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The Use of Caffeine As a Metabolic Probe for Human Drug Metabolizing Enzymes

John O. Miners and Donald J. Birkett*
DEPARTMENT OF CLINICAL PHARMACOLOGY, FLINDERS MEDICAL CENTRE, BEDFORD PARK, SOUTH AUSTRALIA 5042, AUSTRALIA [FAX: 61-8-204-5114]

ABSTRACT. 1. Caffeine (CA) is metabolized extensively and at least 17 metabolites arising from primary and secondary biotransformation pathways are found in urine following CA ingestion. The enzymes responsible for the formation of most of the metabolites derived from CA have been identified.

2. Given the near ubiquitous consumption of CA, this compound potentially constitutes a useful substrate probe for assessment of certain xenobiotic metabolizing enzyme activities *in vivo*. Indeed, various ratios of CA metabolites excreted in urine (urinary metabolic ratios; MRs) are now utilized widely for the population screening of enzyme activities.

3. Excretion of the acetylated secondary metabolite 5-actylamino-6-formylamino-3-methyluracil (AFMU) is dependent on the activity of the polymorphic *N*-acetyltransferase (NAT2), and certain MRs incorporating AFMU may be used for NAT2 phenotyping.

4. The conversion of 1-methylxanthine (1-MX), another secondary metabolite of CA, to 1-methyluric acid (1-MU) is catalyzed by xanthine oxidase (XO), and the urinary 1-MU to 1MX ratio reflects XO activity.

5. N3-demethylation to form paraxanthine (PX), a reaction mediated by cytochrome P4501A2 (CYP1A2), is the dominant primary metabolic pathway of CA. CA *N*3-demethylation activity may be used as a measure of human hepatic CYP1A2 *in vitro*.

6. Plasma CA clearance is considered to reflect CYP1A2 activity *in vivo*. Although a number of MRs are based on the excretion of PX metabolites (PX derived from CA is employed for the assessment of CYP1A2 activity *in vivo*), factors other than enzyme activity may affect these ratios. GEN PHARMAC 27; 2:245–249, 1996.

KEY WORDS. Caffeine, methylxanthines, cytachrome P450, *N*-acetyltransferase, xanthine oxidase, metabolic ratio

INTRODUCTION

Caffeine (CA, 1,3,7-trimethylxanthine; Fig. 1) is one of the most widely and frequently consumed xenobiotics throughout the world. Exposure to CA occurs principally via the diet. The major source of CA, at least in adults, is coffee and tea but varying amounts are found also in cocoa, cola-type soft drinks, maté, and a number of prescription and nonprescription pharmaceutical products. Based on food balance data, per capita daily consumption of CA in Europe and North America exceeds 200 mg/day (James, 1991).

Following ingestion CA is essentially completely absorbed from the gastrointestinal tract. CA is efficiently eliminated, with a mean systemic clearance (viz. 150 ml/min) in the "intermediate" range and mean terminal half-life of 4 h in healthy adults (Lelo *et al.*, 1986a). Hepatic metabolism is the dominant elimination mechanism, with less than 5% of an ingested dose being eliminated unchanged in urine. CA biotransformation is complex, and at least 17 urinary metabolities can be detected following CA consumption (Arnaud, 1993).

As will be described later, it is now known that a number of xenobiotic metabolizing enzyme systems contribute to CA biotransformation in humans. Given the widespread exposure of humans to CA, there has been considerable interest in recent years in the development of this compound as a substrate probe for the investigation of xenobiotic metabolizing enzyme activity *in vitro* and *in vivo*. Here we discuss evidence supporting the use of CA for the assessment of *N*-acetyltransferase (NAT), xanthine oxidase (XO), and certain cytochrome P450 (CYP) activities.

