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COMMENTARIES

The use of caffeine for enzyme assays: A critical appraisal

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Caffeine is useful as an enzyme probe for its ubiquitous consumption, its rapid and complete gastrointestinal absorption, its distribution throughout the total body water, and its low plasma binding, as well as for its short half-life, negligible first-pass metabolism, minimal renal elimination, and biotransformation that is virtually confined to the liver.[1-3] Caffeine has been used to develop liver function tests[4-6] and to support epidemiologic studies.[7-10] The enzymes currently subject to caffeine assay are the inducible P450, CYP1A2, the polymorphic *N*-acetyltransferase, and xanthine oxidase.[5,10-12] Recently some of the older knowledge of caffeine kinetics has been extended through molecular techniques.[13-16] On the one hand, this has led to the introduction of new methods for enzyme assays; on the other hand, it calls for a reassessment of established methods. It is therefore time to give a critical overview of caffeine-based methods currently used in clinical or epidemiologic investigations.

As a basis for review, we need to outline the pertinent aspects of the fate of caffeine and its metabolites in terms of biotransformation, enzyme identification, and renal excretion.

CAFFEINE METABOLISM

The primary metabolism of caffeine

This section deals with the initial metabolism of caffeine (1,3,7-trimethyl xanthine [137X]), primarily the 1-, 3-, and 7-demethylations that lead to the production of 1,7-dimethylxanthine (17X, paraxanthine), 3,7-dimethylxanthine (37X, theobromine), and 1,3-dimethylxanthine (13X, theophylline), respectively (Fig. 1). In addition, there is the 8-hydroxylation to form the 1,3,7-trimethylurate (137U). In humans, this reaction represents only a minor pathway (approximately 3%) in vivo,[7] but becomes prominent in vitro when human liver preparations are tested.[17] We will neglect the uracil formation.

Caffeine 3-demethylation (17X formation) is the most prominent reaction and accounts for 83.9% ± 5.4% of demethylations; by the same measure, the

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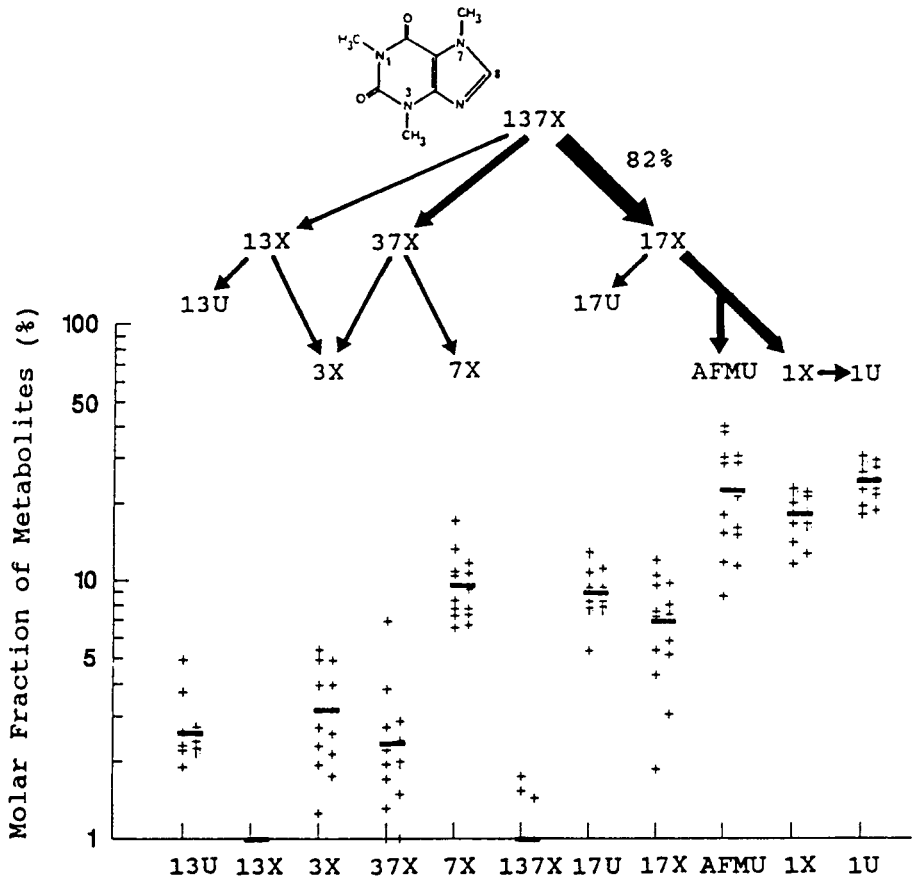

Fig. 1. Mean and distribution of molar fractions of caffeine metabolites in urine collected over 24 hours in 14 subjects after intake of 2 mg/kg caffeine. Caffeine is 137X. The abbreviations for the metabolites are defined in the text. (From Gu L. MSc Thesis. Toronto: University of Toronto, 1992. Reprinted with permission.)

caffeine 1-demethylation accounts for 12.1% ± 4.1% and the 7-demethylation for 4.1% ± 1.4% in human subjects.[18]

