Relationship between the metabolism of antipyrine, hexobarbitone and theophylline in man as assessed by a 'cocktail' approach

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1 Three model substrates for the characterization of drug oxidation activity, antipyrine (AP), hexobarbitone (HB) and theophylline (TH), were administered to 26 healthy volunteers on two different occasions: in the first experiment a combination of AP (250 mg) and HB (250 mg) was given and in the second experiment TH (150 mg) was added to the former combination.

2 Plasma concentrations of AP, HB and TH and urinary excretion of TH and the three main metabolites of AP (3-hydroxymethylantipyrine: HMA, norantipyrine: NORA and 4-hydroxyantipyrine: OHA) were determined and the intrinsic clearance (CL_{int}) of the three substrates and the clearance to the formation of AP metabolites were calculated.

3 The correlation coefficients between CL_{HB} and $CL_{\rightarrow metabolites}$ of AP were highest for $CL_{\rightarrow HMA}$ and $CL_{\rightarrow NORA}$ (>0.80) and lowest for $CL_{\rightarrow OHA}$ (0.63). High correlation coefficients also were found between CL_{TH} and $CL_{\rightarrow OHA}$ (0.89) and $CL_{\rightarrow HMA}$ (0.80).

4 Ideal relationships, defined by a slope of the orthogonal regression line equal to unity, did exist between CL_{HB} and $CL_{\rightarrow HMA}$ as well as $CL_{\rightarrow NORA}$ and between CL_{TH} and CL_{AP} as well as $CL_{\rightarrow OHA}$.

5 Based on the results of correlation and regression analysis it can be concluded that isozymes of the cytochrome P-450 system responsible for the oxidation of HB and formation of HMA and NORA are very closely related and also that isozymes responsible for the oxidation of TH and formation of OHA show a very close relation.

6 With this strategy of simultaneous administration of substrates ('cocktail' approach) it seems possible to characterize and correlate activities of different P-450 isozymes and to investigate their *in vivo* substrate selectivity without the disturbing influence of intra-individual variation in drug oxidation.

Keywords antipyrine hexobarbitone theophylline cytochrome P-450 cocktail approach

Introduction

A major concern in human drug treatment is the existence of large inter- and intraindividual differences in the rate of metabolic drug oxidation. Such differences may amount to a factor 10 or more and are caused by genetic factors, disease,

age as well as environmental influences on the activity of the cytochrome P-450 system in the liver (Breimer, 1983; Sjöqvist, 1985). In clinical practice most problems arise with drugs having a small therapeutic index and when a patient's

drug metabolizing capacity deviates considerably from the mean, e.g. in advanced liver disease, or in poor metabolizers of drugs subject to oxidation polymorphism (Breimer, 1983; Vesell, 1984).

Much effort has been devoted to develop simple methods to predict an individual subject's capacity to oxidize drugs, but most of them did not reach the objective because of the use of incorrect pharmacokinetic parameters, underestimation of occurring intraindividual differences in drug oxidation, or insufficiently taking into account the multiplicity of the cytochrome P-450 system (Breimer, 1983; Greenblatt *et al.*, 1982; Park, 1982). Statistical analysis of data often was limited to calculation of the correlation coefficient (a qualitative parameter) and in most studies the evaluation was not extended to regression analysis in order to obtain a quantitative measure for the investigated relationship (Godfrey, 1985).

Antipyrine (AP) is one of the most widely used model substrates for the assessment of the drug oxidizing enzyme activities (Vesell, 1979) and there is evidence that different isozymes are responsible for the formation of the main metabolites of AP (Boobis *et al.*, 1981; Danhof *et al.*, 1979). Hexobarbitone (HB) and theophylline (TH) are also frequently applied as model substrates and it has been suggested that these two substrates might be used as markers of particular cytochrome P-450 isozymes (Van der Graaff *et al.*, 1983a; Kappas *et al.*, 1976). In rats the feasibility of an approach in which such substrates are administered simultaneously, in order to assess common pathways in metabolic oxidation, has been investigated and proved to be successful (Van der Graaff *et al.*, 1983a,b). In man a preliminary study with the combination of AP and TH has been carried out (Teunissen *et al.*, 1985).

The aim of the present study was to establish relationships between the metabolism of AP, HB and TH in man when administered simultaneously, in order to investigate further the feasibility of such a 'cocktail approach', with more substrates and in a larger population.

Methods

Compounds

AP (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one), aminophylline (1,3-dimethylxanthine ethylenediamine), racemic HB (1,5-dimethyl-5-[1'-cyclohexenyl]-barbituric acid) and phenacetin (PH) were obtained from Brocacef (Maarsse, The Netherlands). Norantipyrine (NORA) and 4-hydroxyantipyrine (OHA) were obtained from Aldrich (Brussels, Belgium), 3-hydroxymethylantipyrine (HMA) was synthesized according to

Buijs *et al.* (1986). 8-Chlorotheophylline (8-CT) and 7- β -hydroxyethyltheophylline (7-HET) were obtained from Sigma Co. (St Louis, MO, USA).

Human experiments

The protocol for the study was approved by the Committee for Medical Ethics of the University Hospital at Leiden, The Netherlands. Twenty-six volunteers (12 males, 14 females) participated after having given their written informed consent. All were healthy according to clinical history, physical examination and routine blood analysis. Except for five females, who were using oral contraceptive steroids, all participants were non medicated. Their average age was 22.8 years (19–29 years). Seven were moderate to heavy smokers (5–40 cigarettes/day).

