]. In this light, thorough interpretation of DBS results often requires comparison with DBS and plasma/serum results, as reference intervals or cut-off values are commonly based on the latter [34]. Apart from these issues, some DBS-specific analytical aspects should be addressed for every DBS method, especially when partial punches are analyzed. These parameters are the impact of the volume of blood spotted onto the filter paper, the punch localization and, undoubtedly the most discussed issue in DBS analysis, the influence of hematocrit. The latter problem, along with strategies to cope with it, has been reviewed recently by De Kesel *et al* [35].

In the last few years, several groups evaluated the utility of DBS for CYP450 phenotyping. By validating five individual LC-MS/MS methods for the determination of caffeine, flurbiprofen, midazolam, omeprazole and rosiglitazone, being substrates of CYP1A2, CYP2C9, CYP3A4, CYP2C19 and CYP2C8, respectively, in 3-mm DBS punches, Lad *et al.* illustrated the idea of using DBS sampling for this purpose. However, specific metabolites of these probe drugs, enabling assessment of metabolic ratios, were not included and the developed methods were not applied in a phenotyping study [36].

Since then, DBS-based phenotyping of single CYP450 isoforms has been investigated in several studies, summarized in Table 1. In a CYP3A4 phenotyping study, de Boer *et al.* determined midazolam concentrations in plasma, whole blood and volumetrically applied venous and capillary DBS of 12 healthy volunteers at different time points following administration of a single 7.5 mg

midazolam test dose. This approach allowed to construct pharmacokinetic (PK) profiles for the different matrices, from which AUC values were derived as CYP3A4 phenotyping metric [37]. Interestingly, while mean midazolam AUC values for venous and capillary DBS punches were comparable, mean AUC obtained from whole blood analysis was considerably lower. Although, based on high regression coefficients for venous DBS, whole blood and plasma, the authors concluded that the results for the evaluated sampling techniques were strongly correlated, we believe that this 'DBS-effect' needs further examination before accepting the presented approach for CYP3A4 phenotyping. Furthermore, strongly deviating results for capillary DBS in some cases negatively impacted the correlation with the other matrices.

More clear findings in favor of DBS sampling were described for other CYP450 enzymes. Déglon *et al.* developed an automated system for on-line desorption of DBS combined with LC-MS/MS analysis. This platform was used for the determination of the CYP2C9 probe substrate flurbiprofen and its metabolite 4-hydroxyflurbiprofen in whole, volumetrically applied 5 µL DBS [29]. The results of a PK study, involving administration of 50 mg flurbiprofen to 10 healthy volunteers, were described in a separate report by Daali *et al.* [38]. Concentration ratios of 4-hydroxyflurbiprofen to flurbiprofen, being measures of CYP2C9 activity, in capillary DBS collected at multiple time points post-administration were in good agreement with corresponding ratios in plasma and urine and with flurbiprofen clearances, as evidenced by Spearman rank correlation coefficients. In addition, it was demonstrated that metabolic ratios in DBS and plasma changed in a similar way following CYP2C9 induction and inhibition. Murphy *et al.* measured cotinine and *trans* 3'-OH-cotinine levels in plasma and non-volumetrically applied capillary DBS of smokers and non-smokers exposed to second hand smoke. As CYP2A6 mediates cotinine conversion to *trans* 3'-OH-cotinine, the resulting metabolite/precursor ratio is often used as CYP2A6 phenotyping metric. Ratios in DBS punches and corresponding plasma samples of 81 subjects were highly correlated, with a Spearman rank correlation coefficient of 0.94 ($p < 0.001$) [39]. We investigated whether DBS were suited for CYP1A2 phenotyping using the established probe drug caffeine and its major metabolite paraxanthine [40]. Six hours after the intake of a 150 mg oral caffeine dose, paraxanthine/caffeine ratios were determined in plasma, whole blood and 3-mm punches of venous and non-volumetrically applied capillary DBS of 73 healthy volunteers. While capillary DBS concentrations were significantly lower than venous whole blood and plasma concentrations and DBS concentrations were influenced by hematocrit and blood volume spotted, both compounds were similarly affected. As a consequence, Bland-Altman and Passing-Bablok plots showed that paraxanthine/caffeine ratios, i.e. the CYP1A2 phenotyping metric, were highly comparable in all matrices [41].