PATHWAYS OF CAFFEINE METABOLISM

Five metabolic pathways contribute to CA clearance in humans (Fig. 2): *N*3-demethylation to form paraxanthine (PX; 1,7-dimethylxanthine); *N*1-demethylation to form theobromine (TB; 3,7,-dimethylxanthine); and *N*7-demethylation to form theophylline (TP; 1,3-dimethylxanthine) account, on average, for 80%, 11%, and 4%, respectively, of CA metabolism *in vivo* (Lelo *et al.*, 1986b) (see Fig. 1 for structures). Formation of the C8-hydroxylated metabolite 1,3,7-trimethyluric acid (1,3,7-TMU) and the C8–N9 bond scission product 6-amino-5-(*N*-formylmethylamino)-1,3-dimethyluracil (1,3,7-TAU, a trimethylaminouracil), which are apparently derived from a common intermediate (Ferrero and Neims, 1983), together account for <5% of CA clearance. A similar pattern of metabolites is observed for incubations of CA with human liver microsomes at substrate concentrations comparable to CA plasma concentrations observed following normal dietary intake (viz. ≤ 100 μM) (Tassaneeyakul *et al.*, 1992, 1994). However, at high CA concentrations (>5 mM) TMU formation dominates *in vitro*.

Once formed, PX, TB, and TP are subject ot extensive metabolism (Grygiel *et al.*, 1979, Lelo *et al.*, 1989; Miners *et al.*, 1982) (Fig. 2). Each of these dimethylxanthines can undergo two separate *N*-monodemethylation reactions to form the corresponding monomethylxanthine (i.e., 1-,3- and 7-methylxanthine [1, 3- and 7-MX]). Further oxidation (viz. 8-hydroxylation) of 1- and 7-MX, but not 3-MX, occurs to produce

*To whom correspondence should be addressed.
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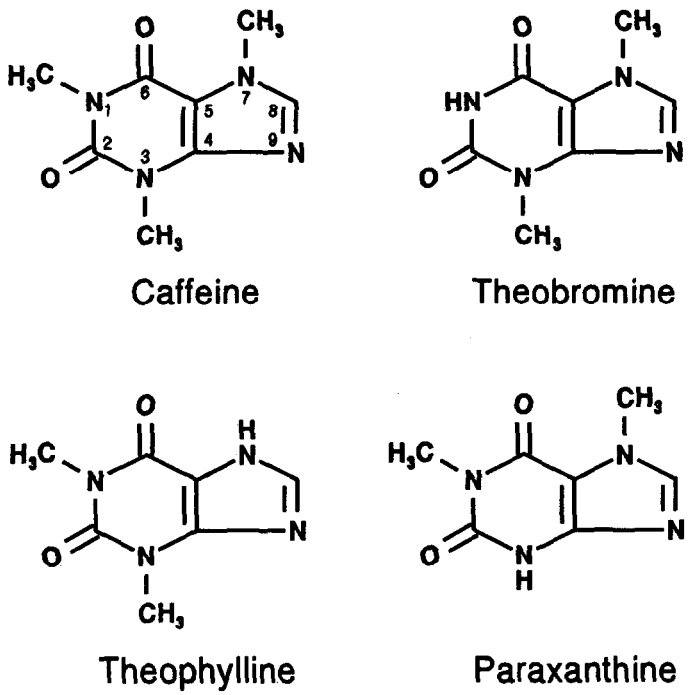

FIGURE 1. Structures of caffeine and its primary monodemethylated metabolites paraxanthine, theobromine, and theophylline.

1- and 7-methyluric acid (1- and 7-MU), respectively. All three dimethylxanthines formed from CA (i.e., PX, TB, and TP) also undergo 8-hydroxylation, giving rise to a dimethyluric acid (1,3-, 1,7-, or 3,7-DMU). Dimethylaminouracil (DAU) formation, arising from C8–N9 bond scission, occurs only for those dimethylxanthines methylated at N7 (i.e., PX and TB). Of particular interest is the conversion of PX to 1-MX and 5-acetylamino-6-formylamino-3-methyluracil (AFMU). It has been proposed that N7-demethylation of PX proceeds by way of an unstable ring-opened intermediate; subsequent acetylation or internal rearrangement of this species results in the formation of AFMU and 1-MX, respectively (Lelo *et al.*, 1989; Kalow and Tang, 1994). Deformylation of AFMU, which may occur spontaneously in urine, produces 5-acetyl-6-amino-3-methyluracil (AAMU).

