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# Caffeine Metabolic Ratios for the *In Vivo* Evaluation of CYP1A2, N-acetyltransferase 2, Xanthine Oxidase and CYP2A6 Enzymatic Activities

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**Abstract:** Phenotyping by probe substrates of cytochrome P450 (CYP) and other metabolizing enzymes is widely used to assess the effects of genes, environment and ethnicity on the *in vivo* metabolism of drugs and environmental chemicals. The caffeine metabolic ratio, in urine, plasma or saliva, has been used extensively as an index of CYP1A2, N-acetyltransferase 2 (NAT2), xanthine oxidase (XO) and CYP2A6 enzymatic activities. Phenotyping using plasma or saliva samples to measure the paraxanthine to caffeine (17X/137X) ratio correlates well with many measures of CYP1A2 activity. Various urinary metabolic ratios for caffeine phenotyping have been proposed, but shortcomings have been demonstrated for all the proposed urinary metabolic ratios. Several groups have proposed the urinary ratio of (1-methylxanthine (1X) + 1-methylurate (1U) + 5-acetylamino-6-formylamino-3-methyluracil (AFMU)) to 1, 7-dimethylurate (17U) i.e. (1X + 1U + AFMU)/17U as the preferred metabolic ratio for CYP1A2 activity (independent of urine flow rate). There is no consensus on the best urinary metabolic ratio for NAT2, XO or CYP2A6 enzymatic activities. Caffeine has been used by different groups to evaluate the *in vivo* activity of CYP1A2, NAT2, XO and CYP2A6 in different populations and the effect of many factors on these activities. Caffeine has been also used as a constituent of a "cocktail" to phenotype several enzymes simultaneously. In conclusion, phenotyping using caffeine as a probe substrate may still provide useful assessment of CYP1A2, NAT2, XO and CYP2A6 activities in epidemiologic and drug-drug interaction studies despite the limitations that are associated with its use.

**Keywords:** Caffeine, CYP1A2, NAT2, XO, CYP2A6, *In vivo*, Urine metabolic ratio, review.

## INTRODUCTION

The cytochrome P450 (CYP) enzymes are responsible for metabolism of many commonly used drugs. Additionally, these enzymes are responsible for the metabolism of several endogenous substrates, dietary compounds, and environmental toxins [1]. The enzyme activities can be assessed by the use of genotyping and phenotyping. In addition mRNA assessment methods provide information regarding gene transcription.

The use of mRNA expression profiling to investigate the CYP activities provides the potential information of the ability of a compound to induce drug metabolizing enzymes [2]. However, the most relevant end point when the induction or the inhibition of CYP is investigated is enzyme activity [2].

Genotyping allows characterization of many significant CYP genes with only a single venous blood sample. However, genotyping is only clinically relevant to the degree with which it affects the enzymatic activity. Genotyping works well to predict enzyme activity if a major fraction of variability is attributable to known polymorphisms. Although it allows cost- and time effective characterization of many significant genes at once, it will fail to provide reasonably exact estimates of enzyme activity when other major factors of influence (e.g. liver disease, enzyme induction/inhibition) are prevalent. To a great extent, this applies to CYP1A2 [3, 4]. Consequently, the optimal method of describing real-time enzyme activity is phenotyping, where metabolism of a carefully selected probe compound is used to estimate the activity of one or more of the enzymes involved in its metabolism [3]. Despite extensive work, there is currently little agreement as to the best method of characterizing CYP enzyme activities [3]. Nevertheless, the optimal therapeutic effect can be achieved by individualizing drug therapy to a patient's genotype/phenotype [5].

The metric for the selective probe used for phenotyping may be systemic clearance of a drug eliminated exclusively by the respective enzyme or partial clearance for a metabolic pathway. Other parameters such as single point concentrations or ratios of metabolite to parent concentrations in plasma, saliva, and/or urine are also often used [5]. Although it is often more difficult than genotyping,

phenotyping provides a measure of the activity of a given drug-metabolizing enzyme, and takes account of not only genetic but also environmental and physiological factors [3, 4, 6]. Several potential phenotyping probes have been proposed for most CYP enzymes, each with unique advantages and disadvantages [3].

Phenotyping studies *in vivo* involve either the administration of one CYP-specific probe drug, or a 'cocktail' of drugs simultaneously to provide independent *in vivo* phenotypic measures for CYP enzymes. However, each method has both advantages and disadvantages [7]. The use of selective phenotyping where only one probe is administered, requires only a small amount of sample, allows rapid analysis and is considered relatively safe, but provides limited information on CYP activity [7]. The mixed, or 'cocktail' approach it can give information on several CYP activities/ pathways in a single experiment, but requires a large amount of sample, involves time-consuming analysis and may involve a considerable number of side effects because of the simultaneous administration [7].

Fuhr *et al* [5] discussed some of the important issues concerned with phenotyping studies. Phenotyping should be simple from technical point of view. Thus, oral administration should be preferred over intravenous administration. The most convenient approach would be the use of dietary constituents (e.g., caffeine for N-acetyltransferase (NAT2) phenotyping) or endogenous substrates (e.g., cortisol or cholesterol for CYP3A4). It is desirable to use a simple metric, e.g., a metabolic ratio in a spot urine sample or a single point plasma concentration is preferred over complete concentration vs. time profile. A short period of observation from administration of the probe drug to the point of time for sampling is an advantage. A long elimination half-life may also be a major obstacle for repeated phenotyping. It is desirable that sampling is non-invasive, and urine and saliva concentrations should be preferred to plasma samples. Non-invasiveness may be essential for epidemiological studies [5].

Coffee is a complex mixture of compounds that may have either beneficial or harmful effects on the human body. Caffeine (1, 3, 7-trimethylxanthine) is by far the best characterized compound in coffee. Caffeine is extensively metabolized in man and at least 17 metabolites have been identified [8].

