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Caffeine as a metabolic probe: a comparison of the metabolic ratios used to assess CYP1A2 activity

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1 Caffeine is widely used as an *in vivo* probe for CYP1A2; the distribution/activity of this enzyme is reported to be reflected by metabolic ratios.
2 Several metabolic ratios using different combinations of urinary metabolites have been used to measure CYP1A2, with varying conclusions on its distribution.
3 A mathematical comparison of five metabolic ratios claiming to reflect CYP1A2 activity was made using data from 237 healthy volunteers.
4 All five metabolic ratios were symmetrically distributed. The five ratios however, measured at least three different parameters, with no one ratio correlating exactly with any other.
5 Data in the literature claiming to measure CYP1A2 using caffeine may reflect other parameters.
6 The complex metabolism of caffeine together with different parameters controlling the renal clearance of each metabolite, makes the use of urinary metabolic ratios an inaccurate probe for assessing the distribution of CYP1A2 activity in populations.

Keywords caffeine metabolic probe cytochrome P4501A2

Introduction

Few drugs utilise CYP1A2 in their metabolic pathways since this enzyme commonly gives rise to reactive intermediates which may be toxic. Caffeine is unusual in that its major routes of metabolism involve demethylation catalysed by CYP1A2 (Figure 1). Owing to its ubiquitous use caffeine has become popular as an *in vivo* probe for phenotyping CYP1A2 activity. To date five different metabolic ratios using various combinations of urinary metabolites have been described, all claiming to reflect CYP1A2 activity. If these ratios are shown to be quantitatively different, any conclusions may depend on the choice of ratio.

The use of metabolic ratios to assess the distribution of the activity of an hepatic enzyme stems from the work of Evans *et al.* [1] who used the ratio of plasma isoniazid to its acetylated metabolite as an indicator of acetylator status. More recently, Maghoub *et al.* [2] used a similar technique where the distribution of CYP2D6 activity was defined using the ratio of debrisoquine to its major metabolite, 4-OH debrisoquine.

Metabolic ratios are acceptable indicators of the activity of an enzyme provided that further metabolism of the product(s) can be accounted for, and renal clearances for the substrate and metabolites utilised have the same dependency on the various factors affecting their renal clearances. The employment of various metabolic ratios for CYP1A2 using caffeine reflects the complexity of the metabolism of the probe drug. Not only is it subject to multiple competing pathways (see below), but the primary metabolites are both products from, and substrates for, the CYP1A2 enzyme under investigation, giving scope for variations in the selection of particular pathways to be used. Additionally, differences in polarity and hence renal excretion of the resulting metabolites is likely, thus introducing an element of error.

Caffeine (137TMX) undergoes *N*-demethylation at three sites forming paraxanthine (17DMX; 84%), theophylline (13DMX: 4%) and theobromine (37DMX; 12%). These reactions have been shown to be catalyzed almost entirely by CYP1A2 [4]. The major fraction of paraxanthine demethylates further

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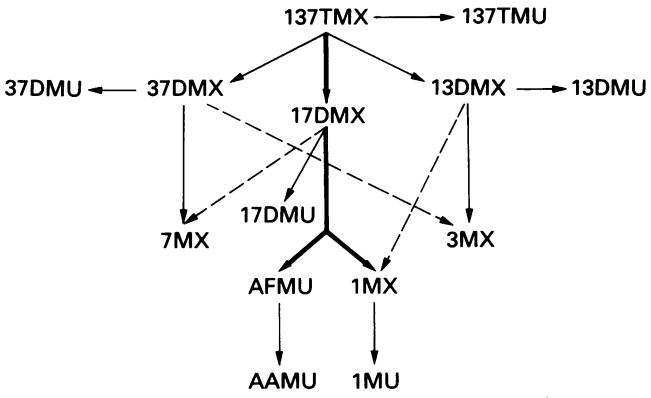

Figure 1 Metabolic pathways of caffeine.