In addition to these single enzyme phenotyping studies, DBS have also been used to measure the activity of multiple CYP450 enzymes simultaneously by the administration of a cocktail of probe drugs. Donzelli *et al.* applied a new phenotyping cocktail, called the Basel cocktail, consisting of low doses of losartan (12.5 mg, for CYP2C9), omeprazole (10 mg, for CYP2C19), midazolam (2 mg, for CYP3A4), caffeine (100 mg, for CYP1A2), metoprolol (12.5 mg, for CYP2D6) and efavirenz (50 mg, for CYP2B6). This cocktail was administered to 16 healthy volunteers followed by non-volumetric collection of capillary DBS at different time points along with saliva samples and whole blood for plasma preparation. As a result of the low drug doses and the moderate sensitivity of the applied LC-MS/MS method, only the pairs caffeine - paraxanthine and omeprazole - 5-OH-omeprazole could be quantified in 3-mm DBS punches. As a measure of CYP1A2 activity, paraxanthine/caffeine ratios at 8 h in DBS correlated with corresponding AUC ratios in plasma. Similar findings were obtained for omeprazole/5-OH-omeprazole ratios at 2 h in DBS, being the CYP2C19 phenotyping metric [42]. Bosilkovska *et al.* conducted a phenotyping study in which another combination of probe drugs, the Geneva cocktail, containing bupropion (25 mg, for CYP2B6), flurbiprofen (25 mg, for CYP2C9), omeprazole (5 mg, for CYP2C19), dextromethorphan (10 mg, for CYP2D6) and midazolam (1 mg, for CYP3A4), was administered to 10 healthy volunteers together with a cup of coffee (100 mg caffeine, for CYP1A2) or coke (25 mg caffeine). Also here, capillary and venous blood were collected at several time points after intake of the drug cocktail. DBS were prepared by spotting 10 µL of capillary blood on DBS cards and whole spots were analyzed. In contrast to Donzelli *et al.*, the higher volume, combined with the use of more sensitive equipment, allowed quantification of all probe drugs and corresponding metabolites in DBS. Single point metabolite/parent drug ratios at 2 h for CYP1A2 and CYP3A4 and at 3 h for CYP2B6, CYP2C9 and CYP2D6, and the $AUC_{2,3,6}$ metabolite/parent drug ratio for CYP2C19 showed acceptable Spearman rank correlation coefficients with AUC_{last} ratios in plasma, both when the cocktail was administered alone or in combination with CYP450 inhibitor and inducer drugs [43,44].

For almost all CYP450 enzymes evaluated in the cited studies, a good agreement between phenotyping metrics measured in DBS and conventional, mostly plasma-based, metrics was found. Therefore, DBS sampling seems to represent a minimally-invasive and patient-friendly alternative for traditional venous blood sampling to obtain high-quality phenotyping data. Given the ease of the sampling technique and the convenient transport, DBS are highly suitable to be implemented in large-scale phenotyping studies or even in home-sampling scenarios. To further accept DBS-based CYP450 phenotyping, other CYP450 isoforms should be investigated and the results of the above-mentioned studies need to be confirmed in other, sufficiently large study populations. Future studies

may also consider to evaluate the impact of known issues, mainly hematocrit and blood volume, on DBS-based phenotyping metrics.

3 Hair

Hair analysis has become an established discipline in bioanalysis in the last decades with numerous published methods, mainly dealing with qualitative or quantitative determination of drugs of abuse, alcohol biomarkers or environmental contaminants. Collection and analysis of hair has been implemented in various fields, including forensic toxicology, investigation of drug-facilitated crimes, workplace drug testing, doping control in sports, detection of perinatal drug exposure and abstinence monitoring in driving license regranting programs or child custody cases [45,46]. The most important feature of hair analysis is probably the possibility to retrospectively assess drug use. Drugs that are incorporated into hair are no longer subject to biotransformation. As a consequence, many drugs proved to be stable in hair for prolonged periods of time, yielding a window of detection of several months or years, depending on hair length. Determination of drugs in specific hair segments even enables retrospective assessment of drug use that can be assigned to a defined time period in the past, taking into account an average hair growth rate of 1.06 cm/month [47]. Furthermore, being a non-invasive sampling technique, collection of hair samples can be performed by non-specialized personnel, although some expertise is required to avoid variation in the amount of hair left on the scalp [47]. Hair strands are typically collected from the posterior vertex region of the head, as hair growth rate shows less variability there [45,46]. Although hair analysis provides distinct benefits compared to traditional bioanalytical matrices, it also holds important limitations, especially concerning the interpretation of hair results. One of the main issues in hair analysis is the potential contribution of external contamination or passive drug exposure to measured hair concentrations. Therefore, hair samples are usually decontaminated prior to further processing, although a general consensus on standard wash procedures is currently lacking [48]. Several strategies have been proposed to distinguish active ingestion of a substance from external contamination, such as determining specific metabolites in hair and calculating metabolite/precursor concentration ratios, which can be compared with established cut-off values [49,50]. In addition, while analysis of hair segments has been widely applied to detect changes in drug consumption over time or to allocate drug intake to a certain period, interpretation of segmental hair analysis may be challenged due to external contamination [51]. On the other hand, analytes may also be removed from the hair as a result of damage caused by cosmetic hair treatment, such as bleaching or dyeing, leading to underestimation of hair concentrations [52]. Another factor impeding the interpretation of hair

results is the fact that, for many compounds, hair concentrations do not correlate with plasma or serum levels and, consequently, with drug dose.