Caffeine is commonly used as a probe drug for the simultaneous assessment of the phenotypes of various drug-metabolizing

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enzymes, including CYP1A2 [9-11], CYP2A6 [9, 12-14], xanthine oxidase (XO; [9, 15]) and N-acetyltransferase 2 (NAT2; [9, 16-18]).

The CYP1A family of enzymes is responsible for the metabolic activation of some known procarcinogenic environmental chemicals and toxins [1]. It is one of the major CYP families and accounts for approximately 13% of the total content of CYP in the human liver [4]. This family contains CYP1A1 and CYP1A2. CYP1A2 plays an important role in the metabolism of several clinically used drugs [4]. In addition, approximately 8.2 % of all drugs used today are metabolized by CYP1A2 [1]. Human CYP1A2 is involved in the metabolism of endogenous compounds and the elimination of environmental toxins as well as the activation of many environmental carcinogens including dietary heterocyclic amines, tobacco-specific nitrosamines and aryl amines [4]. CYP1A2 activity shows both pronounced intra- and inter-individual variability that is based on constitutional and genetic factors and enzyme induction and inhibition [4]. Individuals who are carriers for *CYP1A2\*1A* allele are "rapid" caffeine metabolizers, whereas carriers of the variant *CYP1A2\*1F* are "slow" caffeine metabolizers [19, 20]. Increased CYP1A2 activity will cause more rapid metabolism of drugs and lower *in vivo* levels (with greater exposure to the metabolite) which may lead to sub-optimal response to therapy, while it will shorten the exposure to environmental toxins by the increase in their metabolism and subsequent accelerated elimination. However, for a prodrug that needs biotransformation to form the active metabolite, increased enzyme activity may result in higher concentration of the active (possibly carcinogenic) component [4]. Conversely, decreased enzyme activity slows down metabolism and elimination of drugs and can result in higher *in vivo* concentrations and increased chances of side effects.

CYP2A6 has been detected in liver where it constitutes about 4% of total CYP content and it participates in 2.5% of drug metabolism [1]. Because of its low contribution to drug metabolism, the interactions between xenobiotic substrates is of limited significance. Nevertheless, this is not the case when drugs are primarily metabolized by CYP2A6, for example nicotine and coumarin [1, 21, 22]. Because of the importance of CYP2A6 in nicotine metabolism, it has been suggested that the *CYP2A6* genotype influences the inter-individual differences in smoking behavior as well as lung cancer susceptibility [22]. Coumarin is specifically hydroxylated at position C7 and is used as probe drug for the assessment of enzyme activity both *in vivo* and *in vitro* [1]. CYP2A6 is also responsible for the metabolic activation of several procarcinogens and promutagens such as aflatoxin B1 [1, 21]. CYP2A6 is subject to marked inter-individual and inter-ethnic variability. It is well known that there is a genetic polymorphism in the human CYP2A6 gene [22]. The frequency of the poor metabolizer phenotype in European populations and Asian populations has been reported to be 1 to 3% and 15-20%, respectively [22]. High CYP2A6 activity is associated with increased risk of colorectal cancer [14]. Furthermore it has been shown that CYP2A6 activity is increased in liver cirrhosis [13, 23].

N-acetyltransferase 2 (NAT2) catalyzes acetylation of a variety of aromatic amines and hydrazines, including known carcinogens [24]. NAT2 activity is important for the acetylation of more than 15 drugs, such as isoniazide, hydralazine, procainamide, dapsone and sulfonamides [5, 21]. NAT2 is a cytosolic enzyme with highest activity in the liver and intestine [21]. Rapid and slow NAT2 phenotypes and the associated genotypes have been identified and both rapid and slow acetylation has been associated with the risk of several cancers. Although NAT2 is believed to be predominantly under genetic control, wide ranges exist in enzyme activity and associations have been identified between NAT2 activity and some exposures, such as use of gout medication and intake of heavily browned fish [25].

Xanthine oxidase (XO), is a cytosolic enzyme, involved in the oxidation of endogenous purines and pyrimidines as well as several drugs such as thiopurines and methyl-xanthines with a wide range of substrates [26]. XO activates several clinically important anthracycline antibiotics as well as a number of anticancer drugs into their reactive metabolites [27]. High levels of XO have been associated with tissue injury in certain diseases and is considered to contribute to oxidative damage of cells through the generation of cytotoxic oxygen radicals, which are implicated as important pathological mediators in many clinical disorders, including ischemia-reperfusion injury, myocardial infarction, hypertension, and atherosclerosis [27]. XO is located mainly in human liver and intestine [26]. There is inter-individual variation in human liver XO activity. Population phenotyping studies using caffeine have indicated marked inter-individual and inter-ethnic differences in XO activity [15, 16, 28, 29]. XO activity is approximately 20% higher in men than in women. The molecular basis of these differences remains unclear [30].