to a short-lived intermediate that is subsequently stabilised either as 5-acetylamino-6-formylamino-3-methyluracil (AFMU) [5, 6], or 1-methylxanthine (1MX) and its hydroxylation product 1-methyluric acid (1MU) [5]. This demethylation is also catalysed by CYP1A2 while the step towards AFMU is catalysed by polymorphic *N*-acetyltransferase [NAT2] [5]. The 8-hydroxylation of 1MX to 1MU is catalysed largely by xanthine oxidase although CYP1A2 makes a contribution [7]. About 10% of paraxanthine is excreted unchanged and 10–20% is hydroxylated to 17-dimethyluric acid (17DMU). This step is thought to be mediated largely by CYP2A6 but with a significant contribution of CYP1A2 [5, 6].

This paper compares five metabolic ratios utilising various combinations of caffeine urinary metabolites to assess CYP1A2 activity by mathematically comparing the derived distributions using a single large data set.

Methods

Spot urine samples from 237 healthy volunteers, 121 female, aged between 17 and 90 years (41.7 ± 16.6; mean ± s.d.) were collected. A spot urine sample was collected a minimum of 2 h and a maximum of 6 h after ingesting a cup of tea or coffee (no restriction on strength). Pilot studies indicated that the relative proportions of the metabolites did not vary between overnight, 0–8 h and the above spot urine collections. The urine was stored at −20°C prior to analysis which was performed without extraction by reverse phase h.p.l.c. [8]. The method measures all the metabolites used in the metabolic ratios under investigation.

The molar equivalents of the metabolites were used to calculate the following metabolic ratios which have been used to assess CYP1A2 activity:

(AAMU* + 1MU + 1MX)/17DMU
metabolic ratio 1 [9–11]

(AAMU + 1MU + 1MX + 17DMX)/137TMX
metabolic ratio 2 [13]

(17DMU + 17DMX)/B7TMX
metabolic ratio 3 [13]

(17DMX/137TMX) metabolic ratio 4 [14]

(AAMU* + 1MU + 1MX)/17DMX
metabolic ratio 5 [15, 16]

(*AAMU or AFMU)

Five sets of 'distribution' characteristics were derived for CYP1A2 and statistical analysis was performed to examine their relationship to one another.

Statistical analysis

The distributions obtained with the same data set using five different metabolic ratios were investigated using measures of skewness and kurtosis [17], probability plots and the Shapiro-Wilk statistic [18] to test for normality. Additionally, density estimates [19] of the data were used to check conclusions.

In order to assess to what extent the different variables could be considered as measuring the same quantity, the relationships between them were investigated. The original variables were all transformed to approximate symmetry by taking logarithms (and the square root if appropriate). Scatter plots were then made of every pair using the statistical package 'S' [20] and correlations calculated. In order to give a measure of association unaffected by the choice of monotonic transformation, rank correlations were also calculated.

Results

The mean values and ranges for the individual ratios observed in our population are given in Table 1, and were of similar orders of magnitude to those quoted in the literature [4, 10].

The distribution of each metabolic ratio was examined in turn. The data arising from ratios 2, 3 and 4

Table 1 Summary characteristics of CYP1A2 metabolic ratios

Metabolic ratio	*Mean*	*s.d.*	*min*	*max*
1	12.19	18.20	1.43	180.53
2	103.70	92.09	8.06	640.37
3	21.42	30.96	2.25	199.14
4	8.65	8.09	1.13	69.97
5	12.78	10.46	1.07	110.42

Literature values

1	4.7	1.6 (male non-smokers) [11]
1	4.8	2.5 (male smokers) [11]
1	5.96	0.18 (sem) [10]
3	3.02 (Italian) [4]	
3	5.37 (Chinese) [4]	
3	9.37 (Caucasian) [4]	

were shown to be well modelled by a lognormal distribution. The logarithms of the data from ratio 5 were well fitted by a symmetrical distribution, but had somewhat more extreme tails than a normal distribution would produce. Ratio 1 resulted in a significantly skewed distribution although the square root of the log distribution approximated to normality. The statistics for the various distributions are shown in Table 2.