All volunteers participated twice in the experiment. On the first occasion a combination of AP and HB was administered and on the second occasion, 2 weeks later, a combination of AP, HB and TH. All drugs were administered orally, after an overnight fast, in the following doses: AP 250 mg (aqueous solution), HB 250 mg (capsule) and TH as a freshly prepared solution in water of 190 mg aminophylline (equivalent to 150 mg TH). The volunteers abstained from methylxanthine-containing foods and beverages, starting 1 day prior to and during both experiments.

On the first day of both experiments an indwelling cannula was inserted into an antebrachial vein and heparinized blood samples were collected at 0, 15, 30, 45, 60, 90, 120 and 150 min and 3, 4, 6, 8, 10, 24 and 32 h after drug administration (8 ml each). The samples at 24 and 32 h were collected by separate venous punctures. Two hours after drug administration the volunteers were allowed to take food and beverages again.

During both experiments all urine was collected up to 48 h after drug intake in four portions (0–8, 8–24, 24–32 and 32–48 h) and in advance 1 g of sodium pyrosulphite was added to the urine containers, to prevent further oxidation of the metabolites of AP. All plasma and urine samples were frozen at -20°C , until analysis.

Assays of drugs and metabolites

AP and HB in plasma were simultaneously determined in both experiments by applying a gas chromatographic assay described by Van der Graaff *et al.* (1983a) with some modifications. To 0.5 ml plasma 0.5 μg PH was added as an internal standard. The mixture was extracted

with 6 ml dichloromethane/n-pentane 3/7 (v/v). After extraction, evaporation to dryness and redissolution in 200 μ l ethylacetate, 1 μ l was injected.

TH and AP in plasma were simultaneously analysed based on the method of Teunissen *et al.* (1985) with minor modifications. The eluent consisted of an acetate buffer 0.005 M, pH = 5.0 containing 15% acetonitrile.

The assays of AP, HMA, NORA and OHA were based on methods of Danhof *et al.* (1978) and Teunissen *et al.* (1983), with minor modifications. In the assay of AP and HMA 0.5 ml methanol was added to the extraction mixture to prevent formation of an emulsion. The eluent consisted of a phosphate buffer 0.016 M, pH = 6, containing 17% acetonitrile.

For the analysis of TH in urine to 0.5 ml urine 40 μ g 8-CT and 100 μ l phosphate buffer, 1.0 M and pH = 7.0 was added and the mixture was extracted with 5 ml chloroform. After evaporation 0.5 ml eluent was added and 20 μ l was injected. An acetate buffer of 0.05 M, pH 4.4 containing 12% acetonitrile was used as eluent.

Apparatus

The gas chromatograph was a Hewlett Packard (HP) model 5710A, equipped with a nitrogen selective detector. A capillary column was used (13 m long, 0.3 mm inside diameter), statically coated with OV-1701 (0.2% w/v). The column temperature was kept at 205°C, injection port and detector at 300°C. The flow rate of helium as carrier gas was 3 ml min⁻¹.

The high performance liquid chromatographic system (Waters Ass., Milford, MA, USA) consisted of a M-45 pumping device, a WISP 710B automatic sample injector and a M-440 U.V. detector at 254 nm. A Novopack 8C18 (8 mm internal diameter, C18 coating material) cartridge column was applied for the analysis of TH and AP in plasma (second experiment), AP, AP-metabolites and TH in urine.

All GC and h.p.l.c. data were processed by a Shimadzu CR3A reporting integrator.

Data analysis

Areas under the plasma concentration-time curves (AUC) were determined by applying the linear logarithmic trapezoidal method. Intrinsic clearance (CL_{int}) was calculated by dividing dose by AUC:

$$CL_{int} = \frac{\text{Dose}}{\text{AUC}} \quad (\text{ml min}^{-1}) \quad (1)$$

It was assumed that absorption of all drugs was complete.

Clearance for the formation of metabolites of AP (CL_{->m}) was calculated as:

$$CL_{->m} = f_m * CL_{AP} \quad (\text{ml min}^{-1}) \quad (2)$$

f_m = fraction of the dose excreted as metabolite 'm', after extrapolation to infinity

The plasma elimination half-life was calculated by least-squares regression analysis starting at 2 h for AP and TH and 4 h for HB.

Statistical evaluation

Correlation analysis was performed by calculating the Pearson correlation coefficients. Regression analysis was performed by calculating the 'orthogonal' least-squares regression lines (see appendix). The chi-square test for goodness-of-fit was applied to test all clearance parameters for normality. Two-tailed Student's *t*-test and/or Wilcoxon rank sum test were applied to analyse the influence of smoking, gender and use of oral contraceptive steroids (OC) on clearance values. Wilcoxon's test was preferred when groups were compared with limited numbers of observations. Paired Student's *t*-test was applied to compare parameters of first and second experiment. For all estimations the 95% confidence level was applied.

Results

Drug analysis

It appeared to be quite possible to analyse all drugs and metabolites simultaneously when the substrates were given together, by the outlined procedures.