The relative formation of each precursor dimethylxanthine (i.e., PX, TB, and TP) and the extent of the separate secondary biotransformation reactions determine the proportions of the various CA metabolites excreted in urine *in vivo*. Not surprisingly, those compounds derived from PX, whose formation accounts for around 80% of CA clearance, are the most abundant urinary metabolities. In particular, the N7-demethylation products of PX (i.e., AFMU, 1-MX, 1-MU) account for almost 60% of all metabolites formed from CA *in vivo* (Arnaud, 1993).

ENZYMES CONRIBUTING TO CAFFEINE METABOLITE FORMATION

Cytochrome P450

CYP1A2 is the enzyme of greatest importance for the formation of the primary metabolites of CA. Microsomal kinetic, inhibitor, and correlation approaches along with the use of cDNA-expressed CYP isoforms have provided overwhelming evidence that, at substrate concentrations ≤ 100 μM, CYP1A2 is almost solely responsible for CA N3-demethylation (i.e., PX formation) in human liver (Butler *et al.*, 1989; Campbell *et al.*, 1987a; Fuhr *et al.*, 1992; Tassaneeyakul *et al.*, 1992 and 1994). Human liver microsomal CYP1A2 is also the enzyme which is almost certainly responsible for CA N1- and N7-demethylation (i.e., TB and TP formation) (Campbell *et al.*, 1987; Tassaneeyakul *et al.*, 1994). At higher substrate concentrations, which are of little or no relevance to CA exposure in humans, CYP2E1 and possibly other isoforms additionally contribute to all three CA N-demethylations (Gu *et al.*, 1992; Tassaneeyakul *et al.*, 1994). CYP1A1 also has the capacity to convert CA to PX (Tassaneeyakul *et al.*, 1992) but it is unlikely to contribute significantly to CA elimination *in vivo* given its negligible expression in human liver. In contrast to the N-demethylations, CYP1A2 appears not to contribute to CA 8-hydroxylation. Rather, the results of chemical- and immunoinhibition studies suggest a major role for CYP3A4 in TMU formation (Tassaneeyakul *et al.*, 1992 and 1994). Although the enzymology of TAU formation has not been investigated, the involvement of CYP3A4 in the formation of this compound might be expected since both TMU and TAU are apparently derived from a common intermediate.

CYP1A2 also contributes significantly to the formation of several of the secondary metabolites of CA. Separate studies *in vivo* and *in vitro* indicate that CYP1A2 is probably responsible for the N1-, N3-, and N7-monodemethylations of PX, TB, and TP (Campbell *et al.*, 1987a; Fuhr *et al.*, 1992; Kalow and Tang, 1994; Miners *et al.*, 1985; Robson *et al.*, 1988). In addition, CYP1A2 appears to be involved in the 8-hydroxylation of PX, TB, and TP, although other isoforms (e.g., CYP 2A6, 2E1, 3A4) may be of equal or even greater importance in dimethyluric acid formation (Gu *et al.*, 1992; Kalow and Tang, 1994; Robson *et al.*, 1988). As with CA, the dimethylaminouracils and dimethyluric acids formed from PX and TB are assumed to form via a common intermediate and these same enzymes might therefore be assumed to mediate 1,7- and 3,7-DAU formation. Indeed, a role for CYP1A in the conversion of TB to 3,7-DAU has been established in the rat (Lelo *et al.*, 1990).

