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Genetic variation in rates of antipyrine metabolite formation: A study in uninduced twins

(pharmacogenetics)

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ABSTRACT Adult, male, unmedicated twins received antipyrine orally under carefully controlled environmental conditions. Relative contributions of genetic and environmental factors to 2-fold interindividual variations in rate constants for formation of the three main antipyrine metabolites were compared. Heritabilities for rate constants for formation of 4-hydroxyantipyrine, *N*-demethylantipyrine, and 3-hydroxymethylantipyrine were 0.88, 0.85, and 0.70, respectively. These results suggest that each molecular form of cytochrome *P*-450 that converts antipyrine to a different metabolite exhibits genetically controlled interindividual variations in activity. Unrelated adult male subjects whose environments were also carefully controlled exhibited highly reproducible rate constants for formation of antipyrine metabolites. Because the rate constant for metabolite formation sensitively detects certain variations in the gene product, it should be used in future pharmacogenetic studies on rates of production of multiple metabolites from a single parent drug.

In 1968, causes of large interindividual variations in rates of antipyrine (AP) decay in normal adults were identified in monozygotic (MZ) and dizygotic (DZ) twins (1). Interindividual variations in AP clearance were maintained mainly by genetic factors. The twins lived in different households under nearly basal conditions with respect to multiple environmental factors capable of altering rates of AP clearance. In contrast to large interindividual variations, AP clearance in a given subject was highly reproducible, provided that no significant environmental perturbations occurred.

The twin study reported 13 years ago measured only plasma AP kinetics (1). Because AP is degraded through multiple reactions catalyzed by enzymes independently influenced by numerous genetic and environmental factors, each metabolic pathway needs to be investigated separately. This approach, which was the objective of the present study, permitted a more specific estimate of gene activity than did the earlier study.

In several species, including man, three primary metabolites of AP have been identified: 3-hydroxymethyl-AP, *N*-demethyl-AP, and 4-hydroxy-AP (2–5). Each metabolite is formed by a separate hepatic cytochrome *P*-450-mediated monooxygenase (6, 7). Conjugation then occurs to varying extents with glucuronide, rendering products even more water soluble and hence more easily excreted in urine where metabolites are identified (2–8). In the present study, under carefully controlled environmental conditions, the relative roles of heredity and environment in controlling large interindividual variations in rates of hepatic formation of each AP metabolite were assessed in twins by using new sensitive techniques for metabolite detection. These data were used to test different pharmacokinetic models of AP elimination.

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MATERIALS AND METHODS

Selection of Subjects. Ten unrelated subjects, 10 pairs of MZ twins, and 10 pairs of DZ twins between the ages of 16 and 35 yr without previous serious illness were obtained from Hershey and vicinity through advertisements in local newspapers. The subjects were nonsmoking, unmedicated, Caucasian males. All subjects were in a nearly basal state with regard to numerous environmental factors capable of affecting rates of AP metabolism (9). Zygosity was determined by analysis of 10 erythrocyte antigens.

Sample Collection. At 0800, after a 12-hr fast, all subjects ingested AP (18 mg/kg) dissolved in 150 ml of water. Saliva samples (stimulated by chewing of Parafilm) were collected 3, 6, 9, 12, 15, and 24 hr after AP administration. Urine was collected over the following intervals: 0–4, 4–8, 8–12, 12–16, 16–24, 24–32, 32–40, 40–48, 48–56, 56–64, and 64–72 hr. Sodium metabisulfite (4 mg/ml) was added to each urine sample. Aliquots of urine were frozen at −70°C until analyzed (no longer than 4 weeks). AP was administered again to each subject 2 weeks after the first dose, and again 4 weeks later to the 10 unrelated subjects, to determine the reproducibility of the kinetic values. All subjects abstained from food and drink containing caffeine, methylxanthines, or alcohol during the 4-day study.