Calibration curves for GC-analysis were prepared by simultaneous addition of AP and HB, and for h.p.l.c. of AP and TH, to plasma. These curves were linear for AP (GC- and h.p.l.c.-analysis) in the range of 0.5 to 25 μ g ml⁻¹. For HB (GC) the calibration curves were linear from 0.05 to 10 μ g ml⁻¹ and for TH (h.p.l.c.) from 0.5 to 25 μ g ml⁻¹. Coefficients of variation of the calibration curves were all less than 5%. The variation between GC and h.p.l.c. determination of AP (second experiment) was 6%.

As a consequence of the strict caffeine abstinence no interference occurred of the caffeine metabolites paraxanthine and theobromine with the analysis of TH.

Pharmacokinetics in plasma

In Figure 1 a representative example is shown of the plasma concentration-time curves of the three substrates in one subject obtained when given simultaneously. CL_{int} and $t_{1/2}$ of AP did not differ between the first and second experiment: in the first and second experiment CL_{AP} was $42.5 \pm 12.2 \text{ ml min}^{-1}$ and $40.7 \pm 11.1 \text{ ml min}^{-1}$ and $t_{1/2}$ was $12.8 \pm 3.6 \text{ h}$ and $12.4 \pm 2.5 \text{ h}$ respectively. Also CL_{int} and $t_{1/2}$ of HB did not differ: CL_{int} in the first and second experiment was $406 \pm 163 \text{ ml min}^{-1}$ and $374 \pm 143 \text{ ml min}^{-1}$ and $t_{1/2}$ was $6.4 \pm 2.2 \text{ h}$ and $6.1 \pm 1.8 \text{ h}$ respectively. Total plasma clearance of TH was $50.4 \pm 13.4 \text{ ml min}^{-1}$, metabolic clearance $44.5 \pm 12.7 \text{ ml min}^{-1}$ and $t_{1/2}$ was $7.6 \pm 2.3 \text{ h}$. All pharmacokinetic parameters are given in Table 1.

Urinary excretion and clearance to metabolite formation

The recovery of metabolites of AP and unchanged AP in urine did not differ between both experiments (Table 1). The recovery in the first and second experiment of HMA was $15.1 \pm 3.3\%$

and $13.9 \pm 3.0\%$, of NORA $20.1 \pm 4.8\%$ and $20.8 \pm 5.7\%$ and OHA $29.6 \pm 4.8\%$ and $29.5 \pm 4.5\%$ respectively. The recovery of unchanged AP was $2.4 \pm 0.9\%$ and $3.3 \pm 1.5\%$ in the first and second experiment, hence the contribution of renal clearance to the total plasma clearance of AP was very small and the latter was therefore not corrected (CL_{int} was assumed to be equal to total plasma clearance). The total recovery of AP and metabolites in urine as $67.1 \pm 9.6\%$ and $67.5 \pm 9.5\%$ respectively in first and second experiment. Also the $CL_{>m}$ did not differ between first and second experiment for all metabolites. In the first and second experiment $CL_{>HMA}$ was $6.6 \pm 2.9 \text{ ml min}^{-1}$ and $5.8 \pm 2.5 \text{ ml min}^{-1}$. $CL_{>NORA}$ was $8.8 \pm 3.7 \text{ ml min}^{-1}$ and $8.5 \pm 3.3 \text{ ml min}^{-1}$ and $CL_{>OHA}$ was $12.7 \pm 4.6 \text{ ml min}^{-1}$ and $12.0 \pm 3.6 \text{ ml min}^{-1}$ respectively.

The recovery of unchanged TH was $12.2 \pm 5.0\%$ of the dose and total plasma clearance was corrected for this to obtain a correct estimate for metabolic clearance.

No attempt was made to analyze unchanged HB in urine, because in former studies excretion was found to be negligible (Breimer *et al.*, 1975).

Figure 1 Plasma concentration curves obtained after simultaneous oral administration of antipyrine (250 mg, ○, $t_{1/2}$ 12.1 h), theophylline (150 mg, ●, $t_{1/2}$ 7.3 h) and hexobarbitone (250 mg, △, $t_{1/2}$ 5.4 h) to one subject.

Table 1 Pharmacokinetic parameters in plasma and urinary excretion profile after administration of a cocktail of antipyrine (250 mg), hexobarbitone (250 mg) and theophylline (150 mg) to 26 healthy volunteers

	First experiment	Second experiment
<i>Clearance (ml min⁻¹)</i>		
Antipyrine	42.5 ± 12.2	40.7 ± 11.1
Hexobarbitone	405.5 ± 163.2	373.9 ± 142.6
Theophylline	—	44.5 ± 12.7
<i>Half-life (h)</i>		
Antipyrine	12.8 ± 3.6	12.4 ± 2.5
Hexobarbitone	6.4 ± 2.2	6.1 ± 1.8
Theophylline	—	7.6 ± 2.3
<i>Formation clearance (ml min⁻¹)</i>		
3-hydroxymethylantipyrine	6.6 ± 2.9	5.8 ± 2.5
Norantipyrine	8.8 ± 3.7	8.5 ± 3.3
4-hydroxymethylantipyrine	12.7 ± 4.6	12.0 ± 3.6
<i>Urinary excretion (% dose)</i>		
3-hydroxymethylantipyrine	15.1 ± 3.3	13.9 ± 3.0
Norantipyrine	20.1 ± 4.8	20.8 ± 5.7
4-hydroxyantipyrine	29.6 ± 4.8	29.5 ± 4.5
Antipyrine	2.4 ± 0.9	3.3 ± 1.5
Theophylline	—	12.2 ± 5.0

Data are presented as means ± s.d. No significant differences were observed between variables of both experiments, as assessed by the paired Student's *t*-test.