N-Acetyltransferase

Of all the ring-opened metabolites formed from CA, AFMU (and its degradation product AAMU) is quantitatively of greatest significance. Although there is strong evidence supporting a role for the polymorphic N-acetyltransferease NAT2 in the formation of this compound (Kilbane *et al.*, 1990), direct biochemical evidence linking AFMU formation to NAT2 *in vitro* is lacking. The lack of a molecular mechanisms is not

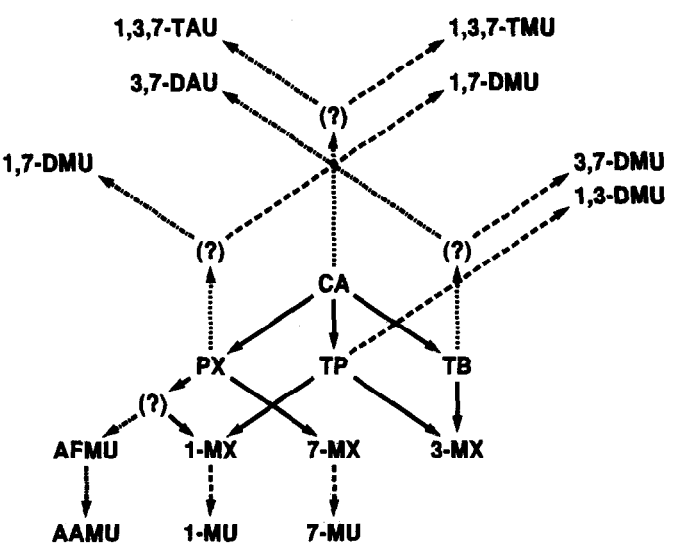

FIGURE 2. Primary and secondary routes of caffeine metabolism in humans. See text for abbrevations.

surprising, however, because the structure of the CYP-generated intermediate which presumably serves as the substrate for NAT2 remains to be identified.

Xanthine oxidase

The 8-hydroxylation of the monomethylxanthines 1-MX and 7-MX to form their respective monomethylurates (i.e., 1-MU and 7-MU) in humans *in vivo* is reduced or abolished by pretreatment with the XO inhibitor, allopurinol. Allopurinol inhibition occurs irrespective of whether the monomethylxanthine is administered directly or is derived from CA, PX, TB, or TP (Birkett *et al.*, 1983; Grant *et al.*, 1986; Grygiel *et al.*, 1979; Lelo *et al.*, 1989; Miners *et al.*, 1982). Recent studies in this laboratory (Miners *et al.*, unpublished data) similarly demonstrated that the conversion of 1-MX to 1-MU by the cytosolic fraction of human liver was abolished by addition of allopurinol (and its active metabolite oxipurinol), whereas 1-MU formation was not detectable in incubations of human liver microsomes supplemented with NADPH. Thus, there seems little doubt that monomethyluric acid production, in particular that of 1-MU, is dependent entirely on XO. Interestingly, 3-MX, whether formed from CA, TB, or TP, is not metabolized to 3-MU.

THE APPLICATION OF CAFFEINE AS A METABOLIC PROBE

CYP1A2

Given the potentially important role of CYP1A2 in chemical carcinogenesis, there has been considerable interest in the identification of substrates which may be used as *in vivo* and *in vitro* probes for the population screening of this enzyme in humans. In this regard considerable attention has focused on CA. Apart from the involvement of CYP1A2 in multiple metabolic pathways, the near ubiquitous consumption, relative safety, and favorable pharmacokinetic characteristics (complete absorption, relatively high clearance, and short half-life) make CA an attractive metabolic probe for use in humans.

There is now little doubt that, under appropriate experimental conditions, CA *N*3-demethylation may be sued as a marker for CYP1A2 activity in human liver *in vitro* (Tassaneeyakul *et al.*, 1992). CYP1A1 may, however, contribute to CA *N*3-demethylation in those extrahepatic tissues which express this isoform. Since the CYP1A2 catalyzed *N*1-, *N*3-, and *N*7-demethylations account for about 90% of CA elimination in humans (Lelo *et al.*, 1986b), CA apparent oral clearance should reflect CYP1A2 activity *in vivo*. Consistent with the expected importance of CYP1A2 to CA elimination *in vivo*, CA plasma clearance is enhanced in cigarette smokers and reduced markedly in subjects coadministered furafylline, a potent and highly selective inhibitor of CYP1A2 (Parsons and Neims, 1978; Tarrus *et al.*, 1987). Collection of multiple blood samples from each subject for the calculation of plasma clearance is however clearly a disadvantage in population-screening studies. Thus, ratios of various CA metabolites excreted in urine (termed urinary metabolic ratios, or MR) rather than plasma clearance have been employed in recent times for phenotyping CYP1A2 activity.

