NAT2 and CYP1A2 phenotyping with caffeine: head-to-head comparison of AFMU vs. AAMU in the urine metabolite ratios

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Aims (i) To compare the phenotyping of healthy subjects for NAT2 and CYP1A2 activities with caffeine, by the simultaneous assay of the urinary metabolites AFMU and AAMU, and (ii) to ascertain whether NAT2 and CYP1A2 phenotyping is influenced by the use of AFMU or AAMU in the metabolite ratio.

Methods Thirty-five healthy subjects (16 men, 19 women) participated to the study. Caffeine metabolite concentrations were measured in urine collected 8 h after 2.5 mg kg^{-1} caffeine intake using a new validated h.p.l.c. method. The metabolite ratios AFMU/1X, AFMU/(AFMU+1X+1U), AAMU/1X, AAMU/(AAMU+1X+1 U), and (AFMU+1U+1X)/17U, (AAMU+1U+1X)/17U were determined as indices of NAT2 and CYP1A2 activity, respectively.

Results Slow and rapid acetylators were similarly identified using the four NAT2 metabolite ratios in 139 out of 140 measurements. An appreciable amount of AAMU was present in urine that was immediately acidified and analysed. Consequently, the ratio using AFMU was lower than that using total AAMU following transformation of AFMU in basic conditions. The proportion of AFMU in urine analysed immediately expressed as AFMU/(AFMU+AAMU) ratio did not correlate with urine pH, but was a function of the acetylation phenotype, with a low intergroup variability ($64 \pm 3\%$ and $32 \pm 5\%$, for rapid and slow acetylators, respectively; $P < 0.00001$, ANOVA). Regarding CYP1A2 activity, a good correlation ($r = 0.99$) was observed between the metabolite ratios calculated from AFMU and AAMU, although the ratios calculated from AFMU were proportionately and systematically lower $P < 0.00001$, paired t-test, slope 1.2).

Conclusions This study demonstrates that both AFMU and AAMU can be used for NAT2 and CYP1A2 metabolite ratio determinations. The reported conversion of AFMU into AAMU is unlikely to explain the large amount of AAMU in urine that was acidified and analysed immediately after voiding. The results suggest that AAMU is formed not solely through a nonenzymatic hydrolysis in urine, but *in vivo* by a NAT2 phenotype-dependent pathway.

Keywords: AAMU, AFMU, caffeine, CYP1A2, NAT2, phenotyping

Introduction

Urinary caffeine metabolite ratios are used in humans to assess the activity of cytochrome P450 1A2 (CYP1A2), xanthine oxidase and N-acetyltransferase 2 (NAT2), which are involved in the activation or detoxification of various xenobiotic compounds, including carcinogens [1, 2]. Investigating the activity of these enzymes is of clinical relevance for assessing intra- and inter-individual differences in NAT2- and CYP1A2-mediated drug metabolism, and for evaluating the risk of developing specific exposure-related diseases.

In phenotyping studies, the enzymatic activities of NAT2 and CYP1A2 are usually expressed as the urinary molar ratio of the caffeine metabolites 5-acetylamino-6-formylamino-3-methyluracil (AFMU), 1-methyluric acid (1U), 1-methylxanthine (1X), 1,7-dimethyluric acid

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(17U). AFMU/1X and AFMU/(AFMU+1U+1X) ratios are used for phenotyping NAT2 status [3-6] and (AFMU+1U+1X)/17U ratio for assessing CYP1A2 activity [5-7], although the validity of the latter ratio has been questioned [8]. As AFMU has been reported to degrade spontaneously to 5-acetylamino-6-amino-3-methyluracil (AAMU), other metabolite ratios have been proposed, notably using AAMU after complete conversion of AFMU under basic conditions [9]. However, AFMU is still widely used for determining CYP1A2 and NAT2 activities [10–14]. The influence on phenotyping results of the presumed decomposition of AFMU in urine has not been formally studied.

A few studies have assessed AFMU stability in various matrices. Lorenzo *et al.* [15] studied the extent of AFMU decomposition at different pH in aqueous buffer solutions at 37°C. They questioned the use of AFMU because of the limited stability they found. Thus, at pH 5 and 8, only 71% and 55%, respectively, of AFMU remained after 4 h at 37°C. The authors recommended caution in the use of the AFMU/1X ratio, suggesting that AFMU decomposition may lead in some cases to misclassification of rapid acetylators as slow acetylators. In contrast, Butler *et al.* [16] indicated that the AFMU/1X molar ratio is a valid index, even in alkaline urine. In a recent study, Wong *et al.* [17] have also reported good stability of AFMU in urine and found that no more than 18–20% of the metabolites is deformylated after 5 h at 37°C.