Table 2 Effect of smoking, gender and oral contraceptive steroids (OC) on clearance of antipyrine, hexobarbitone, theophylline and formation clearance to 3-hydroxymethylantipyrine (HMA), norantipyrine (NORA) and 4-hydroxyantipyrine (OHA).

<i>Clearance (ml min⁻¹)</i>	<i>Smoking</i>		<i>Gender</i>		<i>OC</i>	
	<i>No smoking n = 19</i>	<i>Smoking n = 7</i>	<i>Male n = 12</i>	<i>Female n = 14</i>	<i>No OC n = 9</i>	<i>OC n = 5</i>
Hexobarbitone	361.4 ± 129.3	407.7 ± 181.1	428.4 ± 112.5	327.1 ± 152.7	389.5 ± 148.4	214.9† ± 85.0
Antipyrine	40.3 ± 12.1	41.8 ± 8.5	47.4 ± 8.8	35.0 ** ± 9.8	38.7 ± 10.2	28.2 ± 4.2
Theophylline	43.0 ± 13.0	48.5 ± 11.8	52.0 ± 10.0	38.0 ** ± 11.2	42.2 ± 11.8	30.4 ± 4.2
HMA	5.3 ± 2.4	7.1 ± 2.7	6.9 ± 2.6	4.9 * ± 2.1	5.6 ± 2.3	3.5† ± 0.4
NORA	8.4 ± 3.4	9.0 ± 3.2	9.5 ± 2.9	7.7 * ± 3.5	8.9 ± 3.8	5.5 ± 1.4
OHA	11.2 ± 3.6	14.0 ± 3.2	13.7 ± 3.5	10.5 * ± 3.1	11.5 ± 3.4	8.7 ± 1.4

* *P* < 0.05 *,** Two-tailed Student's *t*-test

** *P* < 0.01

† *P* < 0.05 † Wilcoxon rank sum test

Data are presented on the second experiment as means ± s.d. Smoking is defined as > 5 cigarettes/day. OC = oral contraceptive steroids.

Effect of smoking, gender and contraceptive steroids on clearance values

The clearance parameters of all substrates were evaluated to investigate the influence of smoking, but no significant difference was observed for any of the investigated parameters, although there was a trend for the formation of OHA to be higher in smokers ($P = 0.06$ and 0.08 in first and second experiment respectively). Men compared with all women had higher clearance values for CL_{AP} , $CL_{>OHA}$ in the first and CL_{AP} , $CL_{>OHA}$, $CL_{>HMA}$, $CL_{>NORA}$ and CL_{TH} in the second experiment (all $P < 0.05$), but when men were compared with those women not using contraceptives (OC) no significant differences were detectable anymore between both groups. CL_{HB} , $CL_{>HMA}$ and $CL_{>NORA}$ in the first and CL_{HB} and $CL_{>HMA}$ in the second experiment were lower in females using OC than in non-OC using females (Table 2).

Evaluation of correlation data and orthogonal regression analysis

All clearance parameters for the group of 26 participants were normally distributed (chi-square test: $P > 0.05$ in all cases). The correlation results of the first and second experiment are summarized in Table 3. The correlation coefficient between CL_{int} of HB and AP in the first experiment turned out to be 0.80; it was slightly higher between CL_{HB} and $CL_{>HMA}$ 0.85 and 0.81 between CL_{HB} and $CL_{>NORA}$, but far lower than between CL_{HB} and $CL_{>OHA}$ 0.63. The correlation coefficient between CL_{AP} and $CL_{>HMA}$ was 0.88, $CL_{>NORA}$ 0.87 and $CL_{>OHA}$ 0.88.

In the second experiment the magnitude of the correlation coefficients between these parameters was of the same order (Table 3). The correlation coefficient between CL_{int} of TH and AP was high: 0.89. Between CL_{TH} and $CL_{>HMA}$ 0.80, $CL_{>NORA}$ 0.68 and $CL_{>OHA}$ 0.89, but between CL_{int} of TH and HB far lower: 0.68.

Prior to orthogonal regression analysis log transformation was applied to all variables, to correct for differences in variance. All slopes were calculated between the corresponding parameters of first and second experiment and none was significantly different from 1. Also the slope was not significantly different from 1 for the relationships between CL_{HB} and $CL_{>HMA}$ and $CL_{>NORA}$ (Figure 3), in contrast with the relationships between CL_{HB} and CL_{AP} (Figure 2) and $CL_{>NORA}$ in both experiments. The results for TH were quite different: the relationships between CL_{TH} and CL_{AP} as well as between CL_{TH} and $CL_{>OHA}$ (Figures 4 and 5) were not different from 1, whereas all others were significantly different from 1 (Table 4). In both experiments the OLS had small intercepts (significantly different from zero) for the relationships between CL_{HB} , CL_{TH} and $CL_{>m}$ of all metabolites of AP.

In Table 5 the contribution of intraindividual differences in drug oxidation to the correlation results is summarized when the cross-over design (administration of single substrate probes on different occasions to the same subjects) would have been applied. Correlation coefficients were calculated using the data of AP oxidation (i.e. CL_{AP} and $CL_{>m}$) of the first experiment and the data of TH and HB oxidation (i.e. CL_{TH} , CL_{HB}) of the second experiment. This approach simulates a study design in which AP is administered on a separate occasion. The results of the regression analysis remained the same, but most correlation coefficients were considerably lower.