Assay of AP and AP Metabolites. AP was extracted from 1 ml of saliva by using the procedure of Prescott *et al.* (10). Metabolites were extracted from 1 ml of urine by the method of Danhof *et al.* (8). Metabolites and AP were separated by high-pressure liquid chromatography (Waters 8000A) and quantitated by an integrating recorder (Hewlett–Packard). The columns (10 cm × 2.8 mm) were packed in our laboratory with Lichrosorb RP-2 (mean particle size, 5 μm) by using a commercial column packer (Micromeritics, Norcross, GA). To prevent loss of *N*-demethyl-AP through adsorption to glass, all glassware in contact with AP metabolites was coated with a silicone preparation. Linear standard curves were generated by using commercially available AP (Matheson, Coleman, and Bell) and 4-hydroxy-AP and *N*-demethyl-AP (Aldrich). Yoshimura *et al.* (5) and Danhof *et al.* (8) donated specimens of 3-hydroxymethyl-AP.

We used a new purer standard for 3-hydroxymethyl-AP kindly provided by D. D. Breimer. Breimer's older standards (8) were contaminated with sodium bromide to the extent of 55% by weight. Thus, values for percent AP recovered as urinary 3-hydroxymethyl-AP described in this paper are approximately half those reported in ref. 8.

Pharmacokinetic Analysis. Development of meaningful measurements of the activity of each hepatic enzyme that produces a different AP metabolite requires a parameter that re-

Abbreviations: AP, antipyrine; MZ, monozygotic; DZ, dizygotic.

flects, as specifically as possible, the rate of formation of each metabolite. The rate constant, k, appears to be most appropriate in this regard. According to Brodie and Axelrod (3) and Vesell *et al.* (11), the disposition of AP and its metabolites can be described by a series of monoexponential equations. These and subsequent studies demonstrated that AP and its metabolites conform to a one-compartment model with parallel, simultaneously occurring, first-order reactions in which metabolite excretion is faster than metabolite formation (Fig. 1). Thus:

$$\log(\text{rate of excretion})_i = \log(k_i \cdot \text{dose}) - \frac{k_e t}{2.303} \qquad [1]$$

in which k_i is the rate constant for formation of the ith metabolite and k_e is the overall rate constant describing AP elimination ($k_e = k_x + k_1 + k_2 + k_3 + k_4$) (12).

The first-order rate constant for metabolite production (k_i) can be obtained from urinary excretion data by extrapolating to 0 the linear portion of the curve generated from Eq. 1. Thus:

$$\text{extrapolated time 0 intercept} = k_i \cdot \text{dose} \qquad [2]$$

which, on rearrangement, yields:

$$k_i = \frac{\text{extrapolated time 0 intercept}}{\text{dose}}. \qquad [3]$$

Genetic Analysis. To estimate the relative contributions of genetic and environmental factors to interindividual variations of each kinetic measurement, heritabilities (H_2) were calculated (10):

$$H_2 = \frac{V_{DZ} - V_{MZ}}{V_{DZ}} \qquad [4]$$

in which V_{MZ} and V_{DZ} represent variance amont MZ and DZ twins, respectively. Variance was calculated as follows: $V = [\Sigma(\text{difference between twins})^2]/2N$ in which N is the number of twin pairs of the same zygosity. This equation permits a range of values from 0 (negligible genetic control over variation) to 1 (virtually complete hereditary influence).

RESULTS

Recovery of administered AP as urinary AP and metabolites was 64.3 ± 7.0% (mean ± SD) in unrelated subjects and 64.1 ± 9.4 in twins. Mean (± SD) percentages of ingested AP excreted as 4-hydroxy-AP, 3-hydroxymethyl-AP, *N*-demethyl-AP, and unchanged AP in unrelated subjects were 30.3 ± 3.8, 16.4 ± 2.1, 15.5 ± 1.6, and 2.5 ± 0.7, respectively, and 26.9 ± 3.9, 17.0 ± 3.4, 16.1 ± 3.4, and 2.1 ± 0.7, respectively, in twins. These values are consistent with those reported previously (8, 13, 14).