Apart from the above-mentioned, well-established applications, hair analysis has also been proposed for the assessment of drug metabolism. In 1996, Mizuno *et al.* determined caffeine in hair strands of 6 healthy individuals and 6 patients with liver cirrhosis. Significantly higher caffeine concentrations were found in hair samples of liver cirrhosis patients. In a separate group of volunteers, the authors showed that elimination of a defined caffeine dose, based on saliva concentrations, was slower in patients with liver disease compared to healthy individuals. This led the authors to suggest that caffeine concentrations in hair could be used as an indicator of liver metabolic capacity. It should be noted, however, that no data were available on caffeine intake of the individuals whose hair samples were analyzed and no metabolites of caffeine were measured in hair to make a more conclusive statement on metabolic capacity [53]. To date, 2 studies evaluated the usefulness of hair analysis for the assessment of CYP450-mediated metabolism by correlating metabolic ratios in hair with enzyme genotypes or reference phenotyping metrics (Table 1). Thieme *et al.* analyzed hair samples of 23 children for which illegal, prolonged administration of the antidepressant amitriptyline was suspected. The *N*-demethylation of amitriptyline to nortriptyline is mediated by CYP2C19. While nortriptyline/amitriptyline ratios did not change significantly along individual hairs, large interindividual variations in these ratios were observed. The authors found a clear correlation between nortriptyline/amitriptyline ratios in hair and the number of functional alleles of CYP2C19. In particular, CYP2C19 poor metabolizers, carrying 2 dysfunctional alleles, had significantly lower hair ratios compared to intermediate or extensive metabolizers. Apart from CYP2C19, also CYP2D6 is involved in amitriptyline and nortriptyline metabolism by mediating the hydroxylation of both compounds. Despite wide interindividual variability, no correlation between hydroxylation of amitriptyline and nortriptyline and CYP2D6 polymorphisms was found. This was probably due to the fact that only intermediate and extensive, and no poor or ultrarapid CYP2D6 metabolizers were present in the study population [54]. In a recent study of our group, we evaluated the potential of hair analysis for CYP1A2 phenotyping. Paraxanthine/caffeine concentration ratios were determined in proximal 3-cm hair segments of 60 healthy volunteers. Hair ratios of all subjects were compared with a reference CYP1A2 phenotyping metric, being paraxanthine/caffeine ratios in plasma 6 hours after intake of a caffeine test dose (150 mg). While ratios in both matrices showed a similar range and a statistically significant correlation, large differences between hair and plasma ratios were seen in individual cases. These deviations could not be attributed to factors potentially affecting the incorporation or retention of small molecules in hair, such as hair pigmentation and cosmetic hair coloring, or affecting CYP1A2 activity, such as oral contraceptives, smoking, gender, age and caffeine

consumption. On the basis of these results, we concluded that hair analysis is not (yet) suitable for CYP1A2 phenotyping, as interpreting paraxanthine/caffeine hair ratios on an individual basis was difficult [55].

Hair analysis has also been used as a tool to retrospectively document changes in CYP450-mediated metabolism over time by determining metabolic ratios in consecutive hair segments. In particular, this approach has been applied in several studies to detect pregnancy-induced altered metabolism. Klein *et al.* collected hair samples of 28 smoking women during the postpartum period and divided the obtained samples into segments corresponding to the trimesters of pregnancy. All volunteers reported a consistent smoking behavior throughout pregnancy and concentrations of nicotine and its major metabolite cotinine were determined in all segments. Nicotine/cotinine ratios in the third trimester were significantly decreased compared to those in the first trimester. According to the authors, these findings illustrated that nicotine metabolism to cotinine, mainly catalyzed by CYP2A6, increases as pregnancy advances [56], which was in agreement with previous findings [57]. In a more recent study by the same group, similar results for nicotine/cotinine ratios in hair of 74 smoking women were obtained [58]. O'Brien *et al.* applied an analogous approach to examine changes in antidepressant metabolism during pregnancy. Citalopram/norcitalopram ratios in hair segments corresponding to the first and third trimester of pregnancy were significantly lower than ratios in the postpartum period [59]. Although only 4 women were included, these findings could confirm the elevated metabolism of citalopram [60] and increased CYP2D6 activity during pregnancy [61-63]. Finally, Himes *et al.* analyzed hair samples from 29 opioid-dependent pregnant women enrolled in methadone-assisted therapy. Methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), the major metabolite of methadone, were measured in proximal 3-cm hair segments collected throughout pregnancy. In addition, cumulative methadone doses taken during the time interval corresponding to the analyzed hair segments were calculated. A positive linear trend between this cumulative dose and EDDP/methadone ratios in hair was observed [64]. This could illustrate auto-induction of methadone metabolism, mainly mediated by CYP3A4 [65], or, again, elevated CYP450 activity during pregnancy [61-63]. These studies suggest that applying segmental hair analysis to retrospectively assess drug metabolism may hold promise as an alternative phenotyping approach. Here, hair analysis, due to its distinct characteristics, provides a tool to gain information on a subject's metabolic status that could not have been obtained by analyzing traditional matrices (blood or urine), given their much shorter time window of detection. On the other hand, although altered drug metabolism during pregnancy is a generally recognized concept, it should be noted that in none of the above mentioned studies the metabolic ratios measured in hair

were compared with reference phenotyping indices determined in other matrices to support their validity in reflecting changed metabolism.

In general, in contrast to traditional phenotyping procedures, the probe drugs used to estimate enzyme function in the cited hair methods were not specifically administered for this purpose. To determine metabolic ratios in hair, these methods relied on intake or administration of therapeutic drugs or intake of enzyme substrates via cigarette smoke or nutrition, leading to variation in the duration of exposure and the amount of ingested or inhaled compounds between individuals. Another factor that needs to be taken into account when metabolic ratios in hair are evaluated, is the possibility that metabolites and precursors may be incorporated into hair to a different extent. It is generally accepted that compounds are incorporated into hair from the bloodstream, sweat or sebum or through external contamination [66]. Several compound-dependent factors affect these processes, such as their physicochemical properties and affinity for binding to melanin in hair. In addition, analytical hair decontamination procedures or external influences, such as regular hair washing, influence of UV-light and cosmetic treatment of hair, may potentially cause differential loss of metabolites and precursors from the hair [45]. Therefore, as many variables may impact analyte concentrations in hair, the usefulness of hair metabolic ratios needs to be evaluated on a case by case basis and should be supported by controls in other matrices or data on enzyme genotypes. Futhermore, as hair results are likely to provide an average image of the enzyme phenotype, short-term changes in enzyme activity will not be detected by hair analysis, in contrast to DBS- or oral fluid-based phenotyping procedures.