Table 3 Pearson correlation coefficients between clearances of antipyrine, hexobarbitone, theophylline and formation clearances of 3-hydroxymethylantipyrine (HMA), norantipyrine (NORA), 4-hydroxyantipyrine (OHA)

	HMA	NORA	OHA	Hexobarbitone	Theophylline
<i>First experiment</i>					
Antipyrine	0.88	0.87	0.88	0.80	
Hexobarbitone	0.85	0.81	0.63		
<i>Second experiment</i>					
Antipyrine	0.87	0.77	0.74	0.82	0.89
Theophylline	0.80	0.68	0.89	0.68	
Hexobarbitone	0.87	0.84	0.77		

All $P < 0.01$

Figure 2 Relationship between clearance of antipyrine (CL_{AP}) and clearance of hexobarbitone (CL_{HB}) (first experiment). Orthogonal least-squares regression line and 95% confidence interval of the slope are:

$$Y = 0.11 + 0.59 * X \quad (0.42 < \text{slope} < 0.75), \quad (n = 26).$$

Figure 3 Relationship between the formation clearance of norantipyrine ($CL_{\rightarrow NORA}$) and clearance of hexobarbitone (CL_{HB}) (first experiment). Orthogonal least-squares regression line and 95% confidence interval of the slope are:

$$Y = 1.73 + 1.02 * X \quad (0.78 < \text{slope} < 1.26), \quad (n = 26).$$

Figure 4 Relationship between clearance of antipyrine (CL_{AP}) and clearance of theophylline (CL_{TH}) (second experiment). Orthogonal least-squares regression line and 95% confidence interval of the slope are:

$$Y = -0.17 + 1.05 * X \quad (0.83 < \text{slope} < 1.27), \quad (n = 26).$$

Figure 5 Relationship between the formation clearance of 4-hydroxyantipyrine ($CL_{\rightarrow OHA}$) and clearance of theophylline (CL_{TH}) (second experiment). Orthogonal least-squares regression line and 95% confidence interval of the slope are:

$$Y = -0.96 + 1.19 * X \quad (0.94 < \text{slope} < 1.44), \quad (n = 26).$$

Table 4 Slope of orthogonal regression analysis between clearance of antipyrine, hexobarbitone, theophylline and formation clearance of 3-hydroxymethylantipyrine (HMA), norantipyrine (NORA), 4-hydroxyantipyrine (OHA)

	<i>Antipyrine</i>	<i>HMA</i>	<i>NORA</i>	<i>OHA</i>	<i>Theophylline</i>
<i>First experiment</i>					
Hexobarbitone	0.59** 0.42–0.75	0.96 0.69–1.23	1.02 0.78–1.23	0.69* 0.39–0.99	
<i>Second experiment</i>					
Hexobarbitone	0.61** 0.43–0.80	0.97 0.70–1.24	1.11 0.73–1.49	0.67* 0.39–0.95	0.57* 0.27–0.86
Theophylline	1.05 0.83–1.27	1.49* 1.10–1.88	2.17* 1.07–3.27	1.19 0.94–1.44	

The slope and its 95% confidence interval are shown. All data were calculated after log transformation.

** $P < 0.01$ **, * Two-tailed Student's t -test

* $P < 0.05$

Table 5 Correlation of clearance of antipyrine, hexobarbitone and theophylline of the second (II) with clearance of antipyrine and formation clearance of 3-hydroxymethylantipyrine (HMA), norantipyrine (NORA), and 4-hydroxyantipyrine (OHA) of the first experiment (I): influence of intraindividual variation in drug oxidation

	<i>Antipyrine</i> I	<i>HMA</i> I	<i>NORA</i> I	<i>OHA</i> I
Antipyrine II	0.86 **	0.79 **	0.84	0.74 ***
Hexobarbitone II	0.77	0.86	0.76 *	0.65
Theophylline II	0.78 **	0.78	0.77	0.78 **

Reduction in correlation coefficient:

* 5–10%

** 10–15%

*** 15–20%

Discussion

The aim of the present study was to investigate whether any relationships exist between the rate of metabolic oxidation, represented by the intrinsic clearance and/or clearance to a metabolite, of AP, HB and TH. The three substrates were administered simultaneously in a cocktail approach to overcome the contribution of intraindividual differences in drug oxidation. This experimental strategy has proved to be feasible in previous studies in rats and in man (Van der Graaff *et al.*, 1983a; Teunissen *et al.*, 1985, 1986). For the substrates used in the present study it was assumed that complete absorption after oral administration took place and also that oral clearance equalled intrinsic clearance. Intrinsic clearance reflects the sum of activities

of enzymes involved in the metabolism of parent compounds and formation clearance to metabolites may differentiate between activities of different isozymes (Breimer, 1983).

The administration of more than two substrates together is fairly unusual, hence first the relationships between AP and HB metabolism were studied and subsequently TH was added at a second occasion to the former combination to be able to exclude any influences of TH on the oxidation of AP and HB.