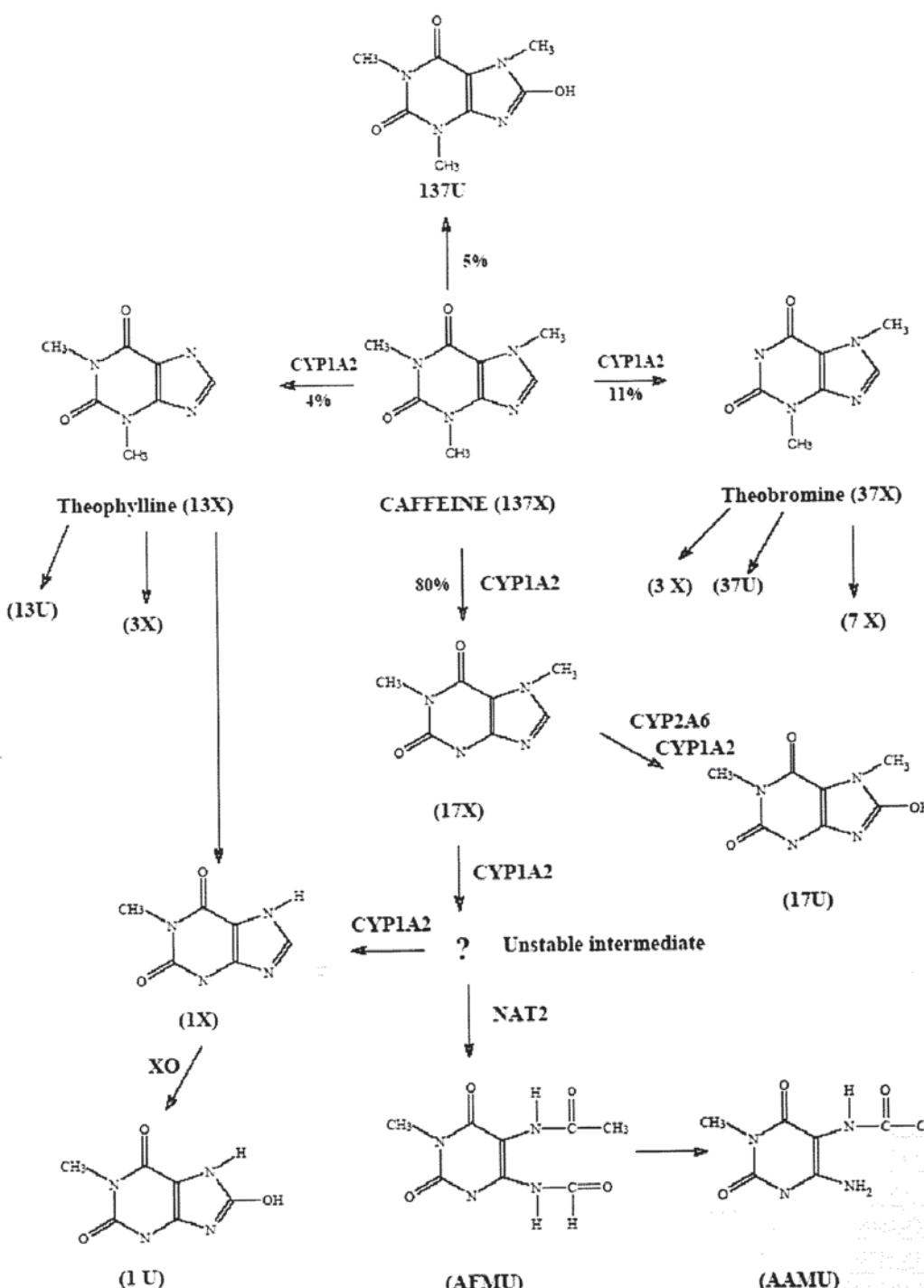
## CAFFEINE METABOLISM

The main metabolic routes of caffeine (1,3,7-trimethylxanthine, 17X) are illustrated in Fig. (1). Caffeine is extensively metabolized in man and at least 17 metabolites have been identified [8].

Five metabolic pathways contribute to caffeine clearance in humans: N<sub>3</sub>-demethylation to form paraxanthine (1,7-dimethylxanthine (17X)); N<sub>1</sub>-demethylation to form theobromine (3,7-dimethylxanthine) and N<sub>7</sub>-demethylation to form theophylline (1,3-dimethylxanthine). The N<sub>3</sub>-, N<sub>1</sub>- and N<sub>7</sub>-demethylation accounts, on average, for 80%, 11% and 4%, respectively [31]. Formation of the C<sub>8</sub>-hydroxylated metabolite 1,3,7-trimethyluric acid (137U) and the C<sub>8</sub>-N<sub>9</sub> bond scission product 6-amino-5-(N-formylmethylamino)-1,3-dimethyluracil, which are apparently derived from a common intermediate, together account for <5% of caffeine clearance [31]. Once formed, 17X, theobromine and theophylline are subject to extensive metabolism; each of these dimethylxanthines can undergo two separate N-monodemethylation reactions to form the corresponding monomethylxanthine [31]. Further oxidation (*via* 8-hydroxylation) of 1- and 7-methylxanthine, but not 3-methylxanthine, occurs to produce 1- and 7-methyluric acid, respectively. All three dimethylxanthines formed from caffeine also undergo 8-hydroxylation, giving rise to a dimethyluric acid. Of particular interest is the conversion of 17X to 1-methylxanthine (1X) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU). It has been proposed that N<sub>7</sub>-demethylation of 17X proceeds by way of an unstable ring-opened intermediate; subsequent acetylation or internal rearrangement of this species results in the formation of AFMU and IX, respectively. Deformylation of AFMU, which may occur spontaneously, and nonenzymatically, in urine, produces 5-acetyl-6-amino-3-methyluracil (AAMU) [21, 31].

Caffeine is commonly used as a probe drug to assess the metabolic activity of various isoforms of CYP and other drug metabolizing enzymes non-invasively and simply by measuring the urinary elimination of its metabolites. CYP1A2 is solely responsible for the caffeine N<sub>3</sub>-demethylation to 17X. Furthermore, CYP1A2 contributes to N<sub>1</sub>- and N<sub>7</sub>-demethylation. Paraxanthine (17X) that is further metabolized by two reactions; 8-hydroxylation by CYP2A6 and CYP1A2 to 1,7-dimethylurate (17U) and another reaction of 7-demethylation by CYP1A2 to form an unstable intermediate. Subsequent acetylation to form AFMU which is catalyzed by NAT2 or internal rearrangement of this intermediate to form IX. AFMU is unstable product that may be deformylated to AAMU. Part of 1X is metabolized to 1-methylurate (1U) by XO [32, 33].