4 Oral fluid

Collection of oral fluid as an alternative to plasma has been applied in diverse areas. For example, as concentrations in oral fluid reflect the free, non-protein bound, pharmacologically active fraction of a drug in plasma, oral fluid sampling has gained attention as a tool for therapeutic monitoring of selected drugs [67]. Currently, the best established applications of oral fluid are probably roadside and workplace drug testing [68,69]. Rapid, simple and non-invasive sample collection are important advantages of oral fluid sampling. As a consequence, samples may be collected by non-experienced individuals and repeated sampling can be done more easily. Compared to blood sampling, the risk of infection is lower. Oral fluid is usually obtained by drooling or spitting in a dedicated collection device or by absorbent swabs. To hasten sample collection and increase the sampled volume, oral fluid flow can be stimulated, e.g. by citric acid or chewing paraffin wax. However, stimulation may affect drug concentrations due to the dilution effect and an altered pH of the oral fluid. Other challenges

associated with oral fluid analysis are adsorption of analytes to collection devices, interferences from food or drinks and contamination with orally ingested or inhaled drugs left in the mouth [70-72].

Oral fluid sampling has been evaluated in phenotyping studies involving several CYP450 enzymes (Table 1). The CYP1A2 substrate caffeine is by far the most investigated and applied probe drug in oral fluid-based phenotyping procedures. Starting from the 1980's, collection of oral fluid has been used for the measurement of caffeine clearance as an indicator of liver function, with several studies showing an excellent agreement between PK parameters derived from oral fluid and serum or plasma samples [73-79]. Fuhr *et al.* evaluated the usefulness of caffeine as CYP1A2 phenotyping probe drug by performing a meta-analysis of 4 studies in which caffeine and paraxanthine had been measured in plasma, urine and oral fluid samples of 78 subjects. It was concluded that caffeine clearance from oral fluid and 3-7 h post-dose paraxanthine/caffeine ratios in both oral fluid and plasma were highly correlated with systemic caffeine plasma clearance, providing a better estimate of CYP1A2 activity than urinary ratios [80]. Furthermore, in a study involving 25 patients undergoing hepatectomy, significant correlations were found between *in vivo* parameters of CYP1A2 activity, being caffeine clearance and paraxanthine/caffeine ratios (3 and 6 h post-dose) in oral fluid or plasma, and *in vitro* parameters, being caffeine 3-demethylation and relative CYP1A2 content in liver microsomes [13]. Since then, the validity of oral fluid for CYP1A2 phenotyping was corroborated by several other studies [81-87] and, consequently, this approach has been widely applied to assess CYP1A2 activity in various settings [61,85,86,88-98].

As urinary dextromethorphan metabolic ratios for CYP2D6 phenotyping are affected by considerable intraindividual variability, Hou *et al.* evaluated the use of oral fluid for this purpose. Dextromethorphan/dextrorphan ratios in oral fluid samples of 61 healthy volunteers, measured 3 h following dextromethorphan administration, showed a significant correlation with 0-8 h urinary ratios and enabled to differentiate all CYP2D6 poor metabolizers from intermediate and extensive metabolizers identified by urinary metabolic ratios. The latter 2 groups, however, could not be separated on the basis of oral fluid results [99]. The applicability of this method and its potential to provide an alternative for urine-based CYP2D6 phenotyping was demonstrated in 100 anuric patients with renal failure [100]. However, in a study with repetitive dextromethorphan administration, Hu *et al.* observed poor repeatability of 3, 4, and 5 h oral fluid dextromethorphan/dextrorphan ratios. The authors proposed to use 6 h ratios instead, as these showed good repeatability and correlated significantly with urinary phenotyping metrics [101]. In addition, Lutz *et al.* were able to differentiate CYP2D6 poor metabolizers in a population of 170 volunteers using 2 h oral fluid dextromethorphan/dextrorphan ratios. Comparison with other phenotyping metrics or genotypic data was not included in this study [102].

While the latter procedures were based on the CYP2D6-mediated O-demethylation of dextromethorphan to dextrorphan, Kuo *et al.* proposed to use another metabolic route of dextromethorphan, N-demethylation to 3-methoxymorphinan, for CYP3A phenotyping. However, dextromethorphan/3-methoxymorphinan ratios in oral fluid of 3 volunteers showed a poor correlation with corresponding urinary ratios [103]. Based on good correlations between midazolam concentrations in plasma and oral fluid, determined in 8 subjects at multiple time points, Link *et al.* suggested that midazolam may provide a suitable probe drug for oral fluid-based CYP3A phenotyping. However, it should be noted that, due to the high plasma protein binding of midazolam, the common CYP3A phenotyping metrics AUC and clearance were considerably lower and higher, respectively, in saliva compared to plasma [104]. As mentioned in the section on DBS, Donzelli *et al.* tested a cocktail of probe drugs to simultaneously measure the activity of multiple CYP450 enzymes. Along with DBS, oral fluid samples were collected from 16 volunteers. Similar to DBS results, oral fluid concentrations of most probe drugs were below the LLOQ. Paraxanthine/caffeine ratios at 8 h in oral fluid, being the CYP1A2 phenotyping metric, showed a significant correlation with corresponding AUC ratios in plasma. As a potential measure of CYP2C19 activity, omeprazole/5-OH-omeprazole ratios at 2 h in oral fluid correlated poorly with corresponding AUC ratios in plasma [42].