It has long been known that caffeine metabolism,[19,20] and the 3-demethylation in particular,[9] are catalyzed by an inducible enzyme, a member of what is now called the CYP1A family; Butler et al.[13] have shown the enzyme to be CYP1A2, and this has been confirmed.[21]

Gu et al.[14] tested complementary deoxyribonucleic acid (cDNA) produced human CYP1A2 and five other P450s (2A6, 2B6, 2E1, 3A4, and 3A5) for their ability to catalyze the biotransformation of caffeine and caffeine metabolites. CYP1A2 was the only enzyme to catalyze the 3-demethylation of caffeine, confirming the data of Butler et al.[13] However, CYP1A2 also catalyzed the 1- and 7-demethylations of caffeine, with rates representing 13.3% and 6.6%, respectively, of the rate of caffeine 3-demethylation. Thus, knowing the rate of caffeine 3-demethylation by CYP1A2, we can calculate how fast the same enzyme catalyzes the 1- and 7-demethylations. If one takes the sum of these three demethylations as 100%, the formation of 17X accounts for 83.4%, 37X accounts for 11.1%, and 13X accounts for 5.5%. These findings are virtually identical with those obtained in vivo and cited above.[18] It means that CYP1A2 activity accounts for most of the systemic caffeine clearance in most subjects. This observation is supported by the in vivo inhibition of caffeine metabolism by furafylline, a specific and potent inhibitor of human CYP1A2.[16,22]

There is a usually small part of the 1- and 7-demethylations that is not explained by CYP1A2 activity; this can be quantitatively accounted for by the catalytic action of CYP2E1, the ethanol-inducible P450[14]; attempts are being made to develop a caffeine

test for CYP2E1. Perhaps a test can also be developed for CYP3A4,[23] which participates in the 8-hydroxylation of caffeine and of theophylline.

The studies with only six cDNA-derived human P450s[14] do not exclude the possibility that other enzymes participate in the metabolism of caffeine or caffeine metabolites. However, the tested enzymes seem to account for most or all of the caffeine metabolizing capabilities of human liver microsomes, making it unlikely that additional enzymes would have major quantitative effects.

Biotransformation of the primary metabolites of caffeine

Each of the three dimethylxanthines—17X, 37X and 13X—can be further demethylated to form the three monomethylxanthines 1X, 3X, and 7X. (Fig. 1) Thus each of these monomethylxanthines has two sources in theory but not necessarily in practice. This introduces some complexity into our subject. Let us therefore first describe the fate of each of the dimethylxanthines.

13X (theophylline).[24,25] Approximately 10% to 15% of 13X is excreted unchanged. The most important pathways for 13X metabolism are 50% formation of 1,3-dimethylurate (13U) and 23% formation of 1-methylurate (1U), with hardly any 1X. This lack of 1X is not yet explained because human liver microsomes and pure CYP1A2 form in vitro 1X and 3X at about the same rate.[15] As always, dimethylurate formation involves more than one enzyme. Thus Gu et al.[14] found the 8-hydroxylation of 13X is catalyzed by CYP1A2, CYP2E1, and CYP3A4; Sarkar et al.[15] named CYP2E1 and CYP3A3 as catalyzing enzymes.

37X (theobromine).[24] Roughly 10% of 37X is excreted unchanged. Approximately 20% is excreted as 3X, and 50% as 7X. The 7X formation was entirely catalyzed by CYP1A2.[14] The 37U formation was catalyzed by CYP1A2 and by CYP2E1, but this hydroxylation and that of the monomethylated products are quantitatively unimportant.

17X (paraxanthine).[26] About 10% of 17X is excreted unchanged. The products of 7-demethylation (i.e., 1X, 1U, and 5-acetylamino-6-formylamino-3-methyluracil [AFMU]) account for 20.1%, 39.2%, and 16.0%, respectively, of metabolites in 24-hour urine; the sum of these is 75.3%, whereas 1,7-dimethylurate (17U) represents 9%. In a comparative study[27] these percentages were 63% and 19%. Under the experimental conditions of Gu et al.,[14] hydroxylation to 17U accounted for about 20% of 17X metabolism and was catalyzed by CYP2A6 and CYP1A2. The rate of 17X hydroxylation by CYP1A2 was 10% of the caffeine 3-demethylation rate and approximately 75% of 17X hydroxylation by CYP2A6. The 1-demethylation represented about 3%[27] or 6%[26] of 17X metabolism.

CYP1A2 is the one enzyme overwhelmingly responsible for the 7-demethylation of 17X. Among the cDNA-produced human P450s available to us, only CYP1A2 catalyzed this reaction.[14] The rate of 7-demethylation of 17X by CYP1A2 was about one third the rate of its 3-demethylation of caffeine. In preparations from 17 kidney donor livers, the two reactions correlated ($r = 0.91$).[28] Thus 17X is both a product and a substrate of CYP1A2.

The 17X 7-demethylation may not be exclusively by CYP1A2: In the same liver preparations, phenacetin *O*-deethylation (an accepted standard for CYP1A2 activity) correlated slightly better with the caffeine 3-demethylation ($r = 0.94$) than with the 17X 7-demethylation ($r = 0.84$).[28] Inhibition data also suggest a small contribution to the 17X 7-demethylation by a still unidentified enzyme in the occasional liver but not in all livers. Nevertheless, this does not change the fact that here is a case in which the same enzyme is responsible for the formation of a product and for its destruction.