In a study performed by Gu *et al* [34] using cDNA-expressed human CYP2E1, CYP2E1 had major influence on the caffeine metabolism: in particular, it catalyzed the formation of theophylline and theobromine from caffeine. This was later rejected by an *in*



**Fig. (1).** Biotransformation pathway for caffeine. Caffeine, 1,3,7 trimethylxanthine (137X); 1,3,7 trimethyluric acid (137U); 3,7-dimethylxanthine (37X) (theobromine); 1,3-dimethylxanthine (13X) (theophylline); 1,7-dimethylxanthine (17X) (paraxanthine); 1,3-dimethylurate (13U); 3,7-dimethylurate (37U); 1,7-dimethyluric acid (17U); 1-methylxanthine (1X); 3-methylxanthine (3X); 7-methylxanthine (7X); 1-methylurate (1U); 5-acetylaminio-6-formylamino-3-methyluracil (AFMU); 5-acetylaminio-6-amino-3-methyluracil (AAMU); N-acetyltransferase 2 (NAT2); xanthine oxidase (XO).

*in vivo* study relating the CYP2E1 immunoreactivity with pharmacokinetic parameters of caffeine *in vivo* [35].

## THE CAFFEEINE METABOLIC RATIOS

### CYP1A2

The N<sub>3</sub>-demethylation of caffeine accounts for between 80 and 90% of the total caffeine metabolism in humans, and the reaction is completely mediated by the activity of CYP1A2. Because of its specificity, this reaction is used to measure the activity of the 1A2 enzyme *in vivo* in humans by determining the ratios of caffeine urinary metabolites [36].

Caffeine is the only CYP1A2 phenotyping agent for which respective metrics are fully validated [3, 5]. Several plasma and salivary ratios have been proposed as valid measures of CYP1A2 activity. The most convenient of the validated metrics is the plasma ratio of paraxanthine (17X) over caffeine 5–7 hr after administration of a 100–200 mg caffeine test dose. Caffeine clearance with full concentration vs. time profile (with somewhat less variation but greater expense) or saliva ratios (slightly higher variation) may also be used [3, 5].

Correlation analyses between immunoreactive CYP1A2 liver content, intrinsic clearance for caffeine-3-demethylation to parax-

thine, and various plasma, saliva, and urine based CYP1A2 metrics have shown the closest correlations between intrinsic clearance and saliva based caffeine clearance estimation, followed by the plasma paraxanthine to caffeine ratio determined 6 hr after caffeine intake and the saliva paraxanthine to caffeine ratio 6 hr after caffeine intake in patients undergoing hepatectomy [35, 37]. A comparison between six methods for caffeine based CYP1A2 phenotyping showed that, besides caffeine systemic clearance as the "gold standard" in direct comparison to CYP1A2 activities in liver biopsies [35], the paraxanthine to caffeine ratio determined in plasma or in saliva as a metric for the CYP1A2 mediated partial caffeine clearance to paraxanthine most completely fulfilled validation criteria [4].

Various urinary ratios for caffeine phenotyping have been proposed (Table 1), but shortcomings have been demonstrated for all the proposed urinary ratios. However, most, if not all of them are prone to be influenced by confounding factors such as renal blood flow, urinary flow and the activities of other enzymes involved in caffeine biotransformation such as NAT2, CYP2A6, XO and CYP2E1 [4].

The caffeine dose may be administered as caffeine containing drinks such as instant coffee or cola. Because of many dietary sources, caffeine abstinence required for at least 12 hr (better for 36 hr) is not always easy to adhere to.

Several groups have studied the influence of various factors on CYP1A2 activity [25, 29, 40, 41]. The enzymatic activity of CYP1A2 is induced by coffee [41], smoking [36, 40, 42-47], consumption of cruciferous vegetables [24, 42, 48-53], chargrilled meat [49, 54] and exposure to various contaminants [19, 21]. In addition, gender, menstrual cycle, race, age, use of oral contraceptives influence CYP1A2 activity but the findings have been inconsistent across all studies [24, 29, 40, 53].

#### CYP2A6

Only a few studies have used caffeine as a probe drug to investigate the activity of CYP2A6 [21]. The Caffeine metabolic ratios used for CYP2A6 activity were 17X/17U [13, 14, 21] and 17U/(AFMU + 1U + 1X + 17X + 17U) [32].

Nowell *et al* [14] studied the effect of smoking, age, gender and race on CYP2A6 activity using the caffeine metabolic ratio of 17X/17U. The same ratio was used by Begas *et al* [21] to study effect of gender and smoking in a Greek population. Krul and Hageman [32] studied the effect of gender, use of oral contraceptives and age on CYP2A6 activity using the metabolic ratio of 17U/(AFMU + 1U + 1X + 17X + 17U). A similar ratio was used by Hakooz and Hamdan [53] (17U/(AFMU + 1X + 17X + 17U)) to study the effect of gender and broccoli supplemented diet on CYP2A6 activity. These studies reported no effect of oral contraceptives [32] or smoking [14, 21] on CYP2A6 activity. Broccoli induced CYP2A6 activity [53], while CYP2A6 activity for African-American subjects was slightly but insignificantly lower than the activity in Caucasians. Conversely, the effect of age [14, 32] and gender [14, 21, 32, 53]) have been controversial.

#### NAT2

Several different urinary caffeine metabolic ratios are included in cocktails, mainly because caffeine is administered anyway as a CYP1A2 substrate (Table 2). Despite the complex secondary metabolism of caffeine, which is not fully understood, all these ratios are useful to identify genetic NAT2 variants. For the metabolic ratio (AFMU + AAMU)/(AFMU + AAMU + 1X + 1U), steady values were reached and the CV was below 16% for any sampling interval after 4 hr. Dietary caffeine sources without administration of a test dose were sufficient for most individuals, making this ratio useful in epidemiological studies. It is not clear how changes in NAT2 activity would relate to changes in the respective metrics.