The scatterplots comparing each ratio with the other four are given in Figure 2, with the correlations and rank correlations listed in Table 3. Ratios 2, 3 and 4 yield reasonably correlated results although even ratios 3 and 4 do not have perfect correlation and can only be measuring the same underlying quantity subject to considerable error. Ratios 1 and 5 are clearly measuring a different entity to ratios 3 and 4.

Table 2 Summary characteristics of each distribution

MR	*Quantity*	*Sample skewness*	*Sample kurtosis*	*Shapiro-Wilk statistic*
1	$\log X_1$	0.75**	0.30**	0.9846***
1	$\surd\log X_1$	0.04	0.49	0.9974
2	$\log X_2$	−0.04	−0.08	0.9980
3	$\log X_3$	0.23	0.13	0.9967
4	$\log X_4$	0.26	0.18	0.9961
5	$\log X_5$	0.18	1.19**	0.9923*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ under the null hypothesis of a normal distribution; MR = metabolic ratio.

Table 3 Correlation of transformed variables between metabolic ratios

Variable	1	2	3	4	5
1		0.28	−0.36	−0.05	0.47
2	0.24		0.76	0.74	0.47
3	−0.04	0.73		0.88	−0.05
4	−0.09	0.73	0.85		−0.24
5	0.48	0.44	−0.06	−0.22	

The values above the diagonal are the correlations between the transformed variables ($\surd\log X_1$, $\log X_{2-5}$) where the subscript relates to the metabolic ratio in question. The values below the diagonal are the rank correlations between the variables.