Five urinary CA MRs which claim to reflect CYP1A2 activity *in vivo* have been reported (Notarianni *et al.*, 1995). The most widely employed is the so-called caffeine-MR (CMR) (Kalow and Tang, 1991a and b, 1993), which measures the ratio (1-MX + 1-MU + AFMU)/1,7-DMU. The numerator is the sum of the products resulting from PX 7-demethylation which, as indicated earlier, is a CYP1A2-catalyzed reaction. However, 1,7-DMU was chosen as the denominator on the basis that its excretion showed the least interindividual variability compared to all other CA biotransformation products. The CMR has been shown in separate studies to correlate significantly ($r = 0.77–0.91$) with plasma CA clearance (Campbell *et al.*, 1986b; Tang *et al.*, 1994) and to increase or decrease in response to factors which induce or inhibit CYP1A2 activity, respectively (Kalow and Tang, 1994). Two other MRs reported to date are variations of the CMR, with either PX as the denominator or with PX included in the numerator and CA as the denominator (Notarianni *et al.*, 1995 [and references therein]). MRs which use the ratios PX or (PX + 1,7-DMU) to CA in urine collected in the period from 4 to 5 h after CA ingestion have been reported by Kadlubar and colleagues (Butler *et al.*, 1992). The time of urine collection is critical, presumably because PX is both a substrate and product of CYP1A2. Interestingly, it has been claimed recently that the ratio of PX to CA in saliva 5–7 h after a CA dose may be used as an index of CYP1A2 activity *in vivo* (Fuhr and Rost, 1994).

All of the CA MRs are partially or completely empirical. From the previous description of the enzymes responsible for the formation of the various CA metabolites it is clear that CYP1A2 activity affects both the denominator and numerator of all MRs to a varying extent. Moreover, the renal clearances or excretion rates of the individual compounds are factors in the various MRs. Inter- and intra-individual differences in these parameters may occur due to the dependence of tubular reabsorption on urine flow (Birkett and Miners, 1991). It is therefore hardly surprising that a recent comparison of the five MRs referred to previously in 237 volunteers administered CA found that the ratios measured at least three different parameters "in the name of" CYP1A2, with no one ratio correlating closely with another (Notarianni *et al.*, 1995). Clearly population distributions of CYP1A2 activity determined from MRs may well be inaccurate. Indeed, both normal and trimodal distributions of CYP1A2 activity have been reported for similar populations using two different MRs (Butler *et al.*, 1992; Kalow and Tang, 1991a). As noted by Tang *et al.*, (1994), "the differences between the results obtained by the two methods might be disregarded as trivial discordance were it not for the potential utility of CYP1A2 measurements in studies of cancer epidemiology."

CYP3A4

Recent work in this laboratory demonstrated that CA 8-hydroxylation (i.e., TMU formation) is catalyzed almost entirely by CYP3A4 (Tasaneeyakul *et al.*, 1992, 1994). This pathway was inhibited by troleandomycin and an anti-CYP3A antibody, activated by α-naphthoflavone, and correlated significantly with other CYP3A4-catalyzed reactions in microsomes from a panel of livers. Thus, at least *in vitro* CA may serve as a substrate probe for both CYP 1A2 and 3A4. Further validation is however necessary before TMU formation can reliably serve as a measure of CYP3A4 activity *in vivo*.