Unless one uses large doses, the metabolism of each of the three dimethylxanthines is about the same whether separately ingested or metabolically formed from caffeine.[29] As quoted above,[18] the average proportions of 13X, 37X, and 17X formed from caffeine are about 4%, 12%, and 84%, respectively. These percentages, multiplied by the proportion of metabolites formed from each of the dimethylxanthines, give an indication of the relative amounts of metabolites obtained from caffeine (Fig. 1). For instance, if the 13X pathway represents less than 5% of caffeine demethylation and if 1U represents 20% of 13X metabolism, the amount of 1U formed from 13X would be less than 1% of caffeine metabolites. Also, although 1X can theoretically be formed from both 17X and 13X, in practice 1X and 1U come virtually all from 17X.

The two theoretic sources of 7X are 17X and 37X but, in reality, most of it comes from 37X. By contrast, 3X has enough double origin that its sources, 37X and 13X, cannot be easily distinguished.

These quantitative relationships have consequences for the interpretation of test data: Because 1X and 1U represent virtually only the 17X pathway and because the 1-demethylation of 17X is negligible, there is a good distinction between the "main pathway" of caffeine metabolism by way of 17X on the one hand, and

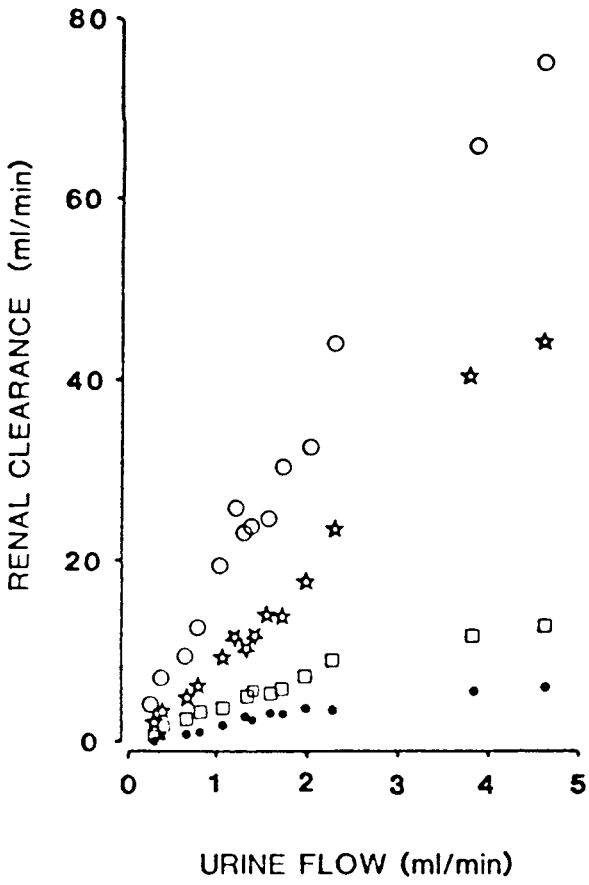

Fig. 2. Relationship between urine flow and the renal clearance of caffeine and three dimethylxanthines in a representative subject after a single dose of caffeine. Caffeine is indicated by *solid circles*, theophylline (13X) by *squares*, paraxanthine (17X) by *stars*, and theobromine (37X) by *open circles*. (From Tang-Liu DDS, Williams RL, Riegelman S. J Pharmacol Exp Ther 1983;224:180-5. Reprinted with permission.)

the "side pathways" by way of 13X and 37X on the other. The distinction between the two side pathways is less clear.

CYP1A2 was capable of catalyzing virtually all oxidative reactions of caffeine and caffeine metabolites,[14] although almost always in combination with other enzymes (except, as already stated, for the 3-demethylation of caffeine and for the 7-demethylation of 17X). Two of these CYP1A2 contributions may have consequences for enzyme testing by way of caffeine: (1) CYP1A2 makes a contribution to the 8-hydroxylation of 1X to form 1U, although this reaction is mainly catalyzed by xanthine oxidase,[30] and (2) CYP2A6 is the main identified catalyst of the 8-hydroxylation of 17X to form 17U; however, CYP1A2 noticeably contributes to this reaction, thereby affecting the interpretation of data that use 17U as a metabolic indicator.[14]

Uracil formation: The acetylation reaction

Several ring-open products have been identified, but only the AFMU by way of 17X pathway is of quantitative importance in urine after caffeine intake.[7,18] With regard to its mechanism of formation, there is no further progress than the original assumption that the 7-demethylation of 17X proceeds by way of an unstable open-ring intermediate that is either stabilized as AFMU by acetylation or by an internal rearrangement that leads to 1X. One of the difficulties is that formation of AFMU has not been shown in any in vitro system. Nevertheless, there is no room for doubt that the acetylating enzyme is the polymorphic *N*-acetyltransferase, currently referred to as NAT2 in distinction from the separately identified NAT1.[31,32]

AFMU may lose the formyl group spontaneously to give 5-acetylamino-6-amino-3-methyluracil (AAMU). The speed of conversion is generally slow but is enhanced at an elevated pH.[33] Clinical observations indicate that a significant conversion within the urinary bladder occurs in exceptional subjects, but it is not clear whether this is merely because of high urinary pH or whether the conversion is amplified by constituents of urine that enhance the pH effect.

