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A simple test for acetylator phenotype using caffeine

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1 A method is presented for the use of caffeine, in the forms commonly ingested by a large proportion of the world's population, to test for the clinically important acetylation polymorphism.

2 Each of 146 subjects provided a spot sample of urine between 2 and 6 h after coffee, tea or cola soft drink consumption, and the molar ratio of 5-acetylamino-6-formylamino-3-methyluracil (AFMU) to 1-methylxanthine (1X) was determined by a simple h.p.l.c. assay.

3 The ratio afforded segregation of three apparent modes of acetylation capacity in this population, in concordance with a standard sulphamethazine phenotyping procedure and with other methods using controlled caffeine intake and urine collections.

4 The day-to-day consistency of the method was established in eight selected subjects.

Keywords caffeine acetylator phenotyping

Introduction

A number of drugs relevant to human therapy are biotransformed by acetylation prior to excretion. The rate of this acetylation is genetically determined and polymorphic in nature—that is, individuals in a population can be distinguished as either 'fast' or 'slow' acetylators on the basis of their ability to convert particular amine and hydrazine drug substrates to their *N*-acetyl derivatives (Weber & Glowinski, 1980). The genetic basis for the polymorphic acetylation capacity resides with the existence of two major alleles at a single autosomal gene locus governing the production of the hepatic *N*-acetyltransferase enzyme (EC 2.3.1.5) (Evans *et al.*, 1960; Evans & White, 1964). Drugs metabolized by this enzyme include isoniazid, procainamide, dapsone, sulphamethazine, phenelzine and hydralazine (Weber & Glowinski, 1980; Lunde *et al.*, 1983).

Many adverse drug and chemical reactions have been associated with acetylator status (Lunde *et al.*, 1983; Drayer & Reidenberg, 1977; Wolf *et al.*, 1980; Cartwright *et al.*, 1982). It can therefore be of some importance in clinical and occupational settings to know the acetylator phenotype of individuals, and a number of screening methods using test drugs (such as isoniazid, sulphamethazine and dapsone) have been developed for this purpose (Evans, 1969; Schroder, 1972; Eidus & Hodgkin, 1973; Weber & Brenner, 1974; Carr *et al.*, 1978; du Souich *et al.*, 1979).

As part of our group's efforts to elucidate determinants of interindividual variability in biotransformation capacity in human populations, we chose to investigate the use of caffeine (1,3,7-trimethylxanthine, 137X) as an *in vivo* 'probe' of hepatic enzyme activities. This choice was based upon its ubiquitous usage, relative safety, and its metabolism by a number of distinct liver enzymes which could hopefully be monitored by measuring urinary metabolite levels after the oral ingestion of caffeine (Figure 1) (Grant *et al.*, 1983b).

In the course of these studies, it was demonstrated that the production of 5-acetylamino-6-formylamino-3-methyluracil (AFMU), a newly discovered metabolite of caffeine (Tang *et al.*, 1983), is mediated by the polymorphic *N*-acetyltransferase (Grant *et al.*, 1983a). Furthermore, it was shown that the molar ratio of AFMU to 1-methylxanthine (1X) excreted in 24

h pooled urine samples after the ingestion of 300 mg of caffeine provided a simple index of acetylator phenotype (Grant *et al.*, 1983b).

We report here the results of studies testing a highly simplified experimental protocol and analytical method using the AFMU/1X ratio after uncontrolled caffeine ingestion for large-scale population assessment of acetylator status. The data include a comparison, in selected subjects, between this simplified method and previous methods used by us, including a standard acetylator phenotyping procedure using sulphamethazine (SMZ) as a test drug.

Methods

a. *Sulphamethazine (SMZ) acetylator phenotyping study* (Grant *et al.*, 1983a)

Subjects ingested 10 mg/kg of SMZ and a plasma sample was collected 6 h later. The percentage of SMZ present in plasma in the acetylated form was determined using the spectrophotometric Bratton-Marshall method, and from this subjects could be classified as genetically 'fast' or 'slow' acetylators.