The effects of several factors have been studied on NAT2 activity using caffeine urinary metabolic ratio [13, 24, 29, 32, 45, 46, 55]. There was no effect of diet [24], coffee consumption [55], cigarette smoking [45, 46, 55], oral contraceptives [32, 45], or alcoholic cirrhosis [13] on the NAT2 activity. Children have lower activity of NAT2 than adults [32]. The effect of ethnic groups has been controversial; Relling *et al* [29] reported higher activity in black subjects compared to white while Tang *et al* [55] reported no difference. Gender was reported to have no effect on NAT2 activity [24, 45, 55] with the exception of one study where it has been reported that males have lower activity of NAT2 than females [32].

**Table 1. Urinary Caffeine Metabolic Ratios Used for the Assessment of CYP1A2 Enzymatic Activity**

Quotient	Author	References
(17X + 17U)/137X	Butler <i>et al</i> 1992	[36]
(AFMU + 1X + 1U)/17U	Campbell <i>et al</i> 1987	[38]
17X/137X	Kadlbur <i>et al</i> 1990	[39]
(AFMU + 1U + 1X + 17U + 17X)/137X	Carrillo and Benitez 1994	[16]
(AFMU + 1U + 1X)/17X	Grant <i>et al</i> 1983	[9]
(1U + 1X + AAMU)/17U	Kalow and Tang 1991	[40]
(1U + 1X + AFMU + AAMU)/17U	Fuhr and Rost 1994	[37]

Abbreviation used: caffeine (137X); 1,7-dimethylxanthine (17X) (paraxanthine); 1,7-dimethylurate (17U); 1-methylxanthine (1X); 1-methylurate (1U); 5-acetylamino-6-formylamino-3-methyluracil (AFMU); 5-acetylamino-6-amino-3-methyluracil (AAMU).

**Table 2. Urinary Caffeine Metabolic Ratios Used for the Assessment of NAT2 Activity**

Quotient	Author	References
AFMU/(AFMU + 1U + 1X)	Bendriss <i>et al</i> 2000	[12]
AFMU/1X	Krul and Hageman 1998	[32]
AAMU/(AAMU + 1X + 1U)	Tang <i>et al</i> 1991	[55]
(AFMU + AAMU)/(AFMU + AAMU + 1X + 1U)	Jetter <i>et al</i> 2004	[56]

Abbreviation used: 1-methylxanthine (1X); 1-methylurate (1U); 5-acetylamino-6-formylamino-3-methyluracil (AFMU); 5-acetylamino-6-amino-3-methyluracil (AAMU).

## XO

The activities of XO, using caffeine as a probe drug, have been studied less extensively than that of CYP1A2 [21]. Most of the Caffeine metabolic ratios suggested for XO activity involve the use of 1U/1U + 1X [15, 32], 1U/1X [13, 23, 45].

It is generally accepted that smoking [15, 40, 45], gender [15, 57], oral contraceptives [45] and diet [24] do not affect XO activity. However, increased XO activity has been reported in smoking subjects [42], in females [29] and after exercise [42]. In addition, putative poor metabolizers of XO have been reported in Ethiopian (4%; [15]), Caucasian (20%; [58]), Japanese (11%; [28]) and Spanish (4%; [16]) populations, whereas XO activity in Greek populations appeared to be unimodal and normally distributed, suggesting that there were no subjects with low enzyme activity [21]. XO activity is increased in liver cirrhosis [13, 23]. The influence of environmental factors on the activity of this enzyme has been studied in Ethiopians [15]. Ethiopians living in Ethiopia showed higher XO activity than those living in Sweden, indicating the influence of other environmental factors such as dietary habits on XO activity [15].

## Use of Caffeine Metabolic Ratio for Phenotyping Enzyme Activities of Different Populations

Several studies have used caffeine as a probe substrate to evaluate the *in vivo* activity of CYP1A2, CYP2A6, NAT2 and XO activities in different populations [15, 18, 19, 21, 53, 59, 60, 62, 63]. An overview of some of these studies is provided in Table 3.

Inter-ethnic variations in genetic polymorphism and enzyme activity have been extensively investigated for the different CYP activities. Ghotbi *et al* [63] carried out the only published comparison of CYP1A2 genetic polymorphism as well as enzyme activity between Swedes and Koreans using caffeine as a probe. One hundred and ninety four healthy unrelated Swedes and 150 unrelated Koreans received a 100 mg oral dose of caffeine. The paraxanthine to caffeine ratio was calculated from plasma samples collected 4 hr

later. CYP1A2 activity based on this metabolic ratio was found to be normally distributed for both Koreans and Swedes. The mean metabolic ratio was higher in Swedes than in Koreans. Smokers had significantly higher metabolic ratio than non-smokers, while Swedish oral contraceptive users had significantly lower metabolic ratio than non users. No effect of gender differences on enzyme activity was observed. Despite the presence of significant variations in CYP1A2 haplotype distribution, none of the genotypes or haplotypes investigated could explain the observed wide variation and differences in enzyme activity between the two populations. Differences in dietary habits between these two populations could in part explain the differences in CYP1A2 activity since dietary substances such as cruciferous vegetables and charcoal-broiled meat are known CYP1A2 inducers [63]. The observed difference in enzyme activity between Asians and Caucasians may be clinically relevant in terms of improving the therapeutic outcome when CYP1A2 substrate drugs with a narrow therapeutic index are prescribed. The authors concluded that further studies are required to explain inter-individual and inter-ethnic variations in CYP1A2 enzyme activity [63].