A similar bimodal distribution for NAT2 has been observed with AFMU and AAMU metabolite ratios, although there was a large numerical difference in the antimode values [4, 18–21], a discrepancy that has not yet been explained.

The few analytical methods reporting the simultaneous measurement of AFMU and AAMU [22, 23] have not been applied to clinical or epidemiological phenotyping studies. The objective of our study was to compare the phenotyping of healthy subjects for NAT2 and CYP1A2 activities using caffeine, and a new analytical method [24] enabling the simultaneous assay of the urinary metabolites AFMU and AAMU, together with 1X, 1U and 17U. A second aim was to ascertain whether phenotyping is influenced by the possible hydrolysis of AFMU into AAMU.

Methods

The study was approved by the Ethics Committee of the University Hospital of Lausanne. Thirty-five healthy subjects (16 men and 19 women) aged between 19 and 52 years participated. All volunteers received detailed oral and written explanations concerning the aims of the study and its implications, and gave written informed consent.

Subjects were instructed not to drink any caffeine-containing beverages from 60 h prior to the beginning of the study until its completion. A blank urine sample was collected on the morning of the study day to test for compliance (absence of caffeine and/or its metabolites). Subjects then drank one cup of instant coffee prepared by the investigator and containing 2.5 mg kg^{-1} bodyweight of caffeine. Urine samples were collected 8 h after caffeine intake, and were analysed immediately by an h.p.l.c. method using a polyamine stationary phase [22]. The limit of determination was 10 μM for all metabolites. The intra- and interassay coefficients of variation for all metabolites were <4.2% and 9.4%, respectively. Accuracy (bias estimated as the deviation from nominal values of spiked samples, expressed in percentage) was within ±11.1%.

Two sample preparation procedures were performed in parallel. The molar concentrations of AFMU, AAMU, 1U, 1X and 17U were measured in one acidified urine aliquot, whereas AAMU, 1U, 1X and 17U were quantified in a second aliquot after complete conversion of AFMU into AAMU under basic conditions. Details of sample preparation are published elsewhere [24].

Additional AFMU stability studies were carried out *in vitro*. Solutions of AFMU (60 μM) were prepared in urine collected from three healthy volunteers following a 60-h xanthine-free diet. The pHs of the urine samples were between 5.7 and 6.6. Spiked urine samples were incubated at 37°C. Aliquots were taken after 0, 1, 2, 4 and 6 h of incubation and AFMU concentration measured as above [24]. The remaining amount of AFMU was expressed as percentage of the initial concentration.

For NAT2 phenotyping, the following four metabolite ratios were calculated: AFMU/1X, AFMU/(AFMU+1U+1X), AAMU/1X and AAMU/(AAMU+1U+1X). For CYP1A2 phenotyping, the following two metabolite ratios were calculated: (AAMU+1U+1X)/17U and (AFMU+1U+1X)/17U.

Statistical analysis

The distribution of measured metabolite ratios was described using histograms complemented with normal probit plots. It was then fitted with a mixture of two Gaussians having a joint probability density of $\pi \cdot N(\mu_1,\sigma_1) + (1-\pi) \cdot N(\mu_2,\sigma_2)$. Maximum likelihood estimates of the parameters were calculated using the Solver module of the Excel software (version 2000; Microsoft). This was compared with a single Gaussian $N(\mu,\sigma)$ using the likelihood ratio test. The antimode of the mixture model was retained as the cut-off value for the classification of cases. χ^2 tests, linear correlations, *t*-tests and analyses of variance ANOVA were performed using Statistix 7.0 (Analytical Software, Tallahassee, FL, USA).