b, c. *Caffeine population study*

This study has been described in detail elsewhere (Grant *et al.*, 1983b). In short, subjects ingested 300 mg of caffeine and pooled urine was collected for 24 h thereafter. High performance liquid chromatographic (h.p.l.c.) procedures were used to quantify a total of 14 urinary caffeine metabolites, of which five were quantitatively important (Figure 1). Two different metabolite ratios (AFMU/(1X + 1U + 17X + 17U + AFMU) and AFMU/1X) providing indices of *N*-acetyltransferase activity were calculated for all subjects.

d. *Simplified acetylator phenotyping study*

Healthy volunteers from the Toronto population, ranging in age from 5 to 66 years, were

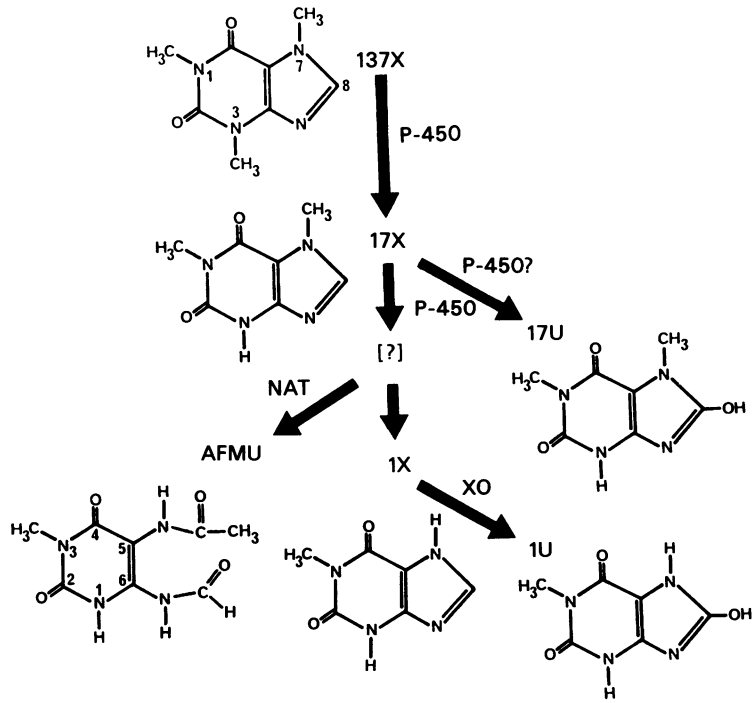

Figure 1 Proposed pathways of formation of the five major metabolites of caffeine in man. Enzymes responsible for the metabolic steps are shown beside the arrows; P-450 = cytochrome(s) P-450, NAT = *N*-acetyltransferase, XO = xanthine oxidase. Question mark denotes a postulated ring-opened intermediate. Abbreviations: 137X = caffeine; 17X = 1,7-dimethylxanthine; 17U = 1,7-dimethyluric acid; 1X = 1-methylxanthine; 1U = 1-methyluric acid; AFMU = 5-acetylamino-6-formylamino-3-methyluracil.

asked to provide a single spot urine sample at any time between 2 and 6 h after consuming at least one cup of coffee or strong tea, or 500 ml of caffeinated cola drink. No restrictions whatsoever were placed upon subjects with regard to food intake or level and frequency of caffeine consumption above the minimum stated above. Urine samples were either analysed immediately or stored at −20°C until the time of analysis, owing to the relative instability of AFMU described previously (Tang *et al.*, 1983). The urinary AFMU/ 1X ratio was determined for a total of 146 subjects using a single simplified h.p.l.c. assay modified from methods already published (Grant *et al.*, 1983b), and performed as follows:

A 0.2 ml aliquot of urine was placed in a 15 ml centrifuge tube and saturated with ~120 mg of ammonium sulphate. Six ml of chloroform:isopropanol (95:5 v/v) was added, followed by 0.2 ml of *N*-acetyl-*p*-aminophenol internal standard solution (120 mg/l in chloroform), and the tube was vortexed vigorously for 30 s. Following centrifugation at 2000 rev/min for 5 min, the organic phase was removed to another tube, taken to dryness under N_2 at 40–50°C, and the residue was resuspended in an appropriate volume (0.8–2.0 ml) of 0.05% acetic acid. A 20 μl volume of the sample was injected onto a reversed-phase h.p.l.c. column (Ultrasphere ODS 5μ, 25 cm × 4.6 mm i.d., Beckman Instruments Inc.), eluted with 0.05% acetic acid: methanol (88:12, v/v) at a flow rate of 1.2 ml/min, and monitored by u.v. absorbance at 280 nm. Quantification was performed by comparison of peak height ratios relative to internal standard with those of blank urines spiked with known amounts of 1X (Pfaltz and Bauer) and AFMU (isolated as described by Tang *et al.* (1983)). These urine standards were stored frozen in separate aliquots and thawed individually for each day's analysis.