In 1970, Kelley and Wyngaarden[34] reported the observation of one hospitalized subject who had large quantities of oxipurinol in his baseline urine sample and who excreted as much as 26.5 mg/day AAMU after 5 days of purine-free diet. Since that time, no AAMU has ever been observed in human urine without previous caffeine intake[33] (and unpublished observations in more than 1000 healthy subjects). The assumption[10] that there is an AAMU source other than AFMU requires confirmation with modern analytic means.

Urinary excretion

For urinary excretion of caffeine and its metabolites, one may distinguish three categories: (1) caffeine itself, (2) the dimethylxanthines 17X, 13X, and 37X, and (3) all other caffeine metabolites.

Caffeine. Caffeine itself is a minor excretion product, accounting for less than 3% of the dose (e.g., see Bonati et al.[35]). The renal clearance of caffeine is on the order of 1 to 3 ml/min for a healthy adult.[35-37] All

investigators observed that the renal clearance of caffeine is flow dependent; it increased by a factor of 3.4 when urine flow rates increased from 0.6 to 3.2 ml/min.[38] The ratios of plasma/urine concentrations of caffeine ranged from 1.10 to 1.74 and were highly correlated, whereas urine caffeine concentrations and urine flow rates were not correlated.[39] These observations indicate that, after glomerular filtration, there is a tendency for a diffusion equilibrium between the caffeine concentrations in renal tubular fluid and in blood plasma.

The three dimethylxanthines. 17X, 13X, and 37X seem to be similar to each other in renal excretion: The excretion of all three is dependent on urine flow, and renal clearance is higher than that of caffeine but still far lower than the glomerular filtration rate (e.g., see Lesko[40]). A set of data suitable for comparison is shown in Fig. 2. Fig. 2 shows that comparable renal clearance values for caffeine, 13X, 17X, and 37X at a urine flow of 2 ml/min are 3, 7, 17, and 32 ml/min, respectively.[41] Flow dependence means that the extent of hydration of a subject may affect the renal excretion of dimethylxanthines. Extensive literature data are available only for 13X (e.g., see Birkett et al.,[24] St. Pierre et al.,[25] Levy and Koysooko,[42] and Gundert-Remy et al.[43]), with quotations of renal clearance values of 13X ranging from 5.3 ± 3.0 to 12.2 ± 6.1 ml/min. The clearance values of 13X and their flow dependence have been interpreted through mathematic modeling as involving glomerular filtration followed by tubular reabsorption, a passive process of diffusion.[44] The conclusion that there is only physicochemical processes is surprising in view of the complex chemistry of methylxanthine diuresis.[45,46] In any case, the involvement of active tubular secretion in addition to filtration and diffusion has not been ruled out.

In contrast to monomethylxanthines, the balance between metabolic and renal factors allows each of the dimethylxanthines to be readily measured in both plasma and urine. This leaves scope for interethnic variability, probably through renal factors.[47,48]

The monomethylxanthines. 3X and 7X and, in particular, the urates and uracils are difficult to measure in blood or plasma because of their low concentrations, but they are readily assessed in urine. Tang-Lui et al.[41] succeeded in determining the renal clearances of 3X, 1U, and 13U. They much surpassed glomerular filtration rates, thereby proving that there is additional elimination by tubular secretion. Renal clearance of these compounds is so fast that any individual difference becomes immaterial in comparison with other rate processes concerning caffeine. These metabolites, once formed, are immediately expelled into the urine and are thereby excellent indicator substances of metabolic processes.

CAFFEINE-BASED ENZYME ASSAYS

Determinations of CYP1A2

Standards of comparison. The high-affinity component of phenacetin *O*-deethylation appears to be entirely attributable to CYP1A2.[13,49] Some years ago, when phenacetin was still a widely used analgesic drug, it could be given to volunteers for metabolic studies. These studies revealed that phenacetin metabolism was increased by cigarette smoking[50] and by consumption of charcoal-broiled beef.[51] The within-subject day-to-day variation of the rate of metabolism was greater with phenacetin than with other drugs.[52] Familial deficiency of phenacetin deethylation was discovered as a rare event.[53] Any assay of CYP1A2 by means of caffeine should show the same characteristics, but direct comparisons are excluded because phenacetin has lost its status as a drug and cannot be given for ethical reasons. There is no longer any equivalent means to assess the relative value of different caffeine-based methods for in vivo CYP1A2 measurements.

CYP1A2 occurs in liver but not in blood cells.[54,55] Hence, assessment of its enzymic presence with the help of Western blot analysis, or its formation by way of messenger ribonucleic acid (mRNA) determinations, is possible by testing liver tissue but not by testing blood or other accessible tissue. It requires medical and ethical decisions before investigations of CYP1A2 in liver biopsy specimens and of in vivo caffeine metabolism can be arranged in the same persons. Validation of current and future assay methods by such in vitro versus in vivo comparisons is most desirable, particularly in view of the fiftyfold liver-to-liver variation of the mRNA of CYP1A2.[56] In our bank of 19 kidney donor livers, rates of phenacetin *O*-deethylation varied 28-fold, and rates of caffeine-3-demethylation varied twentyfold.