The role of nicotine metabolites and corresponding metabolic ratios in various matrices, including oral fluid, as biomarkers of exposure to tobacco smoke, smoking cessation or to guide treatment of nicotine dependence has been thoroughly explored, as indicated in several studies and recent review papers [105-110]. Here, we will discuss studies that specifically evaluated the usefulness of nicotine metabolic ratios in oral fluid for CYP2A6 phenotyping. A first proof was provided by Dempsey *et al.*, who administered deuterium labeled nicotine and cotinine to 62 healthy volunteers. Resulting *trans* 3'-OH-cotinine/cotinine ratios in oral fluid 6 h post-intake were highly correlated with several indices of CYP2A6 activity, including 6 h ratios in plasma and oral clearance of both nicotine and cotinine [111]. CYP2A6 phenotyping based on *trans* 3'-OH-cotinine/cotinine ratios originating from nicotine in tobacco smoke was examined by St. Helen *et al.* Regression analysis revealed high correlations between ratios in oral fluid and both whole blood and plasma of smokers and nonsmokers exposed to tobacco smoke. Bland-Altman analysis confirmed the agreement between oral fluid and whole blood, while this was not the case for the oral fluid-plasma comparison. Potential causes of the latter discrepancy were not further addressed. Overall, the authors concluded that all matrices provided similar measures of nicotine metabolism [112]. In another study, Lea *et al.* addressed the intraindividual variation of oral fluid *trans* 3'-OH-cotinine/cotinine ratios in 6 regular daily smokers and observed that ratios were not affected by the time of sampling during the day [113].

In conclusion, oral fluid may provide a reliable alternative for plasma-based CYP450 phenotyping. Given the easy, rapid and non-invasive sample collection, this technique seems particularly suited for large epidemiological phenotyping studies. From the many available studies it can be stated that oral fluid has been established as a valid alternative sampling method for CYP1A2 phenotyping, with caffeine clearance or the paraxanthine/caffeine ratio as metrics. Although promising data were obtained for other CYP isoforms, such as CYP2D6 and CYP2A6, further studies are needed to confirm the results with these enzymes.

5 Exhaled breath

In the past 40 years, breath analysis has been extensively explored as a diagnostic tool to examine liver function. Typically, these breath tests are based on oral or intravenous administration of an isotopically labeled test compound. While radioactive ^{14}C-labeled compounds were initially used, probe drugs carrying a functional group in which a stable ^{13}C-isotope replaces a ^{12}C-atom have now become standard practice, thereby providing a safer alternative. Upon administration, the probe drug is subject to metabolism and the labeled functional group is enzymatically cleaved. Following additional oxidation reactions, $^{13}CO_2$ is eventually formed. The latter is mixed with the body bicarbonate pool and exhaled in the breath. The $^{13}CO_2/^{12}CO_2$ ratio in breath samples, measured by isotope ratio mass spectrometry or isotope-selective nondispersive infrared spectrometry, has been used as an indicator of liver function. Usually, the ^{13}C-enrichment of breath following administration of the probe drug is compared to the baseline enrichment before ingestion, as several factors (e.g. diet) may cause variations in the natural occurrence of $^{13}CO_2$. Therefore, the final results are expressed as delta-over-baseline (DOB) values [114-116].

Apart from their use to investigate liver function, ^{13}C breath tests have been applied to quantitatively assess the activity of metabolic enzymes. For many probe drugs, the initial metabolic reaction, often a dealkylation step, is catalyzed by CYP450 enzymes and, therefore, the amount of $^{13}CO_2$ in breath should reflect enzyme activity. For a full overview of published breath tests, available probe drugs and their clinical utility, we refer to several recent reviews on this topic [117-121]. Here, we highlight a number of selected studies in which the potential of breath tests for CYP450 phenotyping has been evaluated by comparison with other indices of enzyme activity (Table 1). The N-demethylation of aminopyrine, the first probe drug used in breath tests, is mediated by several CYP450 enzymes, with CYP2C19 being the most important isoform [122]. Kodaira *et al.* found a significant correlation between $AUC_{0\text{-}3h}$ of DOB $^{13}CO_2/^{12}CO_2$ ratios and both plasma [^{13}C]-aminopyrine $AUC_{0\text{-}3h}$ and clearance [123]. [^{13}C]-pantoprazole has been proposed as an alternative CYP2C19 phenotyping probe, as several studies found significantly lower DOB values in CYP2C19 poor metabolizers compared to

intermediate or extensive metabolizers [124-129]. In addition, plasma [^{13}C]-pantoprazole AUC correlated significantly with the AUC of DOB $^{13}CO_2/^{12}CO_2$ ratios [124,127,129].