The correlation coefficients between the metabolic clearance of AP and HB turned out to be 0.80 and 0.82 in the first and second experiment respectively (Table 3) indicating a fairly strong association between the rate of oxidation of both substrates. The correlation between CL_{AP} and CL_{TH} in the second experiment was even better,

again indicating a strong association between the rates of oxidation of these two compounds. Taking into account the formation clearance to the metabolites of AP in relation to CL_{HB} and CL_{TH} a differentiation seems to occur. The correlation coefficient with CL_{HB} in both experiments was highest for $CL_{\rightarrow HMA}$ and $CL_{\rightarrow NORA}$ (both > 0.81) and lowest for $CL_{\rightarrow OHA}$ and therefore the formation of OHA seems to be, at least partly, under different regulatory control. For CL_{TH} the correlation was highest with $CL_{\rightarrow OHA}$ (0.89) and lowest with $CL_{\rightarrow NORA}$ (0.68), indicating a strong association between CL_{TH} and $CL_{\rightarrow OHA}$, which is in agreement with the results obtained by Teunissen *et al.* (1985). Present results however do not demonstrate a clearcut differentiation between AP metabolite formation and the oxidation of HB and TH, and regarding the fairly high correlation coefficients between most of the clearance parameters a certain degree of overlap in substrate selectivity cannot be excluded. It is likely that there is overlap in substrate selectivity for some of the investigated metabolic routes.

More quantitative information was obtained by application of orthogonal regression analysis (Table 4). No ideal relationship existed between CL_{HB} and CL_{AP} indicating differential enzymatic oxidation, although the correlation coefficient showed a fairly high association. The oxidation of HB and formation of HMA and NORA seems to be mediated by closely linked isozymes regarding the ideal relationships. This pattern was highly reproducible in the second experiment (Table 4), which underlines the usefulness of this approach and which is an indication that the addition of TH had no effect on the relationships between the oxidation of AP and HB. Absence of interaction by TH is also supported by the ideal relationship between CL_{AP} in the first and second experiment and also between $CL_{\rightarrow m}$ of AP, CL_{HB} and their equivalents of both experiments. The slope between CL_{TH} and CL_{AP} and $CL_{\rightarrow OHA}$ were both not different from 1 (Figures 4 and 5), which seems to indicate a considerable overlap in substrate selectivity for the oxidation of AP and TH. An ideal relationship was found not only between CL_{HB} and $CL_{\rightarrow HMA}$, but also between CL_{HB} and $CL_{\rightarrow NORA}$, whereas in rats only a strong correlation between CL_{HB} and $CL_{\rightarrow HMA}$ was demonstrated (Van der Graaff *et al.*, 1983a). In the latter experiments no further discrimination in the correlation with AP metabolite formation could be obtained when the metabolite profile of HB was taken into account, indicating that closely linked or the same isozyme(s) are involved in the formation of each metabolite of HB. Also the two enantiomers

of HB seem to be metabolized by the same isozymes(s) (Van der Graaff *et al.*, 1987).

In rats and in a preliminary study in man with fewer subjects (Teunissen *et al.*, 1985, 1986) a high correlation coefficient was found between the oxidation of TH and formation of OHA from AP. In both experiments the TH metabolite profile was taken into account, but this did not discriminate further between the patterns of AP and TH oxidation. It has been suggested (Grygiel & Birkett, 1981; Grant *et al.*, 1983; Birkett *et al.*, 1983; Tserng *et al.* 1981) that more than 1 isozyme is involved in TH oxidation. Rifampicin however gives a non-selective induction of TH oxidation (Robson *et al.*, 1984), in contrast to the selective effect on AP metabolism (Toverud *et al.*, 1981; Teunissen *et al.*, 1984). Dahlqvist *et al.* (1984) found very high correlation coefficients ($r > 0.92$) between the rates of formation of the three main metabolites of TH in a group of 25 volunteers. They concluded that although the formation of the main metabolites of TH may occur partly independently, there is a large degree of overlap in factors regulating their formation. Considering these observations it was judged unnecessary for the present study to analyse the metabolites of TH.

Intraindividual differences in AP and HB oxidation could be observed: several correlation coefficients between CL_{TH} , CL_{HB} of the second experiment and CL_{AP} , $CL_{\rightarrow m}$ taken from the first were lower as compared with the correlation coefficients calculated when all data were obtained from the same experiment (Table 5). One of the advantages of the cocktail approach is that the disturbing influence of intraindividual variability with time is avoided.

The effects of gender, smoking and use of OC on all parameters of drug oxidation were investigated. No differences were observed in kinetic parameters when men were compared with women without use of OC (Table 2). Women using contraceptive steroids showed a decrease in CL_{HB} , $CL_{\rightarrow HMA}$ and $CL_{\rightarrow NORA}$ in first and CL_{HB} and $CL_{\rightarrow HMA}$ in the second experiment in comparison with non-OC using women, possibly indicating a selective inhibition of drug oxidation, which was not found in a previous study (Teunissen *et al.*, 1982). None of the parameters differentiated significantly between smokers and non-smokers (Table 2).

The results of the present study clearly indicate that the formation of HMA and NORA from AP and the oxidation of HB in healthy subjects are mediated by the same or very closely related isozymes of the cytochrome P-450 system. The formation of OHA seems to be mediated predominantly by isozymes which also oxidize TH.

It seems that in general there is a large overlap in isozymes oxidizing AP and TH as might be deduced from the ideal relationship between CL_{AP} and CL_{TH} . It is possible however that the applied regression analysis was not sufficiently powerful under the circumstances of this study, in which neither selective induction nor inhibition of drug oxidation was pursued, to achieve a further discrimination between the involved isozymes.