e. *Reproducibility study*

Of the thirteen subjects who had taken part in all of the preceding studies, eight were selected to test the reproducibility of the simplified test design. These subjects continued their normal daily caffeine consumption, and each provided random afternoon urine samples on five additional occasions over a 3 week period. These samples were analyzed as above for the AFMU/1X molar ratio.

Results

Figure 2 summarizes the results of all of the studies described, and it also illustrates the relationship between the phenotyping methods used. A total of twenty subjects participated in both the SMZ phenotyping study (Figure 2a) and the original caffeine population study, with complete concordance between the methods regardless of whether AFMU production was expressed as a molar fraction of the major recoverable urinary metabolites (Figure 2b) or as a molar ratio to the excretion of 1X (Figure 2c). Thirteen of these subjects were available for further participation in the simplified acetylator phenotyping study, the results of which are shown for 146 subjects in Figure 2d. The population distribution of the AFMU/1X ratio was apparently trimodal in nature. Concordance was again shown, for the 13 common subjects, between this method using 2–6 h spot urine samples after variable caffeine ingestion, and that using a standard high dose of caffeine and pooled 24 h urine collections.

The between-day reproducibility of the AFMU/1X ratio determination is shown in Figure 2e for eight of the thirteen subjects, who provided urine samples after coffee and/or tea ingestion on random occasions over a 3 week period. Between-day coefficients of variation (n = 6 separate days) averaged 14.5% for four lower mode subjects, 11.9% for two intermediate mode subjects, and 11.0% for two upper mode subjects. Each subject's AFMU/1X ratio was confined to one of the three acetylation modes over this period.

Discussion

The method presented here offers a number of practical advantages over those which have been used in the past for determining acetylator phenotype. It requires the oral intake of a very popular and safe drug, caffeine, in a low dose (a single cup of coffee in an average adult, equivalent to 1–1.5 mg/kg of caffeine, is sufficient) and in convenient dosage forms. It does not appear necessary to carefully control either the timing or quantity of caffeine intake or urine collections with this test, and it requires no plasma sampling. It may therefore be widely and easily applied. These considerations may make the test of particular value for large-scale population screening, and also in instances where potential adverse reactions to amine or hydrazine test drugs are anticipated or are to be avoided (i.e. in small children). In addition, the analytical method is simple to perform with standard h.p.l.c. equipment, and only very small urine samples are required for analysis.

Theoretical considerations may also prove the method to be superior to those presently in use.