In the absence of liver biopsy specimens for comparative testing, quantitation of caffeine partial clearance by 17X formation offers itself as the primary standard of CYP1A2 activity, to be used when developing simplified methods. So far this standard has been used neither for method development nor for method comparisons. The partial clearance of 17X formation by means of caffeine can be derived either

from extensive area under the curve data and computer-assisted calculations after a single caffeine dose[35] or from caffeine in steady-state studies and measurements of plasma clearances of 17X, 37X, and 13X.[18]

Measurements of systemic caffeine clearance may serve as a standard of convenience for further method development. As explained earlier (in "The primary metabolism of caffeine"), the contribution of CYP1A2 to 37X and 13X formation is 19.9% of 17X formation.[14] Because 17X formation accounts on average for 80% or more of caffeine plasma clearance,[18] the total involvement of CYP1A2 in caffeine metabolism accounts for more than 95% of caffeine plasma or blood clearance, at least in the great majority of subjects.

A full evaluation of caffeine-based CYP1A2 assays requires future investigations. Nevertheless, it seems useful to comment on the major methods on the basis of recognizable merit and potential pitfalls.

The caffeine breath test. A precise dose of caffeine with a ^{13}C-label at the 3-methyl group is administered, and the 2-hour cumulative exhalation of labeled carbon dioxide is measured.[5,57,58] This process requires a number of steps after the initial caffeine demethylation, including oxidation of the labeled methyl group to formaldehyde, then to formic acid, conversion into carbonic acid with subsequent carbon dioxide formation, and transport into the lungs for exhalation.[4,59] For the system to work, the initial demethylation must be the rate-limiting step, and that seems to be the case. The history of development of breath tests dates from 1921.[5] Breath tests have been used with drugs other than caffeine.[4,5]

Investigations in rats have shown the basic validity of the caffeine breath test.[4] Timing of breath sampling is important to avoid contamination of the measurement by 3-demethylation of 13X and 37X. The subjects should be at rest to ensure a steady basal state.[5] One may expect that measurements of the demethylation rate could be too low but never too high; in practice, this expectation seems not to be borne out because about a third to a half of the ingested label is lost in transit through the one-carbon pool.[5,59] One must expect that this loss will somewhat reduce the discriminative power of the breath test (see the discussion of the caffeine metabolic ratio test).

Extensive tests of reproducibility do not seem to exist, but it has been shown that the ^{13}C-label and the ^{14}C-label give the same result.[59] Furthermore, the 2-hour cumulative caffeine breath test showed excellent correlation ($r = 0.90$[60]; $r = 0.84$[61]) with systemic caffeine clearance, indicating the validity of the method in principle.

The functional utility of the test has been shown convincingly. The caffeine breath test has indicated high CYP1A2 values in smokers,[4] in children,[57] and in subjects exposed to polybrominated biphenyls,[58] as well as after CYP1A2 induction by omeprazol.[62] It is low in subjects on low-protein diets,[63] in patients with various forms of liver disease,[60] in women receiving contraceptive medication,[5] and in women who re pregnant.[5]

The drawback of the test is the requirement for labeled caffeine and the need for specialized equipment for measuring the labeled carbon dioxide in exhaled air.

The caffeine metabolic ratio. The test consists of measuring in urine the metabolites AFMU (or more recently and preferably AAMU), 1X, 1U, and 17U at a time at least 8 hours after caffeine intake, or in overnight urine; the ratio (AAMU + 1X + 1U)/17U is used as a caffeine metabolic ratio and thereby as an index of CYP1A2 activity.[9,11,64,65]

The numerator containing AFMU, 1X, and 1U represents all the metabolites produced by CYP1A2 from 17X by way of 7-demethylation. The denominator 17U has consistently shown the smallest person-to-person variation among all caffeine metabolites in urine if a defined dose of caffeine is given to a group of subjects (e.g., see Grant et al.[7]). Thus 17U in the equation serves to compensate for variations caused by variable caffeine intake or by incomplete urine collection.[11]

However, the 8-hydroxylation of 17X that yields 17U is a reaction catalyzed by at least two enzymes, of which CYP2A6 and CYP1A2 have been identified.[14] The contribution by CYP1A2 was somewhat less than that by CYP2A6, and a contribution by additional enzymes is likely and is being investigated. However, because 17U is used as the denominator, CYP1A2, which is the clear determinant of the numerator, is also a partial determinant of the denominator. Because of this, the range of variation of caffeine metabolic ratio must be smaller than the range of variation of CYP1A2 activity within the liver. That is, there must be a hyperbolic distortion of the true variability, particularly reducing the spread of high values. Such dampening of variances will not affect the comparative ordering of subjects by CYP1A2 activity. For example, although a caffeine metabolic ratio value above average indicates an above-average CYP1A2 activity, the caffeine metabolic ratio difference from the average reflects relative rather than absolute val-

ues. Because the nature and reasons for the distortion are now understood, analytic and mathematic corrections are being explored.