With the approach of simultaneous drug administration the disturbing influence of intra-individual variation in drug oxidation could be eliminated, which allows the detection of common metabolic pathways and hence a more accurate prediction of the rate of oxidation of other drugs. This cocktail approach makes it possible to characterize in one experiment activities of different isozymes of the cytochrome P-450 system, to investigate the substrate selectivity of such isozymes *in vivo* and to study factors which may differentially influence their activities.

Appendix

Orthogonal regression analysis

The technique for fitting lines to data and checking how well the line describes the data is called a linear regression method. Its purpose is often to use one variable (the independent variable, denoted 'X') to predict another (the dependent variable, denoted 'Y'). The least-squares regression line, the line which fits best the given pairs of scores, is the line for which the sum of the squares of the errors of estimation is minimized. In the case of the common least-squares regression analysis it is assumed that only the dependent variable is subject to error, hence slope and intercept of the regression line are found by minimizing the sum of squares of the observed points of the dependent variable (Godfrey, 1985). When regression analysis is applied to variables which both are subject to error, e.g. when the relationship is investigated between the clearance of two substrates or clearance for the formation of metabolites of substrates (Figures 2, 3, 4 and 5), the common least-squares regression analysis is inappropriate, because it underestimates the slope of the regression line and thereby underestimates the magnitude of the existing relationship (Riggs *et al.*, 1978; Bolton, 1984; Van der Velde, 1984; Godfrey, 1985). The model in which the variables X and Y are both subject to error and are related to each other as in eq. 3, is often called a model for a 'functional relationship' (Van der Velde, 1984). The slope and intercept

of the regression line, describing the functional relationship, are found by minimizing the sum of squares of the observed points of both variables (i.e. orthogonal instead of vertical). The relationship between the investigated variables is said to be 'ideal' when the slope of the orthogonal regression line is not significantly different from 1. Because clearance and clearance for the formation of a metabolite reflect enzyme activity it is assumed that when the slope of the relationship is not different from 1 the investigated substrates or metabolites are oxidized, respectively formed, by the same or narrowly related isozymes.

The model can be formulated as follows (Van der Velde, 1984; Patefield, 1977; Kendall & Stuart, 1973):

$$Y_i = A + BX_i \quad i = 1, \dots, N \quad (3)$$

$$y_i = Y_i + \epsilon_i \quad x_i = X_i + \delta_i \quad (4)$$

Where (x_i, y_i) are observations of the 'true' points (X_i, Y_i) on the line; ϵ_i and δ_i are the random deviations. In this model an additional assumption is made, which is often made, i.e. the standard deviations of the disturbances are the same in the two directions X and Y ($\sigma_\delta = \sigma_\epsilon$). The following computational formulae for b and a, to estimate slope B and intercept A of the OLS regression line were applied:

$$b = \frac{S_{yy} - S_{xx} + [(S_{yy} - S_{xx})^2 + 4S_{xy}^2]^{1/2}}{2S_{xy}} \quad (5)$$

$$a = \bar{y} - b\bar{x} \quad (6)$$

With:

$$\bar{x} = (\Sigma x)/N \quad \bar{y} = (\Sigma y)/N$$

N: pairs of observations

$$S_{xx} = \Sigma (x - \bar{x})^2$$

$$S_{yy} = \Sigma (y - \bar{y})^2$$

$$S_{xy} = \Sigma (x - \bar{x})(y - \bar{y})$$

The minimized sum of squared orthogonal distances of the observed points to the fitted line can be computed by:

$$SS = S_{yy} - bS_{xy}$$

The common error standard deviation s ($\sigma_\delta = \sigma_\epsilon$) is estimated by:

$$s = \left[\frac{SS}{N-2} \right]^{1/2}$$

The standard deviation of a and b can be estimated by:

$$s_b = \left[\frac{[(1 + b^2)s_{xy} + s^2b]s^2b}{Ns_{xy}^2} \right]^{\frac{1}{2}}$$