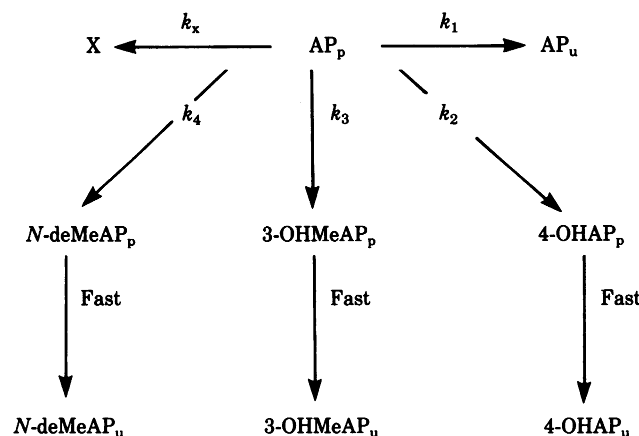

FIG. 1. Proposed pharmacokinetic model for AP metabolism in plasma (p) and urine (u). X is the sum of all unmeasured metabolites of AP; apparent first-order rate constants for the formation of 4-hydroxy-AP (4-OHAP), 3-hydroxymethyl-AP (3-OHMeAP) and *N*-demethyl-AP (*N*-deMeAP) are represented as k_2, k_3, and k_4, respectively.

Reproducibility of rate constant measurements in a given subject was established by analysis of variance designed for repeated measurements in the 10 unrelated subjects who received AP three times. Intraindividual variations remained small and did not reach statistical significance ($P > 0.05$), but interindividual variations exceeded 2-fold for each AP metabolite ($P < 0.05$).

Estimates of heritability from metabolite rate constants, k_i, in 40 twins revealed substantial genetic control over interindividual variations. H_2 was 0.88, 0.85, and 0.70 for 4-hydroxy-AP, *N*-demethyl-AP, and 3-hydroxymethyl-AP, respectively ($P < 0.05$). H_2 of the overall rate constant, k_e was 0.77 (Table 1).

The basic assumptions in the estimation of rate constants for formation of 4-hydroxy-AP, *N*-demethyl-AP, and 3-hydroxymethyl-AP were that (*i*) disposition of AP and its main metabolites followed a series of parallel, simultaneously occurring, first-order reactions with excretion being faster than formation, and (*ii*) absorption of orally administered solutions of AP was rapid and complete such that postabsorption plasma concentrations agreed with plasma concentrations found after intravenous administration of a similar dose (15). Under these conditions, plots of logarithm of rate of excretion of these three metabolites versus time for any individual should yield parallel lines with slope = k_e. Figs. 2 and 3 show that this expectation was fulfilled. Fig. 2 shows high reproducibility of excretion rates in four unrelated subjects. Fig. 3 and Table 1 demonstrate close similarity of excretion rates within most MZ twinships and dissimilarity within most DZ twinships.

DISCUSSION

These results show that genetically controlled variations in plasma AP half-life (1) arise from genetically controlled interindividual differences in rates of hepatic production of each major AP metabolite. The evidence indicates that each AP metabolite is produced by a separate molecular form of cytochrome *P*-450 (6, 7). Our results suggest that each molecular form involved in AP metabolism exhibits genetically controlled differences in activity among normal uninduced adult subjects investigated under carefully controlled environmental conditions.

Several previous pharmacogenetic studies utilized measurement of metabolites rather than of the parent drug alone. For example, twin studies on the urinary metabolites of halothane (16) and nortryptyline (17) led to conclusions similar to those of the present investigation. A study on amobarbital kinetics in twins (18), although not reporting rate constants of metabolite formation, led to a follow-up family study in which rate constants for metabolite formation were measured (19). No previous study (16–18) compared elimination rate constants for metabolite formation in MZ and DZ twins. Expressed as reciprocal time units, the apparent first-order rate constant for metabolite formation relates drug concentration and rate of drug disappearance due to any single metabolic pathway. Our results suggest that, at least for AP, this measurement is a sensitive estimate of certain variations in the gene product. Accordingly, a major conclusion of this study is that the rate constant for metabolite formation can serve in future pharmacogenetic investigations to identify phenotypic variations in the gene product.