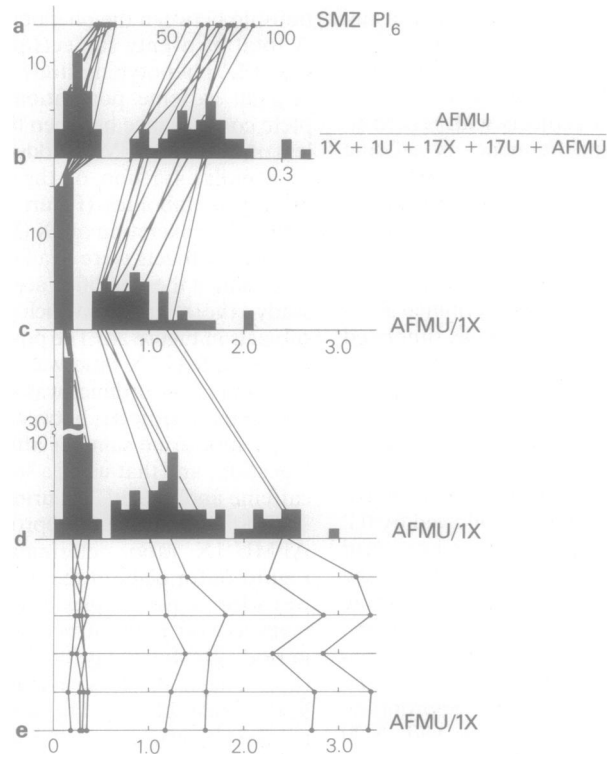

Figure 2 Relationship between methods used for determining acetylator phenotype. Solid lines connect data points from subjects taking part in more than one study, as described in Results. (**a**) sulphamethazine plasma acetylation index, PI_6, denoting the percentage of SMZ in plasma in the acetylated form for 20 subjects. (**b**) Frequency histogram of data (n = 72) from caffeine population study. Abscissa denotes the fraction of the five major recoverable urinary caffeine metabolites present as AFMU in 24 h pooled urine samples after ingestion of 300 mg caffeine. (Data in **a** and **b** adapted from Grant *et al.*, 1983a). (**c**) Frequency histogram of data from study in **b** above; abscissa is the molar ratio of AFMU to 1X. (**d**) Frequency histogram of data from simplified acetylator phenotyping method (n = 146); abscissa is the molar ratio of AFMU to 1X. (**e**) Consistency of the simplified method in **d** for eight selected subjects. Five replicates of the method were performed for each subject over a period of 3 weeks; abscissae as in **c–d**.

As seen in Figure 2d, the test appears sensitive enough to discriminate heterozygous and homozygous fast acetylators in addition to identifying slow individuals. Other workers (Chapron *et al.*, 1978, 1980; Lee & Lee, 1982) have been able to accomplish this degree of segregation only through the calculation of kinetic constants with repeated plasma sampling after a relatively high dose of a test drug such as SMZ. A number of reasons are possible for the sensitivity of the present method despite its uncontrolled nature.

First is the nature of the parameter used. The AFMU/1X ratio approaches no maximum value, and when plotted on a linear scale it tends to accentuate differences at high levels of acetylation. Such is not the case with, for example, the SMZ acetylation indices (Evans, 1969; du Souich *et al.*, 1979) which approach a maximum value (100%) and therefore obscure differences between heterozygous and homozygous fast acetylators.

Secondly, both AFMU and 1X are very polar compounds and are likely rapidly excreted by the kidney without reabsorption, since their plasma levels are negligible (Grant, unpublished observations). Hence the ratio of these will be relatively insensitive to variations in urine flow or kidney function.

Thirdly, the ratio will not be affected by variation in absorption characteristics of the test drug, since the actual substrate for the enzyme is not caffeine itself but is rather a metabolite which is formed locally in the liver. If, as postulated previously (Grant *et al.*, 1983b), the substrate for *N*-acetyltransferase is a ring-opened intermediate which in fast acetylators is quickly acetylated and in slow acetylators has time to reclose to form 1X (see Figure 1), then the ratio

will be a direct reflection of the differing kinetic properties of the allelic proteins mediating the acetylation process. However, it remains to be seen whether alterations in other enzyme activities either giving rise to the immediate substrate (cytochrome P-450) or further metabolizing the products (xanthine oxidase) will affect the determination of the AFMU/1X ratio.

It is also apparent from Figure 2 that the simplified method gives better discrimination of three acetylation modes than determination of the same ratio in 24 h pooled urines after single-dose caffeine ingestion (Figure 2c). This may be because, as noted by Chapron *et al.* (1980) for SMZ, acetylation differences between heterozygous and homozygous fast acetylators are most prominent during early time periods after drug dosing. Therefore, spot urine samples taken 2–6 h after caffeine ingestion will detect these differences better than 24 h pooled urines.

It remains to be proved whether the three observable modes of AFMU/1X excretion truly represent the distinct genotypes of *N*-acetyltransferase activity. In this light, two observations are of interest. Firstly, analysis of the population data among unrelated Caucasian subjects indicates that the observed distribution does not fit with that expected through application of Hardy-Weinberg theory for a single autosomal locus, with an excess of apparent homozygous fast acetylators ($P < 0.01$). Secondly, preliminary studies of partial pedigrees in thirteen families yielded one family which did not agree with the expected segregation of two alleles at a single gene locus. It may be that the method is not able to give consistent discrimination of heterozygous and homozygous fast acetylators. On the other hand, the existence of additional alleles for high *N*-acetyltransferase activity could explain both of these results, and cannot presently be ruled out. More detailed study of these families is necessary to sort out these discrepancies.

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