Leeder *et al.* developed a new breath test for CYP2D6 phenotyping using [^{13}C]-dextromethorphan as probe drug. Single 40 min DOB $^{13}CO_2/^{12}CO_2$ ratios proved equally effective as urinary [^{13}C]-dextromethorphan/dextrorphan ratios in distinguishing between CYP2D6 poor and extensive metabolizers [130]. The utility of this approach was evaluated by Opdam *et al.* in a study involving breast cancer patients taking tamoxifen, a prodrug that is converted to endoxifen by CYP2D6. The correlation between CYP2D6 phenotype, determined by the [^{13}C]-dextromethorphan breath test, and serum endoxifen levels was comparable with the correlation between the latter and sequence-based CYP2D6 activity prediction [131]. For the CYP1A2 substrate [^{13}C]-caffeine, Park *et al.* observed a significant correlation between 1 h single DOB $^{13}CO_2/^{12}CO_2$ ratios and plasma [^{13}C]-caffeine clearance [132]. These results were in agreement with previous studies, in which excellent correlations between caffeine breath test results and caffeine clearance were found [133-136].

One of the most widely applied phenotyping procedures is the erythromycin breath test, although several studies revealed conflicting results concerning its ability to reflect CYP3A4 phenotype, especially in relation to the established CYP3A4 phenotyping probe drug midazolam. While Lown *et al.* found a significant correlation between erythromycin breath test results, expressed as % ^{14}C exhaled in 1 h, and body weight-adjusted clearance of midazolam [137], these results could not be confirmed in other studies applying identical PK parameters [138,139]. Franke *et al.* used $1/T_{max}$ as an alternative erythromycin breath test parameter, but found no correlation with midazolam clearance either [140]. The fact that, in addition to CYP3A4, P-glycoprotein is involved in erythromycin metabolism, whereas this is not the case for midazolam, may explain the discordant results for both probe drugs [141].

In general, breath tests based on the administration of isotopically labeled probe drugs seem to provide an interesting, non-invasive alternative to assess the activity of several CYP450 enzymes. Breath samples are easily collected and do not require extensive sample work-up prior to analysis. However, widespread routine use of breath tests for phenotyping purposes has been limited to date, due to the high cost of isotopically labeled compounds and the highly specialized equipment required for analysis of breath samples.

6 Miscellaneous matrices

In addition to the above-mentioned matrices, many endogenous compounds and xenobiotics are, to a limited extent, secreted into sweat as well. Several applications of sweat testing have been

described, including measurement of chloride ions as a biomarker of cystic fibrosis [142] and detection of drugs of abuse in forensic toxicology, roadside and workplace drug testing [143-145]. Sweat for drug testing is commonly collected using transdermal absorptive sweat patches attached to the skin, representing a non-invasive sampling technique. In most cases, analytes can be removed from these patches using relatively simple extractions protocols, resulting in clean extracts that are subsequently analyzed by GC- or LC-MS. Sweat patches can be worn for several days, enabling continuous drug testing over an extended period of time compared to urine, saliva or blood. Limitations of this sampling strategy are the potential influence of external contamination of the devices or the skin and the unknown collected volume, as sweat production may vary in function of ambient temperature or physical activity [145]. Delahunty *et al.* suggested that sweat could potentially be used as an alternative for urine or plasma to estimate CYP1A2 activity. Following administration of caffeine, the latter compound and its metabolites were determined in transdermal absorptive sweat patches worn for several days by healthy volunteers. Caffeine, paraxanthine and theobromine were readily detected in the patches, allowing to calculate metabolite/parent drug ratios [146]. However, comparison of these ratios with corresponding ratios in plasma or urine, to illustrate their actual usefulness for CYP1A2 activity assessment, was not included in the study. To the best of our knowledge, no other studies on the use of sweat for CYP450 phenotyping are available to date. Kuwaya *et al.* developed a method that allowed to determine caffeine and parxanthine in fingerprints at several time points following consumption of a cup of coffee. Although this concept may hold promise for CYP1A2 phenotyping, paraxanthine/caffeine ratios were not calculated [147].

Another example of using an alternative matrix for measuring CYP450 activity was described by Grosso *et al.* In a study to investigate the association between intrauterine growth restriction (IUGR) and fetal caffeine exposure, caffeine and its primary metabolites were quantified in umbilical cord serum samples of 1606 women. As higher paraxanthine/caffeine ratios were positively correlated with an increased risk of IUGR, it was suggested that CYP1A2 activity may have an effect on fetal growth [148].