Denaro and Benowitz[6] recognized the caffeine metabolic ratio distortion on the basis of observations of Campbell et al.[9] They therefore compared caffeine clearance data with the caffeine metabolic ratio (and with some other infrequently used metabolite ratios of caffeine) using two caffeine doses: With a caffeine dose of 4.2 mg/kg/day, the correlation coefficient was $r = 0.85$ ($p = 0.009$); this dose means an intake of about 4 cups of coffee per day. With a caffeine dose of 12 mg/kg/day, the correlation was reduced to insignificance ($r = 0.5$; $p = 0.139$). For comparison, standardizations of the caffeine metabolic ratio against systemic caffeine clearance yielded $r = 0.91$[66] and $r = 0.82$ in a recent series in this laboratory.[28] The original standardization of the caffeine metabolic ratio test against the caffeine breath test gave a correlation coefficient in 41 adults of $r = 0.74$.[58,64]

In a recent, as-yet unpublished study of 50 subjects, the coefficient of variation of the caffeine metabolic ratio was 39.2%, with a 5.3-fold range of variation. Such ranges are not very robust values, and overinterpretation is undesirable. Nevertheless, here is certainly a much smaller variation than the twentyfold range of caffeine 3-demethylations observed in 19 kidney donor livers in this institution. However, data obtained with the breath test and with the caffeine metabolic ratio show an almost identical range of variation (Fig. 3); if this means that the findings of the caffeine breath test must be somewhat distorted, are data from breath tests with other drugs distorted? Furthermore, in the test on 50 subjects cited above, the coefficient of variation of caffeine clearance was 40.1%. In a list of the ranges of caffeine clearance values from nine different publications, the combined data did not vary by more than 8.7-fold.[6] Thus the relationship between CYP1A2 activity and caffeine clearance may also deserve scrutiny. The fact remains that the caffeine metabolic ratio, caffeine breath test, and systemic caffeine clearance all indicate person-to-person variation of similar magnitude.

Application of a standard dose of either 1 or 2 mg/kg caffeine optimizes the precision of caffeine metabolic ratio determinations. Nevertheless, for epidemiologic studies, we have in the past kept caffeine intake to between 1 and 4 cups of coffee on the test day. This is convenient for volunteer subjects, and the changes of apparent CYP1A2 activity within this dose range[11] are small compared with the commonly observed range of interindividual variation. Furthermore,

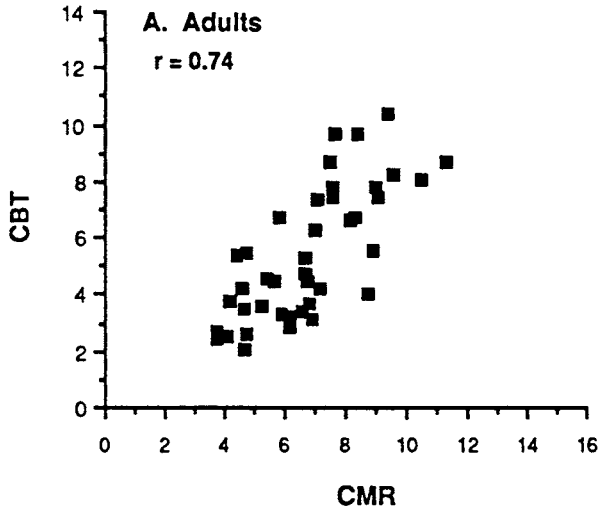

Fig. 3. Results of the caffeine breath test (CBT) and the caffeine metabolic ratio (CMR) in 41 healthy, nonsmoking adults who were not taking any medications. The caffeine metabolic ratio was determined in the form of (AFMU + 1X + 1U)/17U.[9] The caffeine breath tests were made by Dr. D. Schoeller and associates at the University of Chicago in conjunction with Dr. G. Lambert of Loyola University, Illinois. (From Campbell ME. PhD Thesis. Toronto: University of Toronto, 1987. Reprinted with permission.)

because the caffeine metabolic ratio uses only metabolic end products of caffeine metabolism, the timing of urine collection is relatively unimportant as long as there is a sufficient interval between caffeine intake and urine collection and as long as the caffeine intake is large enough for reliable measurements of the metabolites. Only the caffeine metabolic ratio test would allow a population study that completely relied on dietary caffeine intake (such as Vistisen et al.[67]).

The caffeine metabolic ratio correctly indicated increased activity of CYP1A2 in smokers,[65] in children,[66] and in subjects with a body burden of polybrominated biphenyls.[58] Decreased activity was present in pregnant women,[68] in women taking oral contraceptives,[66] and in subjects taking cimetidine.[69] Furthermore, the caffeine metabolic ratio test indicated a dose-effect relationship between number of cigarettes smoked, cotinine levels, and CYP1A2 activity.[65]

The paraxanthine/caffeine test. In urine voided between 4 and 5 hours after intake of a standard dose of caffeine (137X), the concentrations of 17X, 17U, and of caffeine are measured. The ratio (17X + 17U)/137X is used as an index of CYP1A2 activity.[10] (The ratio 17X/137X was used in earlier publications.[70,71]) A detailed account of the features of this

Table I. Caffeine and caffeine metabolites (mean ± SD) in urine of oriental and white subjects who were nonsmokers and rapid acetylators