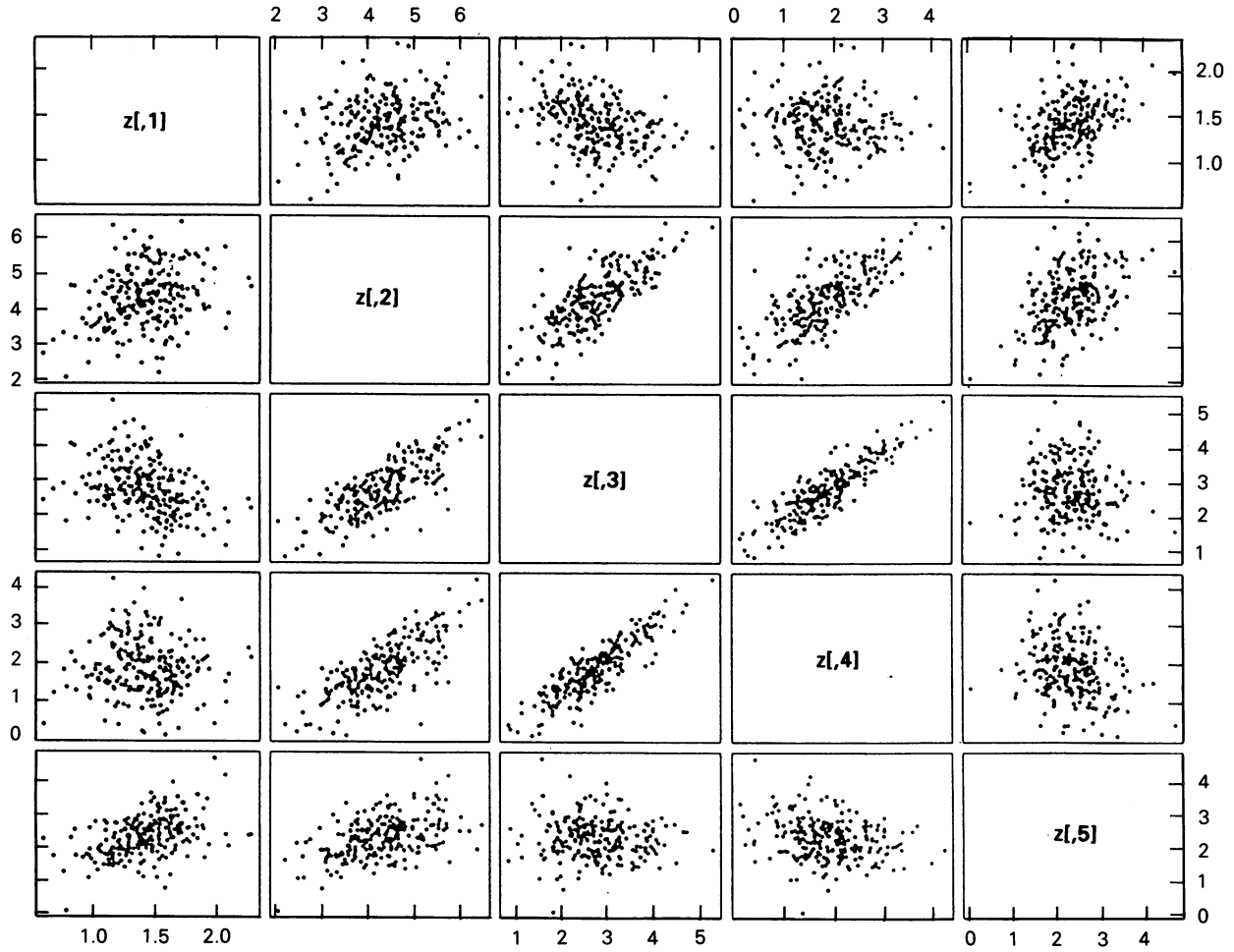

Figure 2 Matrix of scatterplots of the transformed variables. Note the relatively good linear relationship between variables 2, 3 and 4, and the lack of relationships between variables 1 and 5 and the other variables.

Discussion

Five caffeine metabolic ratios all claiming to reflect CYP1A2 activity have been compared. They differ in the metabolites used in the ratios, although all are centred on the demethylation reactions. Ratios 3 and 4 are based on the first (3) demethylation step, ratios 1 and 5 on the second (7) demethylation, while ratio 2 utilises both such reactions. Metabolic ratio 5 uses the major pathway although neglects 17DMU, and caffeine (Figure 1). Ratios 3 and 4 use 'major metabolite(s):caffeine' although these ratios have been criticised because 17DMX is also a substrate for the enzyme, and variable renal excretion between the compounds is likely [5, 10]. Ratio 2 incorporates in its products arising from CYP1A2, substrates which continue to use CYP1A2, thereby putting enzyme activity in both the denominator and numerator. Ratios 1 and 5 use the second part of the major pathway 3 and 4 the first part of the pathway. The results obtained for each metabolic ratio are described below.

Metabolic ratio 1 was first suggested as a CYP1A2 index in 1987 [21] and uses the ratio of all the products from the paraxanthine demethylation to the 8-hydroxylation product of paraxanthine – 17DMU. 17DMU has been reported to have the lowest interperson variation of any metabolite and its use in the ratio was claimed to compensate for variations caused by variable caffeine intake or by incomplete urine collections [5]. This ratio gave the best correlation with caffeine clearance [21] and the results obtained are similar to the caffeine breath test [5]. This index of CYP1A2 has been reported to be lognormal [10, 11] and hence is likely to be under environmental control.

Ratio 2 utilises all paraxanthine and its demethylation products to caffeine [12], making it conform closely to the theoretical ratio of drug:metabolite(s) involved in the major pathway since no selection of metabolites is made.

Ratio 3 was first utilised by Butler in 1992 [13] who compared the correlation of rate constants for 3-demethylation in blood with various urinary metabolite ratios. Of the metabolic ratios tested this metabolic ratio had the best fit ($r = 0.73$). The fit for ratio 1 with their data set using AFMU and AAMU had correlation coefficients of 0.33 and 0.34, respectively. They found that the time of urine collection played an important role in their results largely because 17DMX is both the product and the substrate for the enzyme under study and the aim is to measure the product only.