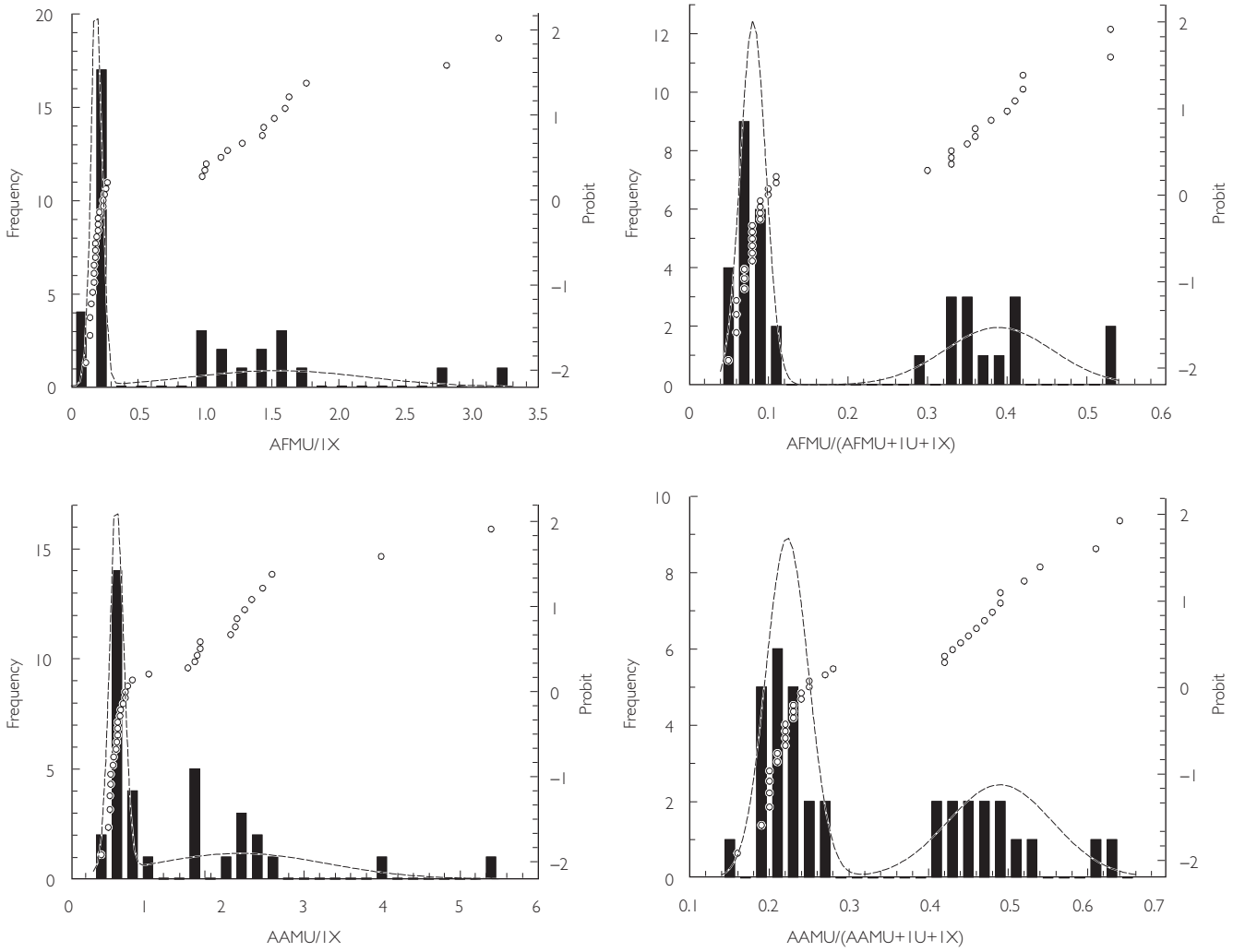

Figure 1 Frequency distribution and probit plots of four NAT2 metabolite ratios ($n = 35$ subjects).

Results

NAT2 phenotyping

The frequency distribution histograms of the four caffeine metabolite ratios are shown in Figure 1. All four NAT2 metabolite ratios displayed a clear bimodal distribution ($P < 0.0001$). The antimode of each ratio, taken as the lowest point of a mixture distribution fitted to the observations, was 0.36, 0.15, 0.94 and 0.32, for AFMU/1X, AFMU/(AFMU+1U+1X), AAMU/1X and AAMU/(AAMU+1U+1X), respectively. The ratios AFMU/1X, AFMU/(AFMU+1U+1X) and AAMU/(AAMU+1U+1X) were concordant in the identification of 21 subjects (60%) as slow acetylators. The cut-off for the AAMU/1X ratio misclassified as rapid acetylator one subject having the highest value in the slow acetylator cluster. However, any cut-off value between 1.1 and 1.5 would have classified this subject as slow acetylator. The AFMU/1X ratio was proportional but systematically lower than that of AAMU/1X (slope 0.71, $P < 0.0001$, $r^2 = 0.97$), as was the ratio of AFMU/(AFMU+1U+1X) compared with that of AAMU/(AAMU+1U+1X) (slope 0.87, $P < 0.0001$, $r^2 = 0.98$).