	Oriental subjects (n = 7)	*White subjects (n = 8)*	*Oriental/white ratio*
137X	3.05 ± 1.77	1.46 ± 1.1*	2.1
17X	10.24 ± 1.70	7.33 ± 3.54	1.4
13X	1.01 ± 0.55	0.25 ± 0.21**	4.0
37X	3.02 ± 2.25	2.05 ± 1.95	1.5
Sum	14.27 ± 2.69	9.63 ± 4.52*	1.5
1X	15.04 ± 1.80	15.97 ± 3.53	0.9
3X	1.91 ± 0.67	1.97 ± 0.81	1.0
7X	8.96 ± 1.51	7.52 ± 1.74	0.9
Sum	25.91 ± 1.72	25.48 ± 3.13	1.0
17U	11.01 ± 1.30	10.82 ± 2.01	1.0
13U	2.04 ± 0.51	2.82 ± 1.17	0.7
1U	18.89 ± 1.64	21.60 ± 2.75	0.9
Sum	31.94 ± 1.57	35.29 ± 3.92	0.9
AFMU	24.83 ± 2.77	28.19 ± 5.69	0.9
CMR	5.42 ± 0.83	7.81 ± 1.33*	0.7

137X, 1,3,7,-Trimethylxanthine (caffeine); 17X, 1,7-dimethylxanthine; 13X, 1,3-dimethylxanthine; 37X, 3,7-dimethylxanthine; 1X, 1-methylxanthine; 3X, 3-methylxanthine; 7X, 7-methylxanthine; 17U, 1,7-dimethylurate; 13U, 1,3-dimethylurate; 1U, 1-methylurate; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; CMR, caffeine metabolic ratio.

Urine was collected 8 hours after intake of 2 mg/kg caffeine. The data represent percent molar fraction of the dose.

Statistical significance of the Oriental-white difference: $*p < 0.05$ $**p < 0.01$. Absence of an asterisk indicates lack of a significant difference.

CMR = (AFMU + 1X + 1U)/17U.

The selection of subjects is referred to in the text.

Note the apparently systematic ethnic difference between those methylxanthines that have in common a low renal clearance, that is, 137X (caffeine) and the three dimethylxanthines. The CMR difference has not been observed in an earlier investigation (Kalow and Tang[11]).

test and the rationale behind its creation have not yet been published, but the underlying idea is simple enough: To use the urinary ratio of primary metabolite versus parent drug as an index of formation of this metabolite. The literature contains many examples of the usefulness of such ratios. However, some special considerations apply in this case.

As explained above, 17X is both product and substrate of the target enzyme, CYP1A2. Thus the time of measurement is crucial because in this test the purpose is a measurement of 17X as product only. Therefore urine collection is restricted to the period between 4 and 5 hours after caffeine intake[10]; this choice of timing may be optimal but caution is advisable. For instance, 8 hours after caffeine intake, the urine of smokers contains on average almost 50% less 17X than the urine of nonsmokers, reflecting the rapid demethylation of 17X through induction of CYP1A2 (Tang BK. Unpublished observation, August 1992).

The renal excretion of caffeine is dependent on urine flow,[38] as is the excretion of dimethylxanthines like 17X. In the paraxanthine/caffeine ratio,[10] a urinary flow-dependent component is in both the numerator (17X) and in the denominator (caffeine); it is possible these flow-dependent factors cancel out, but neither flow dependence nor renal clearance are the same for both caffeine and 17X (Fig. 2). These factors may be immaterial if the urinary concentrations of both chemicals parallel their plasma concentrations; this has been shown for 137X but, to our knowledge, not for 17X. In any case, the renal transport of the xanthines requires further investigation because the renal clearance of dimethylxanthines seems to be subject to interethnic variation.[47,48]

Because of the potential importance of this factor for the interpretation of the data at hand, because of the implied suggestion of active tubular transport, and because pharmacogenetics of renal function represents a novel concept, we checked our existing data bank for pertinent information (Table I). The subjects were healthy volunteers (mostly students at the University of Toronto) recruited during the previous 18 months as control subjects for other projects. Subjects were those recorded as being rapid acetylators (Kalow[47]), nonsmokers, and as either Oriental or white. Because the percentage distribution of metabolites tends to be relatively uniform in a group with the same acetylation capacity and because there were only a few slow acetylators among the group of Oriental subjects, rapid acetylators were compared. It is evident that

there are ethnic differences that would be difficult to explain on the basis of metabolism.

The standard with which to compare the paraxanthine/caffeine ratio should be the partial clearance of caffeine by 17X formation as an index of CYP1A2 activity. Unfortunately, the test was standardized not against this partial metabolic clearance of caffeine by 3-demethylation but against a value that represents a division of this partial metabolic clearance by the renal clearance of caffeine.[10]

The data of Butler et al.[10] must reflect CYP1A2 activity at least in part, but the error components need to be defined. This is particularly important because some findings of the study are unparalleled and have major implications: They showed evidence of enzyme induction by smoking in some cases but not others (e.g., not in Chinese subjects), an apparently trimodal distribution of CYP1A2 activity in some populations, major differences between white subjects in Italy and the United States, and a different CYP1A2 activity in Chinese subjects than in white subjects that was seen neither with the caffeine metabolic ratio[11] nor with the caffeine breath test.[72] Butler et al.[10] emphasized that their paraxanthine/caffeine test showed much larger person-to-person differences than the caffeine metabolic ratio test. This is true, but it remains to be shown to what extent these observations indicate differences in CYP1A2 activity as claimed, and to what extent they are determined or modified by differences of renal function.