## Use of Caffeine as Part of the Cocktail Approach in Phenotyping Studies

Phenotyping procedures may be combined in a "cocktail" to assess a metabolic profile. This is used primarily to examine the drug-drug interaction potential of a new drug as multiple drug therapy is common in clinical practice [5]. Therefore, there is a need to identify possible drug-drug interactions during early drug development [64]. *In vitro* hepatic microsomal studies can provide preliminary information about the CYP isoforms that are likely to be affected clinically. Definitive data for each CYP isoform can be obtained by using an *in vivo* 'probe' drug that is a significant and selective substrate of that enzyme in man [64]. Alternatively, the effect of the test drug on a number of distinct CYP isoforms can be established within one clinical study, the 'cocktail' approach, to

**Table 3. Population Studies Using Caffeine Metabolic Ratios**

Population	Caffeine Dose	Samples, Time	Enzyme (Caffeine Metabolic Ratio Used)	Objective of the Study	Author	References
Japanese	270 ml	Urine, 4-5 hr	CYP1A2 ((17U+17X)/137X)	Genotypes, smoking, gender	Nakajima <i>et al</i> 1994	[59]
Greek	200 mg	Urine, 6 hr	CYP1A2 ((AFMU+1U+1X)/17X) CYP2A6 (17U/17X) XO (1U/(1U+1X)) NAT2 (AFMU/(AFMU+1U+1X))	Smoking, gender	Begas <i>et al</i> 2007	[21]
Greek	200 mg	Urine	NAT2 (AFMU/1X)	Smoking, gender, caffeine-intake habits	Asprodini <i>et al</i> 1998	[18]
Jordanian	100 mg	Urine, 0-5 hr	CYP1A2 ((17U+17X)/137X) CYP2A6 (17U/(AFMU+1X+17X+17U))	Broccoli, gender	Hakooz and Hamdan 2007	[53]
Faroese	200 mg	Urine, 4-6 hr	CYP1A2 ((AFMU+1U+1X)/17U)	Smoking, gender, exposure to polychlorinated biphenyls	Petersen <i>et al</i> 2006	[60]
Northern Mexicans	140 mg	Urine, 0-8 hr	CYP1A2 ((AFMU+1U+1X)/17U)	Polycyclic aromatic hydrocarbons exposure	Castorena-Torres <i>et al</i> 2005	[19]
Chinese	100 mg	Plasma, 5 hr	CYP1A2 (17X/137X)	Genotypes	Chen <i>et al</i> 2005	[61]
Korean	110 mg	Urine, 4-5 hr	CYP1A2 ((17U+17X)/137X) XO (1U/1X)	Smoking, gender, age	Chung <i>et al</i> 2000	[62]
Ethiopians	100 mg	Urine, 0-8 hr	XO (1U/(1X+1U))	Smoking, environment, gender	Akilillu <i>et al</i> 2003	[15]
Swedes and Koreans	100 mg	Urine, 4 hr	CYP1A2 (17X/137X)	Inter-ethnic variations	Ghotbi <i>et al</i> 2007	[63]

Abbreviation used: caffeine (137X); 1,7-dimethylxanthine (17X) (paraxanthine); 1,7-dimethylurate (17U); 1-methylxanthine (1X); 1-methylurate (1U); 5-acetylamino-6-formylamino-3-methyluracil (AFMU); 5-acetylamino-6-amino-3-methyluracil (AAMU).

understand the risk of pharmacokinetic and pharmacodynamic drug-drug interactions [4, 7, 64].

Numerous drug cocktails have been published [3, 6, 61, 64-75]. Earlier cocktails often used less selective marker drugs because the molecular identity of the respective enzymes or transporters was unclear. In all cocktails, the selection of metrics is determined by several factors, including the specific objective of the respective study, the availability of analytical methods, and the balance between expense and validation. Still, even recent phenotyping cocktails often contain substances or use phenotyping metrics that are not optimal. A comprehensive review of the different cocktail approaches has been published recently by Fuhr *et al* [5]. In this review, the authors aimed at providing an overview of the characteristics of typical existing cocktails to provide guidance for users. For identification, the primary affiliation towns of the authors were used. The Pittsburgh and the Cooperstown cocktails have been used most often.

An overview of these cocktails is given in Tables 4 and 5, where the references are also included. In all the cocktail approaches reviewed, caffeine was used as a selective probe for CYP1A2 activity [5]. The dose of caffeine was in the range 100-200 mg with the exception of the Loughborough cocktail that used a caffeine dose of 50 mg [64]. The MR of paraxanthine to caffeine was used with plasma samples collected at 4 [70], 6 [65, 66, 74], 6.5 [64], 8 hr [71, 72] after an oral dose of caffeine or the AUC from plasma samples [68, 73]. In others the urinary MR was used when urine samples were collected at the following intervals 0-8 [6], 0-12 [67, 68, 73].

NAT2 activity (Table 5) was estimated from caffeine [6, 66, 67], sulfamethazine [73] and dapsone [71]. XO activity using urinary caffeine MR of 1U/(1U + 1X) in urine samples collected at 0-8 [6] and 0-12 hr [67].

Fuhr *et al* [5] discussed the published information on cocktail safety. A thorough review of the data shows that for most of the enzymes addressed, there are phenotyping procedures that provide valid information on individual activity. However, selection of an appropriate test drug and metric is essential. Most existing phenotyping cocktails are appropriate for their intended use. Still, there is ample room for improvement by replacement of individual probe drugs, by use of a better validated metric, by reducing the doses, and/or by simplification of the procedures. Low doses and successful validation of limited sampling strategies make the use of cocktails increasingly convenient. Therefore, phenotyping drug cocktails are valuable, safe, cheap, and scientifically sound tools for characterizing drug metabolism. Specifically, they may find place in phase I of drug development to assess the metabolic interaction profile of drugs. It is desirable to register and market phenotyping agents also with the indication of phenotyping in order to make phenotyping procedures independent of the current availability of the respective substances as therapeutic agents [5].