To disclose genetic factors, studies on drug disposition in twins require maximal uniformity of the environmental conditions under which the subjects live. Heterogeneous environmental conditions can minimize or completely conceal these

Table 1. Rate constants for metabolite formation (k_i) and overall elimination (k_e) in 10 sets of MZ and 10 sets of DZ twins

	k_i hr^{-1} × 10^2			k_e, hr^{-1} × 10^2
Subjects	4-OHAP	3-OHMeAP	*N*-deMeAP	AP
		MZ twins		
RH	2.44	1.92	0.61	5.20
WH	2.04	1.30	0.67	5.00
RC	1.81	1.15	0.94	7.20
TC	1.73	0.67	1.06	7.70
DsM	2.16	1.85	0.77	5.10
DnM	1.69	1.23	0.46	5.00
JB	2.32	1.05	0.81	5.80
TB	1.53	0.81	0.56	6.20
RE	1.12	0.45	0.48	4.70
DE	1.22	0.64	0.42	5.60
MeC	2.10	2.12	1.18	6.00
MkC	1.85	1.35	0.84	6.40
JL	2.13	1.54	1.17	6.10
DL	2.66	1.91	1.17	6.20
AyB	1.54	0.51	0.86	7.50
AwB	1.68	0.95	0.63	6.20
DsL	1.88	1.18	0.69	6.30
DdL	1.39	0.97	0.63	5.60
LyT	1.14	0.61	0.61	5.10
LeT	1.41	0.91	0.68	6.20
Δ^2	1.7×10^{-4}	2.1×10^{-4}	3.5×10^{-5}	4.8×10^{-4}
		DZ twins		
JH	2.61	1.88	0.94	6.66
SH	1.35	0.95	0.95	6.24
FM	1.03	0.76	0.61	4.44
TM	1.52	1.26	1.07	5.10
MS	1.56	0.64	0.43	4.81
NS	1.37	1.00	0.56	4.13
CR	2.22	0.79	0.72	7.14
TR	1.24	0.54	0.83	4.05
DBd	1.56	1.09	1.15	7.60
MBd	1.49	0.95	0.95	8.30
JnF	2.13	1.50	1.06	6.30
JfF	1.90	1.45	1.03	6.13
CE	1.59	1.18	0.91	5.21
EE	4.98	2.90	2.07	6.48
RC	2.17	1.36	1.83	7.62
DC	1.36	2.37	1.12	4.88
JT	2.10	1.92	1.54	5.21
PT	2.81	2.81	1.37	4.85
ST	2.52	2.13	1.14	4.71
DT	2.84	1.26	1.58	5.68
Δ^2	1.6×10^{-3}	6.9×10^{-4}	2.4×10^{-4}	2.1×10^{-3}
Heritability	0.88	0.70	0.85	0.77

k_i is based on urinary excretion and k_e is based on AP decay in saliva.