Determinations of NAT2

Grant et al.[73] described a test to determine the genetic acetylator status by testing in urine the ratio AFMU/1X within a few hours after drinking coffee. This test is convenient for the test subjects and for the analyst; it has been recently praised.[10] Misclassification by this test is sufficiently infrequent that Grant et al.[73] and other investigators[74,75] continue to use it. However, it must be realized that the concentration of 1X can be affected by xanthine oxidase activity, which may greatly vary under pathologic conditions.[11]

There is published criticism of this method.[76,77] To us, it was embarrassing when a colleague with particularly rapid acetylation capacity brought his family for testing by the AFMU/1X method, and one daughter registered as a slow acetylator—a genetic impossibility in that family. She registered as a fast acetylator when tested with AAMU instead of AFMU.

The AAMU method consists of exposing the voided urine to alkaline pH to convert all AFMU into AAMU, which is then measured by high performance exclusion chromatography. The ratio AAMU/(AAMU + 1X + 1U) was chosen as index of NAT2 activity[12,78] because its within-subject variability was least (less than 7% coefficient of variation) among potential ratios. Calibration of the method against tests with sulfamethazine and potential pitfalls of the testing procedure with AAMU have been described recently.[12]

Determinations of xanthine oxidase activity

The in vivo conversion of 1X to 1U by xanthine oxidase has been shown by the fact that this conversion is selectively inhibited by allopurinol.[30] The ratio 1U/1X or 1U/(1X + 1U) has therefore been used as a measure of xanthine oxidase activity.[79] A critical appraisal of the procedure and of its results has been recently published.[11]

There were some suggestions of discrepancies regarding age and sex differences, which had registered differently in measurements of xanthine oxidase activity in a set of surgical liver biopsy specimens measured in the Mayo Clinic.[80] Furthermore, CYP1A2—in addition to xanthine oxidase—has the capacity for 8-hydroxylation of 1X.[14] New studies are necessary to assess the relative importance and significance of this observation for the testing of xanthine oxidase activity with caffeine.

CONCLUSIONS

The pharmacologic and biochemical properties of caffeine make it a probe capable of revealing activities in humans of the polymorphic *N*-acetyltransferase (NAT2), of xanthine oxidase, and of the P450 cytochromes CYP1A2 and, after further investigation, probably of CYP2E1.

There are several different procedures available for assays of NAT2; the technically simplest procedure, with an error deemed to be acceptable when investigating healthy subjects, is to measure the ratio AFMU/1X in urine. In borderline cases or in the presence of disease, wrong classification will be avoided by use of AAMU/(AAMU + 1X + 1U).

There are three principally different methods in use for assays of CYP1A2. The caffeine breath test uses a ^{13}C-label of the methyl group at the *N*-3 position of caffeine to measure the exhalation of labeled carbon dioxide for 2 hours after intake. A third to a half of the ingested label is lost in transit through the one-carbon pool, and repeat measurements in the same subjects seem to be missing. Nevertheless, the test has excellent credentials for clinical observations. It is expensive for epidemiologic studies.

The caffeine metabolic ratio measures in 8-hour or overnight urine the ratio (AAMU + 1X + 1U)/17U. This is essentially an assessment of the main pathway

of caffeine metabolism with paraxanthine (17X), which is both formed and metabolized by CYP1A2. Because CYP1A2 activity determines the numerator and is a component of the denominator of this equation, caffeine metabolic ratio represents a reduced (dampened) picture of the true magnitude of CYP1A2 variation without affecting the comparative ordering of values. This test has credentials for clinical and epidemiologic observations that are similar to the caffeine breath test. For epidemiologic purposes, the caffeine metabolic ratio test could be operated without strict standardization of the caffeine dose and without narrow control of the time of urine sampling.

The paraxanthine/caffeine ratio determines the ratio (17X + 17U)/137X in urine that is collected between the fourth and fifth hour after intake of a standard dose of caffeine. This test aims to assess 17X formation without interference by 17X disappearance; it still needs to be shown that the substantial urinary flow dependence and tubular reabsorption of both 17X and 137X (caffeine) are canceled out by the ratio and that the apparent interethnic differences in the tubular transport of these substances are not affecting the ratio. This is the newest test with presentation of a number of important observations; however, these observations require confirmation.

Because all the tests have potential shortcomings, a comparison with a generally accepted standard will be useful. Most informative will be direct comparisons between the functional CYP1A2 tests in human beings in vivo and the enzyme in liver biopsy specimens. If this standard is unattainable, the partial metabolic clearance of caffeine by way of 3-demethylation should be an acceptable substitute. Systemic caffeine clearance may serve as a standard of convenience because CYP1A2 accounts for more than 95% of the primary caffeine metabolism in the great majority of subjects.

Because at least 11 caffeine metabolites in urine are analytically accessible, the development of new tests on the basis of additional metabolite determinations deserves consideration.

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