[13] Assays for CYP1A2 by Testing *in Vivo* Metabolism of Caffeine in Humans

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Introduction

Caffeine (1,3,7-trimethylxanthine, 137×) undergoes *N*-demethylation, ring hydroxylation, and acetylation to give di- and monomethylxanthines, urates, and an acetylated uracil in humans (Fig. 1). The major enzyme involved in the biotransformation of caffeine and its metabolites is CYP1A2, a cytochrome P450.[1,2] Since the contribution of CYP1A2 to systemic caffeine clearance is estimated to be more than 95% of the total, clearance is the "gold standard" for an index of CYP1A2 activity for most subjects.[3] Other enzymes with contributions to caffeine metabolism are CYP2E1 (the ethanol-inducible cytochrome P450), the polymorphic *N*-acetyltransferase (NAT2), xanthine oxidase (XO), and, to a minor extent, CYP3A4 and CYP2A6. For subjects with induced CYP2E1 activity (e.g., workers in solvent factories, subjects with ethanol dependency), its contribution to systemic caffeine clearance may be more than 5%.

The urinary caffeine metabolite ratios established in this laboratory as indexes for the activity of CYP1A2, *N*-acetyltransferase, and xanthine oxidase are summarized as follows[3–5]:

In blood: CYP1A2 index = systemic caffeine clearance = Cl_{CAF}
In urine: CYP1A2 index = CMR = (AFMU + 1X + 1U)/17U
N-Acetyltransferase index = NAT2 = AFMU/(AFMU + 1X + 1U)
Xanthine oxidase index = XO = 1U/(1X + 1U)

A caffeine test for CYP2E1 is still being evaluated based on the shifting of the metabolite profile of caffeine 3-demethylation to the 1- and 7-demethylation pathways by CYP2E1.[2] Thus the caffeine test promises to become a single test for monitoring multiple liver enzymes identified in carcinogen activation, i.e., CYP1A2, CYP2E1, NAT2, and XO. The caf-

[1] M. A. Butler, M. Iwasaki, F. P. Guengerich, and F. F. Kadlubar, *Biochemistry* **86,** 7696 (1989).
[2] L. Gu, F. J. Gonzalez, W. Kalow, and B. K. Tang, *Pharmacogenetics* **2,** 73 (1992).
[3] W. Kalow and B. K. Tang, *Clin. Pharmacol. Ther.* **53,** 503 (1993).
[4] M. E. Campbell, S. P. Spielbery, and W. Kalow, *Clin. Pharmacol. Ther.* **42,** 157 (1987).
[5] B. K. Tang, D. Kadar, L. Qian, J. Iriah, J. Yip, and W. Kalow, *Clin. Pharmacol. Ther.* **49,** 648 (1991).

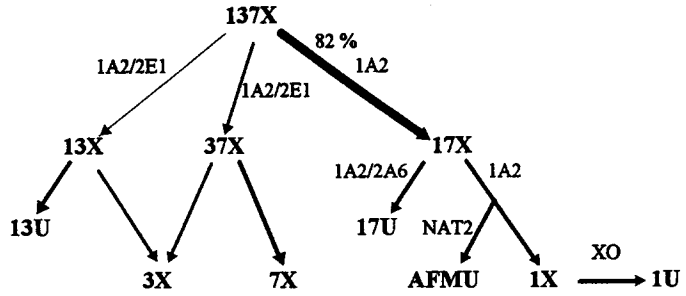

FIG. 1. Proposed caffeine metabolic pathways in humans and the major enzymes involved. 137X, caffeine = 1,3,6-trimethylxanthine; 13X, theophylline = 1,3-dimethylxanthine; 13U, 1,3-dimethylurate; 37X, theobromine = 3,7-dimethylxanthine; 17X, paraxanthine = 1,7-dimethylxanthine; 17U, 1,7-dimethylurate; 3X, 3-methylxanthine; 7X, 7-methylxanthine; AFMU, 5-acetylamino-6-formylamine-3-methyluracil; 1X, 1-methylxanthine; 1U, 1-methylurate; 1A2, CYP1A2; 2E1, CYP2E1; 2A6, CYP2A6; NAT2, the polymorphic *N*-acetyltransferase; XO, xanthine oxidase.

feine-based assays have been reviewed.[3] This chapter describes one blood and one urine test in detail that have served as indexes of CYP1A2 activity in clinical and epidemiological studies.