7 Conclusion

A substantial number of studies has evaluated the usefulness of DBS, hair, oral fluid or exhaled breath as alternative sampling strategies for CYP450 phenotyping. A large body of evidence supports the validity of oral fluid for CYP1A2 phenotyping, using caffeine as a probe drug. While this method has already been implemented in several phenotyping studies, promising results were also obtained for other oral fluid-based phenotyping approaches, especially for nicotine metabolic ratios as a

measure of CYP2A6 activity and the CYP2D6 probe drug dextromethorphan. CYP3A phenotyping in oral fluid following midazolam or dextromethorphan administration needs further investigation. Concerning the utility of exhaled breath as an alternative matrix for CYP450 phenotyping, the available data are, in some respect, comparable to those of oral fluid. While [^{13}C]-caffeine and [^{13}C]-dextromethorphan breath tests proved to be reliable procedures for CYP1A2 and CYP2D6 phenotyping, respectively, conflicting results were obtained for the assessment of CYP3A4 activity using the widely applied probe drug [^{14}C]-erythromycin. [^{13}C]-pantoprazole-based breath tests, on the other hand, showed promise to assess CYP2C19 metabolizer status. Recently, several groups proposed DBS sampling as an alternative strategy for CYP450 phenotyping. Procedures for several clinically relevant CYP450 isoforms, including CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19 and CYP2D6, revealed good agreement between phenotyping metrics determined in DBS and in plasma. Also here, inconclusive data were obtained for CYP3A4 phenotyping using midazolam as probe drug. Although hair sampling may provide an interesting tool in case a standard phenotyping procedure is not feasible (e.g. post-mortem), the actual value of hair analysis in this field remains to be established, given the limited number of studies available to date.

Based on the studies discussed in this review, it can be concluded that reliable phenotyping procedures for selected CYP450 enzymes are currently available for either oral fluid, breath and DBS. Important to note is that it should be decided on a case-by-case basis what phenotyping metric is to be used. While in many of the described alternative procedures metabolic ratios at one given time point have been used as phenotyping metric, other metrics (e.g. AUC, clearance, etc.) may be more appropriate in other scenarios. Also here -especially when repeated sampling is to be performed- these alternative matrices offer the benefit that they can be collected in a non- or minimally invasive way, causing minimal discomfort for the patient. Table 2 summarizes selected advantages and limitations of the alternative sampling strategies. To date, phenotyping of drug metabolizing enzymes has been mainly applied in research settings, for example in drug-drug interaction studies or to assess the impact of various factors, such as gender, age, diet or cigarette smoke, on enzyme activities. The availability of simple, convenient and patient-friendly protocols may hasten the acceptance and routine application of CYP450 phenotyping in clinical practice as well. There, phenotyping could play a role in predicting optimal drug dose prior to the start of the therapy or to guide therapy in case of unexpected changes in plasma levels of (or response to) a given drug. Examples of therapeutic drugs for which phenotyping using alternative strategies may be applied are the CYP2D6 substrates imipramine, nortriptyline (tricyclic antidepressants), haloperidol, risperidone (antipsychotics), codeine and tramadol (opioid analgesics), the CYP2C19 substrates omeprazole, lansoprazole (proton-pump inhibitors) and clopidogrel (platelet aggregation inhibitor), the CYP1A2

substrates clozapine and olanzapine (antipsychotics) and the CYP2C9 substrate warfarin (anticoagulant) [3,6,8]. For several of these, it remains to be fully established if and to what extent phenotyping (and resulting dose adaptation) indeed results in a better clinical outcome.

Furthermore, in contrast to venous blood sampling, collecting DBS, oral fluid or breath does not require a phlebotomist and can be performed by instructed patients themselves. In this light, a phenotyping scenario involving home-sampling may be envisaged, in which samples are transferred to the laboratory by mail, provided that the analytes of interest are stable under the transport conditions. In our opinion, DBS sampling may be the preferred approach for this purpose, given the ease of transport and storage of DBS cards compared to oral fluid and, especially, breath samples collected in dedicated bags. The required analytical instrumentation is another factor that may determine which alternative sampling strategy is best suited to allow a more widespread use of phenotyping. While DBS and oral fluid can be analyzed using techniques that have become standard in the modern clinical laboratory, such as LC-MS/MS, measurement of $^{13}CO_2/^{12}CO_2$ ratios in breath requires highly specialized equipment not commonly available in such laboratories. In recent years, microdosing of probe drugs has been applied in phenotyping studies to reduce the risk of adverse drug reactions or interferences with other drugs. Since doses are at least 100-fold lower than regular doses, highly sensitive, accurate and precise analytical procedures are required [149]. While this already represents a challenge for microdosing approaches using plasma, it may especially pose a problem when alternative sampling strategies are to be applied. DBS methods, for example, typically analyze only a few µl of dried blood. For oral fluid, other issues, such as drug adsorption to sampling devices, may play a role. Alternative sampling strategies have -to the best of our knowledge- not yet been applied in microdosing studies. This may, however, be an interesting future application taking into account the increasing sensitivity of the latest generations of LC-MS/MS instruments. Finally, for alternative sampling techniques to become fully accepted as valid phenotyping strategies, the results obtained to date need to be confirmed in future studies, including trials in subjects with deviating enzyme activity and large population studies, allowing to define reference ranges of phenotyping metrics in the various matrices.

Compliance with Ethical Standards

Pieter M.M. De Kesel, Willy E. Lambert and Christophe P. Stove have no conflict of interest to declare. This study was financed by the Laboratory of Toxicology, Ghent University, Belgium. Pieter M.M. De Kesel, Willy E. Lambert and Christophe P. Stove received no additional funding directly related to the content of this study.

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Table 1. CYP450 phenotyping procedures using dried blood spots (DBS), hair, oral fluid and exhaled breath.

