Potential artifacts in the use of caffeine to determine acetylation phenotype

The polymorphic drug acetylation phenotype has been determined by measuring the metabolism of several drugs. Grant *et al.* (1984a) have suggested that acetylator phenotype can be determined by measuring the ratio of the caffeine metabolites 5-acetylamino-6-formylamino-3-methyluracil (AFMU) to 1-methylxanthine (1X) in urine from people after they have consumed a caffeine-containing beverage. The ease of phenotyping in this way gives this method great appeal. However, studies have shown AFMU to be unstable *in vitro* at ambient temperature in aqueous solution at a pH greater than 3.5 (Grant *et al.*, 1984b). We were concerned that AFMU degradation at the pH and temperature of urine in the bladder could lead one to misclassify some rapid acetylators as slow acetylators if their urine pH was high. Therefore, we evaluated the stability of 1X and AFMU at the temperature and variety of physiological pHs present in the bladder.

A 0.01 M phosphate buffer was adjusted to pH 5.0, 6.0, 7.0, and 8.0, used to make separate standards containing 40 μg ml^{-1} 1X (Adams Chemical Co., Round Lake, Illinois) and 40 μg ml^{-1} AFMU (courtesy of Dr W. Kalow of University of Toronto, Toronto, Canada) and incubated at 37° C. Duplicate aliquots of 0.5 ml were drawn at time 0 (less than 1 min), and after 1, 2, 3, and 4 h of incubation. Each aliquot was immediately adjusted to pH ≤ 3.0 and stored in a Revco freezer at −80° C. The samples were thawed at room temperature and vortexed prior to analysis.

To 200 μl aliquots of sample were added 20 μl of a 1 μg μl^{-1} aqueous solution of *N*-acetyl-*p*-aminophenol (Sigma, St Louis, Missouri) as the internal standard and a large spatula tip of ammonium sulphate. Samples were extracted into 6.0 ml of a 95:5 solution of chloroform: isopropanol. The organic phases were evaporated to dryness in a water bath under a stream of dry nitrogen. The residues were dissolved in 100 μl of a 0.05% v/v acetic acid solution and injected into a chromatograph comprising a Waters 6000A pump, a Waters U6K injector, a 5 micron 150 × 4.6 mm i.d. stainless steel Supelcosil LC-18 column and a Waters Model 450 variable wavelength detector set at 280 nm. The mobile phase was 6% methanol/94% 0.05% acetic acid (V/V) pumped at 1.0 ml min^{-1}.

1X remained stable under all tested conditions. However, the 1X samples which had been adjusted to pH ≤ 3.0 and stored at −80°C contained precipitates. These samples were analyzed from a vortexed suspension. Sample extracts stored overnight in a refrigerator again formed precipitates. In these sample supernates, the ratio of peak height of 1X to peak height of APAP, which is directly proportional to concentration, fell 33% from 2.62 the previous day to 1.75. The precipitates were redissolved by warming to 60° C and peak height ratios returned to the previously observed levels indicating that the precipitate was 1X.

AFMU was labile under all conditions tested (Figure 1). After 4 h at 37° C, AFMU levels had fallen 29% at pH 5, 35% at pH 6, 39% at pH 7, and 45% at pH 8. To ascertain whether the observed concentration loss was due to decomposition alone or a decomposition product co-chromatographing with the internal standard, the 4 h samples were also analyzed without the internal standard. No decomposition peaks were detected outside of the void volume.
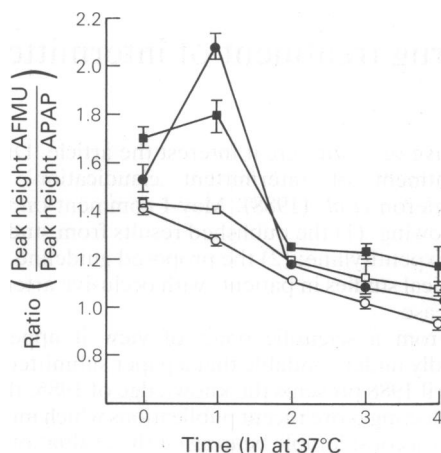

Figure 1 Ratio of AFMU peak height to APAP peak height at various times during incubation (mean ± s.e. mean). The reason for the high 1 h values has not been determined but may represent the presence of intermediary decomposition products. ■ pH 5.0, ● pH 6.0, □ pH 7.0, ○ pH 8.0.

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The caffeine metabolite AFMU appears to be very labile under physiological conditions. Even if specimens are adjusted immediately to pH ≤ 3.0 and frozen, there would still be a loss of AFMU in the more basic urines prior to voiding. Acidifying the urine by giving ammonium chloride might help but would add additional steps to the process of using caffeine to determine acetylation phenotype.

The poor aqueous solubility of 1X is also a potential problem. Achieving reproducible results may be difficult when measuring concentrations of a suspended rather than a dissolved substance. Redissolution of 1X in the urine by raising temperature or pH would accelerate AFMU degradation if both are measured from the same sample. This instability of AFMU and poor aqueous solubility of 1X may lead to a fall in the concentration of both compounds during the analysis with fortuitously little change in their ratio much of the time. The instability of AFMU and poor solubility of 1X may also explain the intra-subject phenotype variability (Hardy *et al.*, 1988) when caffeine was used as a test marker and suggests caution in the use of this phenotyping method.

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Drug treatment of intermittent claudication

I have read with great interest the article 'Drug treatment of intermittent claudication' by Cameron *et al.* (1988). May I comment on the following: (1) the published results from studies of oxpentifylline; (2) the proposed guideline for clinical studies in patients with occlusive arterial disease.

From a scientific point of view it appears hardly understandable that a paper submitted in April 1988 presents the knowledge of 1985, thus neglecting more recent publications which might have a considerable bearing on the evaluation of the drugs concerned.

In relation to the oxpentifylline study done by Porter *et al.* (1982) it is claimed that exclusion of 36% of patients from final analysis resulted in bias. In 1987, Gillings *et al.* published an intention-to-treat analysis of the data in question which confirmed the earlier findings from endpoint analysis, thus refuting the bias presumed. The list of placebo-controlled studies omits two studies in which oxpentifylline was tested against pharmacologically active material (adenosine, nylidrin). However, no mention was made of the dosage for these substances, both of which were deliberately used at a level as low as 3 mg three times daily so that they may be considered equal to placebo. The FDA accepted the nylidrin study (Acetto, 1982) as pivotal, after they had inspected the study centre and examined the original data.

One would expect that a review article appearing in 1988 would give now generally used evaluation statistics such as confidence intervals, in addition to mean values. This type of information was presented for ketanserin but not for oxpentifylline. The respective data were reported by Roessner & Mueller in 1987. Based on 252 patients (seven studies), Cameron *et al.* (1988) reported a therapeutic effect of 65% for oxpentifylline, as compared with placebo. With a variation of results of similar order, the respective 95% confidence interval would be in the range of