Blood Test for CYP1A2 Activity

Gu[6] has compared standard AUC-based measurements of systemic caffeine clearance with a two-point method measuring caffeine concentration in blood and he found a high correlation ($r = 0.98$). Thus, systemic caffeine clearance (CL_{CAF}) can be estimated by a two-point method after a single oral dose of caffeine.

$$CL_{CAF} = K_{el} \times V_d$$

$$K_{el} = -2.303 \times \text{slope}$$

$$\text{Slope} = \frac{\log C_2 - \log C_1}{t_2 - t_1}$$

$$V_d = \frac{f \times \text{dose}}{C_0 \times \text{BW}}$$

$$\log C_0 = \frac{\log C_2 + \log C_1}{2} - \text{slope} \times \frac{t_2 + t_1}{2},$$

[6] L. Gu, M.S.c. Thesis, p. 76. University of Toronto (1992).

where K_{el} is the elimination rate constant; V_d is the apparent volume of distribution; C_0, C_1, and C_2 are blood or plasma caffeine concentration at time zero, at time 1 (t_1), and at time 2 (t_2), respectively; f is the bioavailability fraction of the oral dose, which is assumed to be unity; and BW is body weight in kilograms.

A. Experimental Protocol

After abstinence from caffeine- and xanthine-containing foods or drinks (including coffee, tea, cola, chocolate products) for 2 days, the subject is asked to consume a cup of coffee containing 100 mg of caffeine (1 to 2 mg/kg body weight) in the morning. At 4 and 8 hr after coffee intake, 1 ml of blood is taken from the antecubical vein and stored in a heparinized vial (7 units/ml) at −20° until analysis. Samples stored at −20° are stable for at least a year. At time zero, a saliva sample (0.2 ml) may be taken for a check of compliance.

B. Analytical Determination of Caffeine Concentration in Blood, Plasma, and Saliva

To 0.1 ml of sample in a glass tube (13 × 100 mm), add 0.04 ml of 0.05% acetic acid, 0.025 ml of the internal standard solution [7-(β-hydroxypropyl)theophylline, 5 μg/ml], and 3 ml of dichloromethane : isopropanol (90 : 10, v/v). The mixture is vortexed for 30 sec and centrifuged at 2500 rpm for 5 min. The organic phase is collected and dried under a gentle stream of nitrogen at 37° for 20 to 30 min. The residue is dissolved in 0.25 ml of the HPLC mobile-phase solvent (3.0% isopropanol, 0.5% acetonitrile, 0.05% acetic acid) and 0.05 ml of the solution is injected onto an Ultrasphere ODS column (5 μm, 25 cm × 4.6 mm, Beckman Instruments, Fullerton, CA). The caffeine and metabolites are eluted at 1 ml/min and are detected by ultraviolet absorbance (AUF = 0.02) at 280 nm.[7]

All samples should be warmed to room temperature and mixed well before sampling. Saliva samples, if foaming, should be centrifuged. The standard samples were made by mixing pure chemical (concentration ranged from 1 to 40 μM) with blank plasma. A 3% solution of bovine serum albumin could be used instead of blank plasma if it is free of interfering substances.

Under these conditions, the retention times for caffeine and the internal standard [7-(β-hydroxypropyl)theophylline] were 25 and 29 min, respectively. The retention times for 17X, 37X, and 13X were 7, 11, and 13 min, respectively. The mean recovery of caffeine was over 95% at 10 μM with

[7] B. K. Tang, Y. Zhou, D. Kadar, and W. Kalow, *Pharmacogenetics* **4,** 117 (1994).

TABLE I
SYSTEMIC CAFFEINE CLEARANCE, MEAN (SD) AS CYP1A2 INDEX IN 39 HEALTHY SUBJECTS

Subject	N	CL_{CAF} (ml/min/kg)	$T_{1/2}$ (hr)	V_d (liter/kg)
Non-OC[a] users	31	1.16 (0.49)	5.76 (2.46)	0.55 (0.24)
OC users	8	0.71 (0.20)[b]	7.26 (1.61)	0.43 (0.12)
All subjects	39	1.07 (0.48)	6.20 (2.09)	0.52 (0.22)

[a] Oral contraceptives.
[b] $P < 0.05$.

a CV of about 6%. The recovery of caffeine was reduced by drying the organic phase at a temperature higher than 40° and/or for more than 30 min.