DBS: dried blood spots, CYP: cytochrome P, AUC: area under the curve, LLOQ: lower limit of quantification, PM: poor

Matrix	CYP450 isoform	Probe drugs	Phenotyping metric	Comments
DBS	CYP3A4	Midazolam	Midazolam $AUC_{0\text{-}12h}$	Unexplained diffe whole blood AUC
	CYP2C9	Flurbiprofen	OH-flurbiprofen/flurbiprofen ratio (multiple time points)	Significant correla urine ratios and fl
	CYP2A6	Nicotine (from tobacco smoke)	*trans* 3'-OH-cotinine/cotinine ratio	Significant correla
	CYP1A2	Caffeine	6 h paraxanthine/caffeine ratio	Significant correla and plasma ratios specific paramete
	CYP2C19, **CYP1A2**, CYP3A4, CYP2C9, CYP2D6, CYP2B6	**Omeprazole**, **caffeine**, midazolam, losartan metoprolol, efavirenz	2h omeprazole/OH-omeprazole & 8 h paraxanthine/caffeine ratio (other probe drugs or metabolites < LLOQ)	Significant correla ratios
	CYP3A4, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP2C19	Midazolam, caffeine, bupropion, flurbiprofen, dextromethorphan, omeprazole	2 h OH-midazolam/midazolam & paraxanthine/caffeine ratio, 3 h OH-bupropion/bupropion, OH-flurbiprofen/flurbiprofen & dextrorphan/dextromethorpha n ratio, $AUC_{2,3,6h}$ OH-omeprazole/omeprazole ratio	Significant correla ratios
Hair	CYP2C19	Amitriptyline	Nortriptyline/amitriptyline ratio in entire hair samples	Significant differe IM/EM
	CYP1A2	Caffeine	Paraxanthine/caffeine ratio in proximal 3-cm segments	Overall significant paraxanthine/caff large deviations in

metabolizer, IM: intermediate metabolizer, EM: extensive metabolizer

Table 1 continued. CYP450 phenotyping procedures using dried blood spots (DBS), hair, oral fluid and exhaled breath.

Matrix	CYP450 isoform	Probe drugs	Phenotyping metric	Comments
Oral fluid	CYP1A2	Caffeine	Caffeine clearance and 3-10 h paraxanthine/caffeine ratios	Significant correla plasma/serum cle paraxanthine/caff *in vitro* CYP1A2 ac
	CYP2D6	Dextromethorphan	3 or 6 h dextromethorphan/dextrorpha n ratio	Significant correla dextromethorpha and differentiatio IM/EM
	CYP3A	Dextromethorphan	8 and 12 h	Poor correlation v

		dextromethorphan/3-methoxymorphinan ratio	dextromethorpha ratios
	Midazolam	Midazolam AUC and clearance	Large differences clearance
CYP2C19, **CYP1A2**, CYP3A4, CYP2C9, CYP2D6, CYP2B6	**Omeprazole**, **caffeine**, midazolam, losartan metoprolol, efavirenz	2h omeprazole/OH-omeprazole & 8 h paraxanthine/caffeine ratio (other probe drugs or metabolites < LLOQ)	Correlation with p significant for CYP
CYP2A6	Nicotine-d_2, cotinine-d_4	6 h *trans* 3'-OH-cotinine-d_4/cotinine-d_4 ratio	Significant correla and oral clearanc
	Nicotine (from tobacco smoke)	*trans* 3'-OH-cotinine/cotinine ratio	High correlation v not consistently s

CYP: cytochrome P, AUC: area under the curve, LLOQ: lower limit of quantification, PM: poor metabolizer, IM: intermediate metabolizer, EM: extensive metabolizer

Table 1 continued. CYP450 phenotyping procedures using dried blood spots (DBS), hair, oral fluid and exhaled breath.

CYP: cytochrome P, AUC: area under the curve, DOB: delta-over-baseline, PM: poor metabolizer, IM: intermediate

Matrix	**CYP450 isoform**	**Probe drugs**	**Phenotyping metric**	**Comments**
Exhaled breath	CYP2C19	[^{13}C]-aminopyrine	$AUC_{0\text{-}3h}$ of DOB $^{13}CO_2/^{12}CO_2$ ratios	Significant correla aminopyrine AUC
		[^{13}C]-pantoprazole	DOB $^{13}CO_2/^{12}CO_2$ ratio (multiple time points & AUC)	Significant differe IM/EM and correl pantoprazole AUC
	CYP2D6	[^{13}C]-dextromethorphan	Single 40 min DOB $^{13}CO_2/^{12}CO_2$ ratio	Comparable with dextromethorpha distinguish betwe
	CYP1A2	[^{13}C]-caffeine	Single 1 h DOB $^{13}CO_2/^{12}CO_2$ ratio	Significant correla caffeine clearance
	CYP3A4	[^{14}C]-erythromycin	% ^{14}C exhaled in 1 h or $1/T_{max}$	Poor correlation v clearance

metabolizer, EM: extensive metabolizer,

T_{max}: time point when maximum concentration is reached following intake of a given dose

Table 2. Authors' view on main advantages and limitations of dried blood spot (DBS), oral fluid and exhaled breath sampling.

	DBS	Oral fluid	Exhaled breath
Patient comfort (incl. ease of sampling, invasiveness)	++	++	+++
Sampling in absence of specialized	+++	+++	+++
Transport & storage	+++	++	-
Home-sampling potential	+++	++	+
Analysis using standard analytical equipment	+++	+++	-