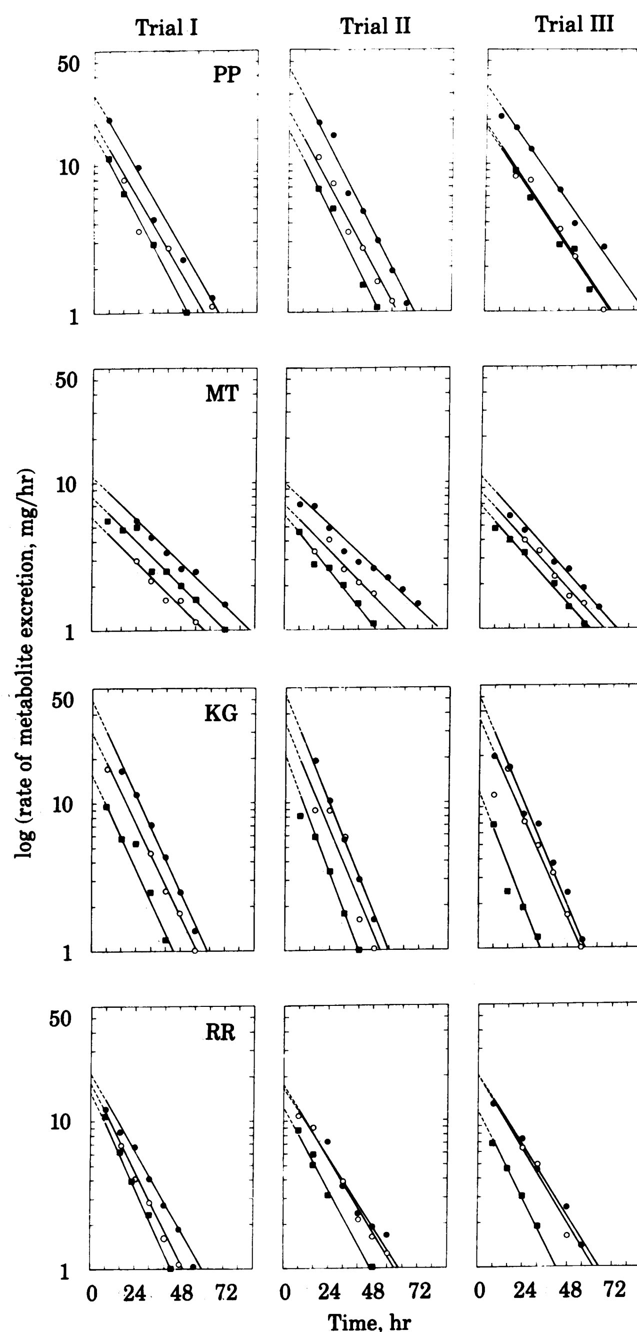

FIG. 2. Excretion of three metabolites of AP plotted against time after oral administration of AP (18 mg/kg) to four unrelated subjects in three separate trials. ●, 4-Hydroxy-AP; ○, *N*-demethyl-AP; ■, 3-hydroxymethyl-AP.

genetic factors. If both genetic and environmental factors vary simultaneously, valid conclusions cannot be drawn from twin studies. Under markedly different conditions, environmental factors can be varied, but only when genetic factors remain constant as in studies of phenotypic variation between MZ twins reared apart. The twin method has been criticized for overemphasizing genetic factors and underestimating the impact of environmental variables on rates of drug elimination (20). This criticism misses the main objective of the twin method which consists in identification of even small or subtle genetic factors which frequently involve multiple loci. The twin method is inappropriate and inefficient for investigating environmental factors. The AP test (21, 22) is preferable. It measures AP kinetics in each subject before and after imposition of a single environmental variable. Contributions of genetic factors to differences among subjects are completely eliminated because genetic factors remain constant when each subject serves as his own control. Thus, only environmental effects on AP metabolism are measured and all changes in AP kinetics that occur can be attributed to the single environmental condition altered independently of others.

The twin method can identify efficiently certain subtle genetic factors that control phenotypic pharmacokinetic vari-