C. *Results of a Population Study*

The frequency distribution of systemic caffeine clearance was log normal.[7] The mean (SD) in arithmetic terms was 1.07 (0.48) ml/min/kg for 39 healthy subjects who were not cigarette smokers (Table I). The variability of the apparent volume of distribution (CV = 46%) was similar in magnitude to that of other kinetic parameters. Oral contraceptive users showed significantly decreased caffeine clearance.

Urine Test for CYP1A2 Activity

The urinary caffeine metabolism ratio (CMR) reflecting 7-demethylation of 17X, which is catalyzed by CYP1A2, showed a high correlation with systemic caffeine clearance (Cl_{CAF}) ($r = 0.92$).[4] The high correlation has been confirmed with $r = 0.77$ to 0.87.[7,8] The advantage of the urine test is that it involves collection of a single urine sample without strict control of caffeine intake.

There are some disadvantages in using CMR as an index for CYP1A2 activity. First, 17U, the denominator of CMR, was chosen because its urinary excretion is not flow dependent and because it showed only a limited person-to-person variation. However, CMR could be subject to error in persons or populations with CYP2A6 variability since this cytochrome is now known to contribute to 17U formation (Fig. 1). An association of CMR and NAT2 ratios may arise because the numerator in the CMR is the same as the denominator in the NAT2 index. The CMR values for fast and slow acetylators were reevaluated using published data from one of

[8] K. L. Rost and I. Roots, *Clin. Pharmacol. Ther.* **55,** 402 (1994).

our previous studies.[9] The mean CMR was slightly higher ($P < 0.05$) in Caucasian subjects who were fast acetylators than in slow acetylators (Table II). This trend was similar in Oriental subjects. A peculiarity of caffeine and its dimethylxanthine metabolites (e.g., theophylline) is their flow-dependent urinary excretion, a factor probably related to the diuretic effect. The CMR as the CYP1A2 index is not urine flow dependent, but some published caffeine-based ratios for CYP1A2 activity are [e.g., (17X + 17U)/137X by Butler *et al.*[10]]. To different degrees, 17X and 137X are urine-flow-dependent components so that the ratio may be distorted by renal factors.[7] Furthermore, the magnitude of the ratio is sensitive to the time of urine collection versus the time of caffeine intake. Therefore, this and some similar ratios are not dealt with in this chapter.

A. Experimental Protocol

After abstinence from chocolate (caffeine is allowed) and acetaminophen for 2 days and during the day of study, the subject is asked to consume a cup of coffee containing caffeine (60 to 90 mg) in the afternoon. No more than four cups should be consumed on the day of study. About 1 to 10 ml of the overnight urine (the first morning urine) is collected in a sample vial containing ascorbic acid (10 mg/ml) and is kept frozen at −20° until analysis. Alternatively, the cup of coffee may be consumed in the morning and one sample of 8-hr-pooled urine will be collected.

It is necessary to abstain from chocolate and acetaminophen because chocolate may inhibit CYP1A2 activity and acetaminophen may interfere with the analytical assay. The reliability of CMR as an index for CYP1A2 declines as the collection periods decrease to below 8 hr.

B. HPLC Analysis of Urinary Caffeine Metabolites

Two separate assays are used routinely in our laboratory for the determination of CMR: one for xanthines and urates and one for AFMU. Prior to the assay, the labile AFMU is converted to the more stable AAMU (5-acetylamino-6-amino-3-methyluracil). This conversion has led to the most reliable results.[11] Measuring AFMU without this conversion will lead to an underestimate of CMR values, as is clearly illustrated in Table II.

Since the conversion is pH dependent, spontaneous transformation is difficult to avoid, and may even take place sometimes in the bladder urine prior to voiding.