NAT2

There is good evidence *in vivo* that the urinary ratios of AFMU to 1-MX and AFMU to (AFMU + 1-MX + 1-MU) are reliable indicators of NAT2 phenotype. The use of these CA metabolite ratios for NAT2 phenotyping has been validated by demonstrating concordance with both dapsone and sulphamethiazine *N*-acetylation (Kilbane *et al.*, 1990; Tang *et al.*, 1991). In addition, family pedigree analysis by the CA NAT2 phenotyping procedure was consistent with a Mendelian segregation pattern (Kilbane *et al.*, 1990). Although it has been argued that more reliable results are obtained using the ratio of AFMU to 1-MX, use of (AFMU + 1-MX + 1-MU) as the denominator precludes XO activity as a variable affecting the CA NAT2 phenotyping test (see next section).

Xanthine oxidase

As described earlier, it is well established that XO catalyzes the 8-hydroxylation of 1-MX *in vitro* and *in vivo*. The conversion of 1-MX to 1-MU

in vivo may be abolished by coadministration of allopurinol, irrespective of whether the 1-MX is administered directly or derived from CA, PX, or TP. Following the administration of 1-MX itself, the ratio of 1-MU to 1-MX in urine was shown to reflect XO activity (Day *et al.*, 1988a). This ratio decreased with increasing allopurinol dose in six subjects, and in each subject there was a sigmoidal relationship between the 1-MU/1-MX ratio and the plasma concentration of oxipurinol (the active metabolite of allopurinol). Similar concentration-responsible relationships have been demonstrated using 1-MX derived from both CA and TP (Birkett *et al.*, 1991; Birkett *et al.*, unpublished results).

In a study investigating the relationship between allopurinol dose and plasma oxipurinol and urate concentrations in patients with gouty arthritis it was found that many patients were taking unnecessarily high doses of allopurinol (Day *et al.*, 1988a). Measurement of the urinary 1-MU/1-MX ratio following a single dose of CA (as tea or coffee) may well provide a useful adjunct for otpimizing allopurinol dosage in such patients.

SUMMARY AND CONCLUSIONS

Although complex, CA metabolism in humans is now well characterized. In most instances, the enzymes responsible for the formation of the various primary and secondary biotransformation products have been identified. CYP1A2 catalyzes the three CA *N*-demethylations, which account for more than 90% of CA clearance *in vivo*. In addition, this isoform contributes significantly to the metabolism of the three primary *N*-demethylation products of CA (i.e., PX, TB, and TP). Additional CYP isoforms also appears to be involved in the formation of certain secondary metabolites while CYP3A4 catalyzes the 8-hydroxylation of CA itself. Apart from CYP, NAT2, and XO contribute to the formation of secondary metabolites. The urinary excretion of AFMU is dependent on NAT2 activity while the ratio of 1-MX to 1-MU in urine is determined by XO. Population studies of NAT2 and XO activities have been undertaken using the appropriate CA MRs (Kalow and Tang, 1991a, 1994; Tang *et al.*, 1991).

Knowledge of the enzymology of CA metabolism has provided a basis for the use of this compound as a substrate probe for a number of xenobiotic metabolizing enzymes *in vitro* and/or *in vivo*. AFMU excretion (relative to other *N*7-demethylation products of PX) and the ratio of 1-MU to 1-MX appear to provide reliable estimates of NAT2 and XO activity, respectively. CYP1A2 catalyzes CA *N*3-demethylation and the conversion of CA to PX is a valid measure of the activity of this isoform in human liver microsomes under appropriate conditions. Partial metabolic clearance by *N*3-demethylation should accurately reflect CYP1A2 activity *in vivo*, but determination of this parameter is difficult experimentally given the extensive secondary metabolism of PX and complications arising from common pathways of dimethylxanthine metabolism (Fig. 2). However, plasma CA clearance is an acceptable alternative to this parameter since all three *N*-demethylations are catalyzed by CYP1A2 and together they account for more than 90% of the primary metabolism of CA.

Since collection of multiple blood samples limits the feasibility of plasma CA clearance in population screening studies, methods based on MRs have been developed for CYP1A2 phenotyping *in vivo*. However, the theoretical basis underlying each of the MRs is flawed to varying extents and it is now apparent that factors other than CYP1A2 activity contribute to all of the urinary ratios utilized to date. The value of *in vivo* CYP1A2 activities estimated from MRs therefore requires careful consideration.

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