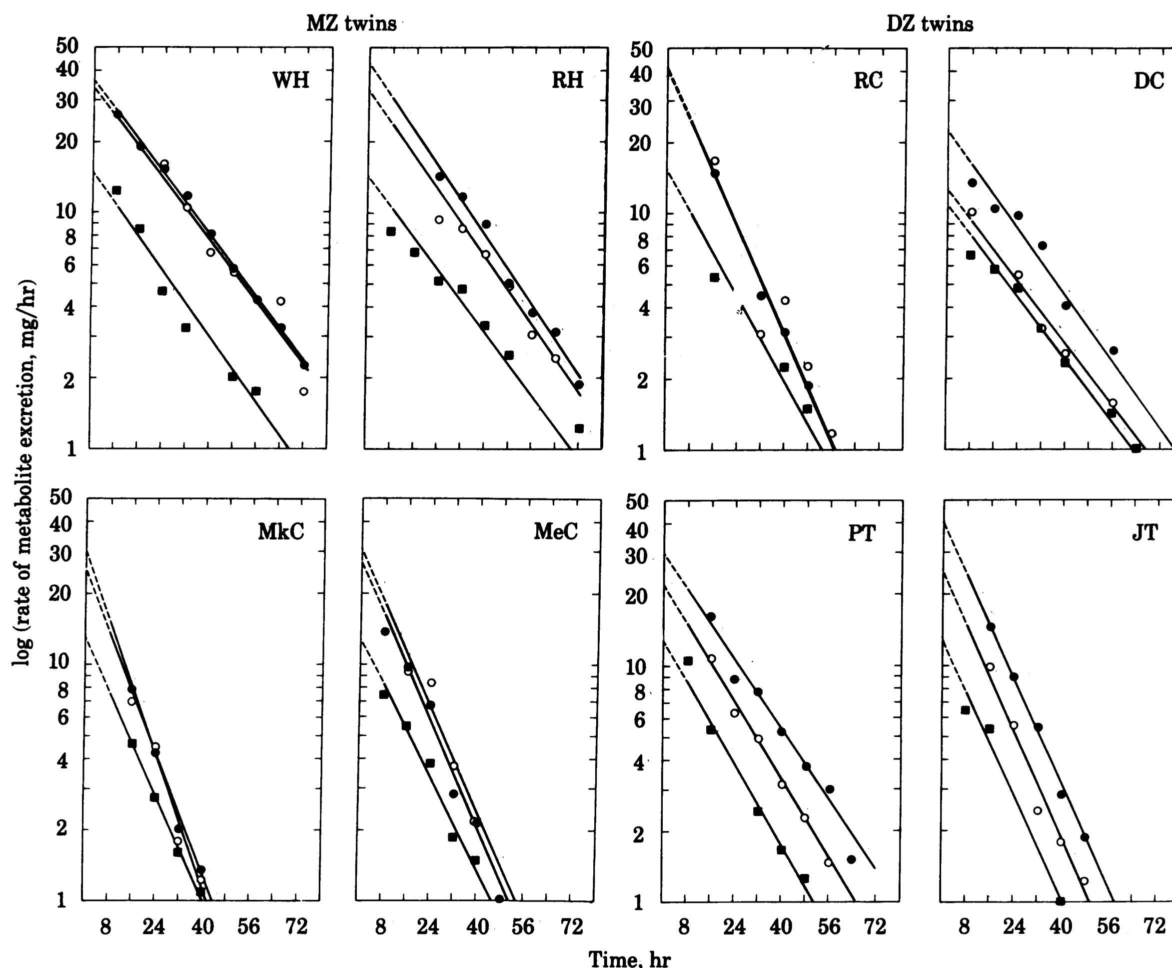

FIG. 3. Excretion of three metabolites of AP plotted against time after oral administration of AP (18 mg/kg) in two sets of MZ and two sets of DZ twins. Symbols as in Fig. 2.

ations. Pedigree analyses or distribution curves of unrelated subjects may suffer much more than twin studies from masking of genetic factors by environmentally induced pharmacokinetic differences among subjects, including differences in age, diet, smoking, ethanol ingestion, and exposure to other inducing or inhibiting chemicals at home or at work. These considerations led to the suggestion that pharmacogenetic studies use a tier system beginning with screening in twins to determine whether a genetic factor exists and concluding with investigations in families selected from the screening procedure for unusual values whose clear-cut segregation would help to identify their Mendelian mode of transmission (9).

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