## DOSE OF CAFFEINE TO BE USED FOR PHENOTYPING STUDIES

Caffeine metabolism is dose dependent, resulting in non-linear accumulation of methylxanthines in the body [76]. A study by Cheng *et al* [77] demonstrated a significant decrease in caffeine clearance with increasing dose from 70 to 300 mg, indicating saturable caffeine metabolism. Hence, it has been recommended to use a low oral dose of caffeine ( $\leq 3$  mg/kg) for phenotyping studies [29, 76]. An oral dose of 1-2 mg/kg has been suggested in order to optimize the precision of the phenotyping test [11]. Investigators have used a variety of caffeine sources for caffeine phenotyping (aqueous solution, coffee, soda, tablets and random dietary sources), this dosing flexibility is a significant advantage using caffeine as a probe, allowing easier administration to special populations such as pediatrics [3].

## LIMITATIONS AND CONCERN ABOUT CAFFEINE PHENOTYPING STUDIES

### Urine

The consensus between different authors is that the timing of urine collection is of little importance as long as enough time is allowed for metabolism of caffeine into its tertiary and quaternary metabolites, and as long as the ratio does not include measurement of 17X, as it is metabolized to the corresponding tertiary and quaternary metabolites [3]. Sinués *et al* [80] studied five of the MRs reported in literature to select the most sensitive MR/timing schedule to discriminate between non exposed and exposed with respect to inducers of CYP1A2 activity. The authors recommended the use of (AFMU + 1X + 1U)/17U which was proposed by Campbell *et al* [38] in the period of 4-5 hr after caffeine intake. Conversely, they concluded that there are no differences between several of the other MR reported in literature in detecting induction of CYP1A2 if urine samples are collected 4-5 hr after ingesting caffeine [78]. Rost and Roots [44] found that correlations between the urinary ratio of (1X + 1U + AFMU)/17U and other measures of CYP1A2 activity such as the caffeine breath test and the plasma caffeine clearance were improved using a 5-8 hr versus a 8-24 hr urine collection.

### Plasma or Saliva

The timing of the sample collection for the saliva and plasma ratio of 17X/137X is important due to the inclusion of 17X, but there is a large window within which to collect a sample. This ratio is highly correlated to other *in vitro* and *in vivo* standards of CYP1A2 activity with urine collections ranging from 3 to 7 hr after the caffeine dose [3, 37].

Earlier studies demonstrated that both CYP2A6 and CYP1A2 catalyzed the conversion of 17X to 17U, and as a result, 17U is included in both the numerator and denominator of most ratios, this would particularly affect large values of CYP1A2 activity, and as a result, would be greater in patients with increased CYP1A2 activity (i.e. smokers) [3]. Therefore, the range of measured CYP1A2 variability would be smaller than the actual range of variability and, as evident in the study by Tang *et al* [79], the standard deviation of the metabolic ratio with 17U would be smaller than the corresponding caffeine systemic clearance standard deviation. Conversely, Nowell *et al* [14] demonstrated that CYP2A6 selectively catalyzes the conversion of 17X to 17U with additional support coming from the observation that this ratio is not influenced by smoking status, a condition that has a profound effect on CYP1A2 activity [14]. Thus, the best urinary metabolic ratios appear to be those using 17U as the denominator.

Streetman *et al* [3] discussed the different urinary metabolic ratios that have been reported in literature. The strongest urinary ratio appears to be the ratio proposed by Campbell *et al* [38] in the period of 4-5 hr after caffeine intake which is (1U + 1X + AFMU)/17U. This ratio has correlated well with caffeine systemic clearance in several studies [35, 38]. This ratio has been shown to vary closely and directly with CYP1A2 activity [80], to vary indirectly with CYP2A6 activity [80], to be independent of urine flow rate [11, 78, 80], and to be independent of any influence of CYP2E1 [80].

Instead of using 17U as the denominator, some studies have used 17X as the denominator [3]. However, this raises two unique problems. First, with 17X as both a product and a substrate of CYP1A2, timing of urine collection is critically important. Urine collection 4-5 h after the caffeine dose has been recommended [11, 78], but the optimal collection time may vary in subjects with induced CYP1A2. The second problem is 17X flow-dependent renal clearance [9]. Denaro *et al* [81] demonstrated a 30-fold variation in CYP1A2 phenotyping results with the ratio of (1U + 1X + AAMU)/17X that was hypothesized to be a consequence of urine flow-dependence on 17X renal clearance. The authors also found

Table 4. The CYP1A2 Activity Assessment in the Common "Cocktail" Approaches that Use Caffeine

Cocktail Identification	Caffeine Dose	Samples and the Time of Samples Used	Caffeine Metabolic Ratio Used	Author	References
Changsha	100mg	Plasma, 6 hr	Paraxanthine/caffeine	Chen <i>et al</i> 2003	[65]
Cologne	150mg	Plasma, 6 hr	Paraxanthine/caffeine	Tomalik-Schärtel <i>et al</i> 2005	[66]
Cooperstown (5+1)	2 mg/kg	Urine, 0-12 hr	(1X + 1U + AFMU)/17U	Chainuvati <i>et al</i> 2002	[67]
Darmstadt	100mg	Plasma, AUC (0-24 hr)		Krosser <i>et al</i> 2006	[68]
Indianapolis	100-200mg	Plasma, 6 hr	Paraxanthine/caffeine	Bruce <i>et al</i> 2001	[69]
Karolinska	100mg	Plasma, 4 hr	Paraxanthine/caffeine	Christensen <i>et al</i> 2003	[70]
Loughborough	50mg	Plasma, 6.5 hr	Paraxanthine/caffeine	Blakey <i>et al</i> 2003	[64]
Pittsburgh	100mg	Plasma, 8 hr	Paraxanthine/caffeine	Zgheib <i>et al</i> 2006 Frye <i>et al</i> 1997	[71] [72]
Quebec	100mg	Urine, 0-8 hr	(AAMU + 1X + 1U)/17U	Sharma <i>et al</i> 2004	[6]
Jena	200mg	Plasma, AUC (0-12 hr)		Henschel and Hoffmann 1991	[73]

Abbreviation used: 1,7-dimethylurate (17U); 1-methylxanthine (1X); 1-methylurate (1U); 5-acetylamino-6-formylamino-3-methyluracil (AFMU); 5-acetylamino-6-amino-3-methyluracil (AAMU).