Ratio 3 required further investigation to determine the extent of its dependence on differences in renal function. Urinary flow dependence and renal clearance for both caffeine and 17DMX are not the same, and urinary concentration of 17DMX does not always parallel its plasma concentration [5]. Additionally, renal transport seems to be subject to inter-ethnic variation [5]. Butler *et al.* [13] reported that this ratio when applied to three different populations strongly indicated a trimodal distribution.

Ratio 4 has been used by various authors [13, 14] and does not involve 17DMU, utilising only the first metabolic step – the ratio of 17DMX to caffeine. The correlation coefficient between urinary metabolite ratios and plasma 3-demethylation coefficient was 0.54 [13]. The distribution for CYP1A2 using ratio 4 has been described as bimodal with 77% of the population being described as slow metabolisers [14].

The fifth ratio was first quoted in 1983 [15] as a measure of P448-dependent activity and subsequently by Relling and co-workers [16]. The use of 17DMX has been criticised because of variability in its renal elimination and its presence as a minor caffeine metabolite [10]. It has been suggested that there is a possible effect on differential renal excretion of 17DMX between races which accounts for the variation in the CYP1A2 activity between Caucasians and Orientals [10]. No racial differences however have been observed in individual metabolite concentration, which suggests that the difference is not caused by altered renal excretion or urine stability [16]. This is also supported by the fact that no difference has been observed in total excretion of all four measured caffeine metabolites [16]. The frequency distribution of the log of this ratio has been reported to be skewed although the double log showed a more symmetrical distribution.

The present comparison of metabolic ratios which have been reported to measure CYP1A2 activity has been made using a large ($n = 237$) data set. Of the five metabolic ratios used, 2, 3 and 4 were fitted by a lognormal distribution. The data did not display any significant signs of bi- or tri- modality with ratios 1 and 5. This suggests that, assuming one of these caffeine ratios accurately reflects CYP1A2 activity, the enzyme is likely to be predominantly under environmental control.

The correlations between the five ratios indicate clearly that at least three different 'activities' are being measured in the name of CYP1A2. Ratios 2, 3 and 4 are likely to be measuring a similar entity, although without perfect correlation. Ratios 1 and 5 are measuring a different parameter. The literature therefore contains values for CYP1A2 which are inaccurate since three different parameters are being measured, and in the three exhibiting a degree of correlation, extrapolation from one ratio to another is not possible.

Assuming that caffeine can reflect CYP1A2 activity, the choice of metabolic ratio is crucial to the conclusions drawn concerning the distribution of the enzyme. A normal distribution as found by several authors [10, 11, 16] would suggest that the enzyme is under environmental control whereas a non-normal pattern [13] would indicate either genetic control with implications of inherited risk to chemicals activiated by this enzyme, or variable environmental influences within the population.

In contrast to the distribution of the enzyme within populations, urinary metabolic ratios may validly reflect intra-subject variation. Metabolic ratios 1 and 4 have been found to correlate with caffeine plasma clearance and the [^{13}C]-caffeine breath test after

CYP1A2 induction by omeprazole [22]. The decrease in caffeine metabolism following oral contraceptive use is reflected by metabolic ratio 1 but not metabolic ratio 3 [23]. It may be that metabolic ratios are suitable for observing intra-variation changes in CYP1A2 activity rather than its distribution within populations.

Many papers have implied the suitability of caffeine as a probe for CYP1A2. This work indicates that further data on physiological factors controlling clearance of caffeine metabolites arising from CYP1A2 pathways are required. The complex metabolism of caffeine, together with different parameters controlling the renal clearance of each metabolite, make urinary metabolic ratios an inaccurate probe for assessing the distribution of CYP1A2 within populations.

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