[9] W. Kalow and B. K. Tang, *Clin. Pharmacol. Ther.* **50,** 508 (1991).
[10] M. A. Butler, N. P. Lang, J. F. Young, N. E. Caporaso, P. Vineis, R. B. Hayes, C. H. Teitel, J. P. Massengill, M. F. Lawsen, and F. F. Kadlubar, *Pharmacogenetics* **2,** 116 (1992).
[11] B. K. Tang, T. Zubovits, and W. Kalow, *J. Chromatogr.* **375,** 170 (1986).

TABLE II
BIOLOGICAL FACTORS THAT MAY AFFECT CMR AS CYP1A2 INDEX

Factor	Aspect	*N*	CMR mean (SEM)	Ref.
Gender	Male	30	4.8 (0.3)[b]	4
	Female	30	4.7 (0.3)[b]	4
	Male	106	6.36 (0.24)	9
	Female	54	5.85 (0.31)	9
Ethnic	White adults	42	4.7 (0.4)[b]	4
	Oriental adults	26	4.6 (0.4)[b]	4
	White adults	130	6.2 (0.2)	9
	Oriental adults	21	5.8 (0.4)	9
Age	3–11, average 7 years, white	21	7.9 (0.4)[b]	4
	6–11, average 8.9 years, white	10	8.86 (1.06)	16
	Adults 18–30	45	4.4 (0.2)[b]	4
	Adults 30–65	15	4.7 (0.4)[b]	4
	Adults, average 57 years,	94	5.9 (0.3)	17
	Adults, average 26 years,	178	5.96 (0.18)	9
	Adults, average 56 years,	125	7.3 (0.4)	17
OC user		9	3.6 (0.5)[b]	4
		18	3.90 (0.32)	9
		8	4.23 (0.79)	7
Pregnancy, epileptic		7	3.33 (0.3)	
Epileptic, nonpregnant female		7	8.08 (0.9)	18
Schistosomiasis and other factors				
12–16, average 13.5 years, black		45	3.78 (0.4)	16
Polycyclic aromatic hydrocarbons				
Smokers: 19/day		26	9.4 (0.7)[b]	4
Smokers: 10–24/day		8	8.52 (1.04)	9
Smokers: 44 pack/year		31	11.6 (1.0)	17
PBB exposed (35 ppb)		43	6.96 (0.32)[b]	19
TCDD (140 pg/g)		45	6.8 (0.7)	17
TCDD (217 pg/g) and smoker		13	10.9 (0.9)	17
Acetylation status				
Caucasian				
Slow		79	5.82 (0.26)	*c*
Fast		51	6.78 (0.38)	*c*
Oriental				*c*
Slow		5	5.26 (0.72)	*c*
Fast		16	5.93 (0.54)	

[a] PBB, polybrominated biphenyls; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; OC, oral contraceptives.
[b] CMR estimated by measurement of AFMU without convertion to AAUM.
[c] CMR values recalculated using published data from Kalow and Tang.[9]

Determination of 1X, 1U, and 17U Concentrations. This assay is similar to that of caffeine in blood described earlier. To 0.1 ml of urine in a glass tube (13 × 100 mm), 0.025 ml of the internal standard solution (1.2 mg of *N*-acetyl-*p*-aminophenol dissolved in 10 ml of 50% isopropanol water) and 3 ml of dichloromethane/isopropanol (88:12, v/v) are added. The mixture is vortexed for 30 sec. The organic phase is then separated and dried under a gentle stream of nitrogen at 37° for 20 to 30 min. The residue is dissolved in 0.25 ml of the HPLC mobile-phase solvent (1.3% isopropanol, 0.1% acetonitrile, 0.05% acetic acid) and 0.05 ml of the solution is injected onto an Ultrasphere IP ODS column (5 μm, 25 cm × 4.6 mm, Beckman Instruments). The caffeine and metabolites are eluted at 1 ml/min and detected by ultraviolet absorbance (AUF = 0.02) at 280 nm. Under these conditions, the retention times of 1U, 1X, and 17U and the internal standard (*N*-acetyl-*p*-aminophenol) were 8.5, 11.5, 29, and 14.5 min, respectively.