AFMU hydrolysis in urine samples

A comparison of rapid vs. slow acetylator subjects indicates that rapid acetylators have, as expected, higher total concentrations of AFMU +AAMU in their urine, but with a large variability in both groups (197.0 ± 118 μM vs. 95 ± 80 μM, respectively; $P = 0.01$).

The molar ratio AFMU/(AFMU+AAMU) indicates that AFMU accounts for 21–70% of the total uracil species (median 37%), showing that an appreciable amount of AAMU is already present in urine immediately after voiding.

Figure 2 shows the AFMU/(AFMU+AAMU) ratio plotted as a function of native urine pH. There was no

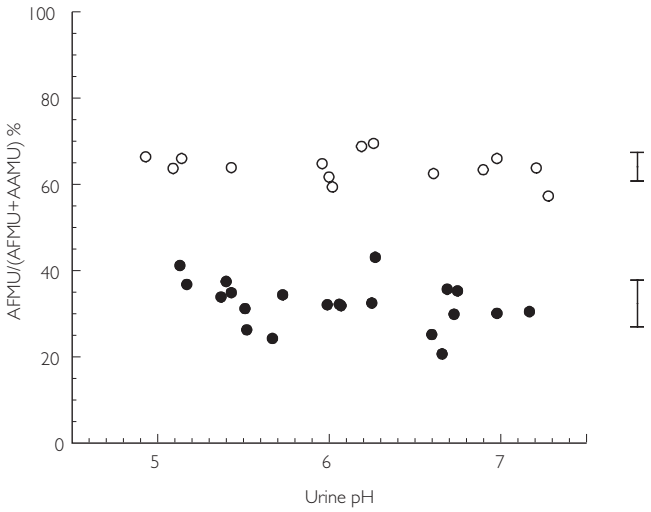

Figure 2 AFMU as a percentage of total AAMU and AFMU present in urine samples, in relation to urine pH. ○, Rapid acetylators; ●, slow acetylators. The error bars represent s.d.

relationship ($r = 0.023$, $P = 0.90$) between urine pH and the extent of AFMU decomposition. The AFMU/(AFMU+AAMU) ratios appeared as two clusters with mean values around 30% and 60%. These two clusters correspond to NAT2 phenotype (Figure 2), with the AFMU percentage being less in slow (32 ± 5%) than in rapid acetylators (64 ± 3%) ($P < 0.00001$, ANOVA) (29, 35%, 95% CI on the difference).

In vitro *stability of AFMU in urine samples*

After a 4-h and 6-h *in vitro* incubation at +37°C 86–90% and 77–85% of AFMU, respectively, remained in urine.

CYP1A2 phenotyping

There was a good correlation ($r = 0.99$, $P < 0.0001$) between the two ratios (AAMU+1U+1X)/17U and (AFMU+1U+1X)/17U, although the (AFMU+1U+1X)/17U ratio was consistently lower ($P < 0.00001$, paired t-test) (slope of the regression line 1.2).

Discussion

A recently developed analytical method [22] made it possible to quantify simultaneously AFMU and AAMU in urine, enabling the study of the AFMU conversion into AAMU both *in vitro* and *in vivo*.

We found that AFMU is relatively stable in urine *in vitro*, with 86–90% still present after 4 h at 37°C. Its rate of decomposition in urine is slower than previously reported by Lorenzo *et al*. in aqueous solution [15], but in good agreement with a recently published study [17]. These data contrast with our study where AFMU corresponds to only 21–70% of the sum of AFMU + AAMU *in vivo*. We also found that *in vivo* the extent of AFMU hydrolysis did not correlate with urine pH (within the 4.9–7.3 pH range). AFMU corresponds to only 21–70% of the sum of AFMU + AAMU *in vivo*.

On the other hand, AAMU was already present in appreciable amounts in urine and immediate acidification and analysis could not prevent its formation. Furthermore, we discovered that the AFMU/(AFMU+AAMU) ratio is a function of acetylation phenotype, being significantly higher in rapid acetylator individuals.