Table 5. The NAT2 Activity Assessment in the Common "Cocktail" Approaches

Cocktail Identification	Drug and Dose	Samples and the Time of Samples Used	MR Used	Author	References
Cologne	Caffeine, 150mg	Urine, 6 hr	(AFMU + AAMU)/(AFMU + AAMU + 1X + 1U)	Tomalik-Schärtel <i>et al</i> 2005	[66]
Cooperstown (5+1)	Caffeine, 2 mg/kg	Urine, 0-12 hr	AFMU/(1X + 1U)	Chainuvati <i>et al</i> 2002	[67]
Pittsburgh	Dapsone, 100mg	urine, 0-8 hr	Monoacetyldapsone/dapsone	Zgheib <i>et al</i> 2006	[71]
Quebec	Caffeine, 100mg	Urine, 0-8 hr	AAMU/(AAMU + 1X + 1U)	Sharma <i>et al</i> 2004	[6]
Jena	Sulfamethazine, 500mg	Urine, 0-6 hr; plasma, 6 hr		Henschel and Hoffmann 1991	[73]

Abbreviation used: 1-methylxanthine (1X); 1-methylurate (1U); 5-acetylamino-6-formylamino-3-methyluracil (AFMU); 5-acetylamino-6-amino-3-methyluracil (AAMU).

no correlation between that ratio and caffeine systemic clearance and recommended that the (1U + 1X + AAMU)/17X ratio should not be used [81]. In a study of 59 non-smokers and 66 smokers, Sinués *et al* [78] additionally demonstrated that the (1U + 1X + AFMU)/17X ratio did not correlate well with the (1U + 1X + AFMU)/17U ratio but did significantly correlate with urine flow.

Due to the non-enzymatic spontaneous degradation of AFMU to AAMU in the urine, some modifications of the above ratio have been proposed. One recommended ratio simply adds urinary AAMU to the numerator to account for any AFMU degradation. This ratio has been shown to correlate significantly with the intrinsic clearance of caffeine 3-demethylation *in vitro* [35], but the ratio appears to explain only approximately 28% of the variability in intrinsic clearance. Another proposed ratio is (1U + 1X + AAMU)/17U, which is calculated after the urine pH is increased to promote the conversion of all AFMU into AAMU [11]. In a study by Denaro *et al* [81] this ratio was shown to correlate with caffeine systemic clearance at low doses (4.2 mg/kg per day), and was also significantly correlated in smokers with induced CYP1A2 [3].

Other proposed urinary ratios are 17X/137X and (17X + 17U)/137X measured in urine collected 4-5 h after the caffeine dose [3]. Correlations between these ratios and caffeine systemic clearance have varied considerably [35, 37, 79]. Tang *et al* [79] found that the (1U + 1X + AFMU)/17U ratio was a significantly better predictor of caffeine systemic clearance than was the 17X/137X ratio. In the same study, the 17X/137X ratio was not significantly different in eight oral contraceptive users, while the caffeine systemic clearance and the (1U + 1X + AFMU)/17U ratio showed significantly different results [79]. In addition, the inclusion of 17X

in both ratios raises similar concerns to those discussed previously. Another concern is the flow-dependent renal clearance of 137X. Although having flow-dependent variables in both the numerator and denominator may negate the effect, renal clearance is different for each compound, making the validity of such an assumption unlikely [11]. In a comparison of different caffeine urinary ratios in 125 subjects, Sinués *et al* [78] found that all ratios with either 137X or 17X as the denominators were correlated with urine flow, despite the inclusion of a flow-dependent variable in the numerator. Overall, these ratios do not appear to be as useful as other currently available urinary ratios [3, 35].

## SUMMARY

Caffeine phenotyping has been extensively studied, discussed, and debated [3, 4, 78]. The caffeine test is a non-invasive, safe, well-accepted, easily accessible method and can give valuable information on the *in vivo* activity of the CYP1A2, CYP2A6, NAT2 and XO enzyme activities simultaneously. Phenotyping using plasma or saliva samples to measure the 17X/137X ratio correlates well with many measures of CYP1A2 activity. The preferred urinary ratio is (1X + 1U + AFMU)/17U, which correlates well with CYP1A2 activity and is independent of urine flow rate, but questions regarding the validity of caffeine urinary ratios in general have recently been raised [3]. The preferred urinary ratio for CYP2A6 is 17U/17X [21]. The preferred metabolic ratio for NAT2 is (AFMU + AAMU)/(AFMU + AAMU + 1X + 1U), as it is more reliable than other formulas and, besides being robust against XO activity, it is not influenced by caffeine dose [56]. However, in most studies the molar ratio of AFMU/1X suggested by Krul and

Hageman [32] is used mainly because of analytical limitations [56]. The metabolic ratio used in many studies for evaluation of XO was  $1U/1U + 1X$  [15, 32].

In conclusion, phenotyping using caffeine as a probe substrate may provide useful guidelines on cancer susceptibility, drug toxicity and drug interactions in population studies of healthy subjects [21].

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