For the preparation of standard samples, 1U (1 mg) is first dissolved in about 10 ml of water of pH 9 and the pH is obtained by adding a drop of sodium hydroxide (10 *N*), which is then neutralized to pH 7 by 12 *N* HCl. This solution is mixed with other xanthines and urates and is diluted to 100 ml with blank urine (pH 3.5). The final concentrations of the metabolites are 5 to 10 mg/liter. Several 10-ml aliquots of this standard mixture can be stored at −20° until use. Precautions and pitfalls of the assay have been described.[5]

Determination of AFMU by Conversion to AAMU. To convert AFMU to AAMU, 0.05 ml of sodium hydroxide (0.25 *N*) is added to 0.05 ml of urine in a glass tube (13 × 100 mm). The pH should be at least 10. After reaction for 10 to 20 min, 0.05 ml of 0.25 *N* HCl is added to neutralize the excess sodium hydroxide. Then 0.1 ml of the internal standard solution (10 mg of benzyloxyurea dissolved in 10 ml of water) is added. An aliquot of 0.02 ml of the mixture is injected onto the TSK-20 column (G200PW, Toso Haas, Philadelphia, PA; 10-μm particle size, 300 × 7.5 mm i.d.). It is eluted with 0.1% acetic acid at a flow of 0.8 ml/min and is monitored by UV absorbance at 263 nm.[11] Under these conditions, the retention times of AAMU and the internal standard (benzyoxylurea) were 15 and 29 min, respectively.

For continuous analysis, the TSK column should be washed with 20% methanol in 10% acetic acid at a flow of 0.8 ml/min for 1 hr after 20 to 30 determinations and then conditioned for 1.5 hr before use.[5]

The use of one chromatographic system for all four metabolites has been reported.[12] The reliability of this assay needs to be defined because

[12] P. Dobrocky, P. N. Bennett, and L. J. Notarianni, *J. Chromatogr.* **652,** 104 (1994).

the AAMU peak overlapped by over 30% with that of an endogenous substance in the chromatogram from one urine sample of one volunteer.

Other methods using capillary electrophoresis (CE) have been published for AFMU, 1X, and 1U.[13–15] By a combination of HPLC and CE techniques, a complete baseline separation of AFMU, 1X, 1U, and 17U was achieved.[15]

C. Factors That May Affect CMR as an Index of CYP1A2 Activity

Table II[16–19] summarizes the results from 10 years of our laboratory work to detect biological factors which may have an influence on CMR as a CYP1A2 index. Significant differences were found between males and females, whites and Orientals, and of female epileptics. CYP1A2 activity was lowered by the use of oral contraceptives and was reduced in pregnancy. It was induced by cigarette smoking. Children between 3 and 11 years of age had CMR values as high as some cigarette smokers. Factory workers exposed to TCDD 20 to 30 years prior to test but with still high concentrations of TCDD in their tissues (140–217 pg/g) showed CMR values not significantly different from controls; in the smokers among these subjects, the CMR was elevated. Farmers tested not too long after exposure to PBB in Michigan showed significantly higher CMR values than controls.

[13] J. Caslavska, E. Hufschmid, R. Theurillat, C. Desiderio, H. Wolfisberg, and W. Thormann, *J. Chromatogr.* **656,** 219 (1994).

[14] R. Guo and W. Thormann, *Electrophoresis* **14,** 547 (1993).

[15] N. Rodopoulos and A. Norman, *Scand. J. Clin. Lab. Invest.* **54,** 305 (1994).

[16] C. M. Misimidrembwa, M. Beke, J. A. Hasler, B. K. Tang, and W. Kalow, *Clin. Pharmacol. Ther.* **57,** 25 (1995).

[17] W. Halperin, W. Kalow, M. H. Sweeney, B. K. Tang, M. Fingerhut, B. Timpkins, and K. Willie, *Occup. Environ. Med.* **52,** 86 (1995).

[18] M. Bologa, B. Tang, J. Klein, A. Tesoro, and G. Koren, *J. Pharmacol. Exp. Ther.* **257,** 735 (1991).

[19] G. H. Lambert, D. A. Schoeller, H. E. B. Humphrey, A. N. Kotake, H. Lietz, M. Campbell, W. Kalow, S. P. Spielberg, and M. Budd, *Environ. Health Perspect.* **89,** 175 (1990).