It may be argued that the urine collected after 8 h postdose could be associated with 0.5–8 h accumulation, depending on the last time urine was voided. The time that urine stayed in the bladder was not strictly controlled in our and previous studies. However, the uncertainty in the time that AFMU stayed in bladder cannot account for the highly significant difference observed between slow and rapid acetylators, given the very small within-group variability (less than 6%), in the AFMU/(AFMU+AAMU) ratio. This observation is an additional argument indicating that neither the residence time nor urine pH are likely to explain the presence of such high concentrations of AAMU in urine, especially in slow acetylator subjects.

Taken together, these observations question whether AAMU in urine is formed solely from AFMU through a nonenzymatic hydrolysis. If AFMU was deformylated only via a nonenzymatic step in the bladder, the percentage of AFMU should be similar at equilibrium in both groups. Alternately, if the equilibrium was not attained at the time of analysis, a higher or similar degree hydrolysis, and hence AAMU concentration, should be observed in the samples containing high concentrations of AFMU, depending on whether or not the hydrolysis rate is concentration-dependent.

As more AFMU is formed in a given period of time in rapid acetylator subjects, correspondingly more AAMU should be found in this group, if the subsequent deformylation reaction was solely a nonenzymatic hydrolysis. However, our findings indicate that this is not the case and that comparatively more AAMU was found in slow acetylator subjects. Other mechanisms should therefore be considered.

The fact that both acidification and immediate analysis of the urine samples could not prevent the formation of AAMU prompted us to evaluate whether this phenomenon could influence to a significant extent the AFMU/1X, AFMU/(AFMU+1U+1X) ratios and the (AFMU+1U+1X)/17U ratio widely used for NAT2 and CYP1A2 phenotyping, respectively.

We confirmed that 139 out of 140 acetylation phenotype assignments were concordant whatever the metabolite ratio used (AFMU, or AAMU after complete transformation of AFMU in basic condition). The overall shapes of the frequency distribution histograms and antimode values calculated from the mixture model compare well with that previously reported in the literature, mostly determined by visual inspection of the ratio distributions [4, 18–21]. The single subject in our study who was incorrectly phenotyped, based on our calculated antimode value for AAMU/1X, would have been assigned to the correct phenotype using previously published cut-off values of 1.23 and 1.4 [20, 21]. More generally, the previously published antimodes correspond well with those determined by visual examination of our frequency-distribution histograms.

The numerical difference between the ratios incorporating AFMU or AAMU is in line with previously published studies [4, 18–21] and reflects the presence of AAMU in urine even after immediate acidification and analysis. Smaller ratios are therefore expected when AFMU alone is measured than when AAMU is determined after conversion of AFMU. The somewhat better segregation observed between the slow and rapid acetylator groups using AFMU is a consequence of the lower proportion of AFMU found in the urine of slow acetylators.

Our results also indicate that both the AFMU/1X and AAMU/1X ratios can be used for NAT2 phenotyping of a healthy population, as the interindividual variation in the xanthine oxidase activity is small (data not shown). However, the use of AFMU/(AFMU+1U+1X) (reportedly the best marker for NAT2 activity [6]) and AAMU/(AAMU+1U+1X) ratios is to be preferred in patients taking xanthine oxidase inhibitors, as these ratios are not affected by the activity of this enzyme [25].

The comparison of CYP1A2 metabolite ratios showed lower values with the (AFMU+1U+1X)/17U ratio than with the (AAMU+1U+1X)/17U ratio. However, as the correlation between the two ratios is good, and because relative rather than absolute values are of interest for CYP1A2 activity assessment, the use of AFMU, measured after immediate analysis of acidified samples for metabolite ratio determination, does not influence CYP1A2 phenotyping. (AFMU+1U+1X)/17U is not only the most sensitive to changes in CYP1A2 activity, but not influenced by the renal clearances of caffeine and paraxanthine, which have been reported to be affected by urine flow [5, 6].

In general, it is important that all products downstream of a particular pathway be measured and summed for the estimation of metabolite ratios. Therefore, conversion of AFMU to AAMU and using total AAMU as an indicator of all downstream products of NAT2 may be advantageous [5, 6]. This study shows that, although the assessment of NAT2 and CYP1A2 activities using AAMU together with all downstream products appears to be more robust, the use of AFMU can also be considered valid. However, in this case urine samples should be acidified and analysed immediately to minimize any hydrolysis of AFMU, although this is unlikely to explain by itself the presence of large amounts of AAMU in urine.

The latter finding suggests that AAMU may not be formed solely through a nonenzymatic hydrolysis of AFMU as previously thought, but that a NAT2 phenotype-dependent pathway may contribute, directly or indirectly, to its formation *in vivo*.

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