With: $s_{xy} = S_{xy}/N$

$$s_a = \left[\frac{(1 + b^2)s^2}{N} + \bar{x}^2s_b^2 \right]^{\frac{1}{2}}$$

References

- Birkett, D. J., Miners, J. O. & Attwood, J. (1983). Evidence for a dual action of sulphinpyrazone on drug metabolism in man: theophylline-sulphinpyrazone interaction. *Br. J. clin. Pharmacol.*, **15**, 567-569.
- Bolton, S. (1984). Linear regression and correlation, In *Pharmaceutical statistics. Practical and clinical applications*, ed Bolton, S., pp 181-202. New York: Marcel Dekker Inc.
- Boobis, A. R., Brodie, M. J., Kahn, G. C., Toverud, E. L., Blair, I. A., Murray, S. & Davies, D. S. (1981). Comparison of the *in vivo* and *in vitro* rates of formation of the three main oxidative metabolites of antipyrine in man. *Br. J. clin. Pharmacol.*, **12**, 771-777.
- Breimer, D. D. (1983). Interindividual differences in drug disposition. Clinical implications and methods of investigation. *Clin. Pharmacokin.*, **8**, 71-377.
- Breimer, D. D., Honnhoff, C., Zilly, W., Richter, E. & Van Rossum, J. M. (1975). Pharmacokinetics of hexobarbital in man after intravenous infusion. *J. Pharmacokin. Biopharm.*, **3**, 1-11.
- Buijs, W., Van Meeteren-Walchi, B., Breimer, D. D. & Van der Gen, A. (1986). An improved synthesis of 3-hydroxymethylantipyrine using antipyrine as starting material. *Arzneimittel Forsch./Drug Res.*, **36**, 419-421.
- Dahlqvist, R., Bertilsson, L., Birkett, D. J., Eichelbaum, M., Sawe, J. & Sjöqvist, F. (1984). Theophylline metabolism in relation to antipyrine, debrisoquine, and sparteine metabolism. *Clin. Pharmac. Ther.*, **35**, 815-821.
- Danhof, M., De Groot-Van der Vis, E. & Breimer, D. D. (1978). Assay of antipyrine and its primary metabolites in plasma, saliva and urine by high performance liquid chromatography and preliminary results in man. *Pharmacology*, **18**, 210-223.
- Danhof, M., Krom, D. P. & Breimer, D. D. (1979). Studies on the different metabolic pathways of antipyrine in rats: influence of phenobarbital and 3-methylcholanthrene treatment. *Xenobiotica*, **9**, 695-702.
- Godfrey, K. (1985). Simple linear regression in medical practice. *New Engl. J. Med.*, **313**, 1629-1636.
- Grant, D. M., Tang, B. K. & Dalow, W. (1983). Variability in caffeine metabolism. *Clin. Pharmac. Ther.*, **33**, 591-602.
- Greenblatt, D. J., Divoll, M., Abernethy, D. R., Harmatz, J. S. & Shader, R. I. (1982). Antipyrine kinetics in the elderly: prediction of age-related changes in benzodiazepine oxidizing capacity. *J. Pharmac. exp. Ther.*, **220**, 120-126.
- Grygiel, J. J. & Birkett, D. J. (1981). Cigarette smoking and theophylline clearance and metabolism. *Clin. Pharmac. Ther.*, **30**, 491-496.
- Kappas, A., Anderson, K. E., Conney, A. H. & Alvares, A. P. (1976). Influence of dietary protein and carbohydrate on antipyrine and theophylline metabolism in man. *Clin. Pharmac. Ther.*, **20**, 643-653.
- Kendall, M. G. & Stuart, A. (1973). *The advanced theory of statistics*. 3rd ed, Vol. 2, pp 375-418. London: Griffin.
- Park, B. K. (1982). Assessment of the drug metabolizing capacity of the liver. *Br. J. clin. Pharmacol.*, **14**, 631-651.
- Patefield, W. M. (1977). On the information matrix in the linear functional relationship problem. *Appl. Statistics*, **26**, 69-70.
- Riggs, D. S., Guarnieri, J. A. & Addelman, S. (1978). Fitting straight lines when both variables are subject to error. *Life Sci.*, **22**, 1305-1360.
- Robson, R. A., Miners, J. O., Wing, L. M. H. & Birkett, D. J. (1984). Theophylline-rifampicin interaction: non-selective induction of theophylline metabolic pathways. *Br. J. clin. Pharmacol.*, **18**, 445-448.
- Sjöqvist, F. (1985). Interindividual differences in drug responses: an overview. In *Variability in drug therapy. Description, estimation and control*, eds Rowland, M. & Sheiner, L. B., pp 1-11. New York: Raven Press.
- Teunissen, M. W. E., Bakker, W., Meerburg-Van der Torren, J. E. & Breimer, D. D. (1984). Influence of rifampicin treatment on antipyrine clearance and metabolite formation in patients with tuberculosis. *Br. J. clin. Pharmacol.*, **18**, 701-706.
- Teunissen, M. W. E., Brorens, I. O. N., De Langen, H. J., Geerlings, J. M. & Breimer, D. D. (1986). Correlation between *in vivo* antipyrine metabolite formation and theophylline metabolism in rats. *Pharmac. Res.*, **3**, 156-161.
- Teunissen, M. W. E., De Leede, L. G. J., Boeijsing, J. K. & Breimer, D. D. (1985). Correlation between antipyrine and theophylline metabolism in man after simultaneous single dose administration and at steady state. *J. Pharmac. exp. Ther.*, **233**, 770-775.
- Teunissen, M. W. E., Meerburg-Van der Torren, J. E., Vermeulen, N. P. E. & Breimer, D. D. (1983). Automated HPLC-determination of antipyrine and its main metabolites in plasma, saliva and urine, including 4,4'-dihydroxyantipyrine. *J. Chromatogr.*, **278**, 367-378.

- Teunissen, M. W. E., Srivastava, A. K. & Breimer, D. D. (1982). Influence of sex and oral contraceptive steroids on antipyrine metabolite formation. *Clin. Pharmac. Ther.*, **32**, 240–246.
- Toverud, E. L., Boobis, A. R., Brodie, M. J., Murray, S., Bennett, P. N., Whitmarsh, V. & Davies, D. S. (1981). Differential induction of antipyrine metabolism by rifampicin. *Eur. J. clin. Pharmac.*, **2**, 155–160.
- Tserng, K. Y., King, K. C. & Takieddinne, F. N. (1981). Theophylline metabolism in premature infants. *Clin. Pharmac. Ther.*, **29**, 594–600.
- Van der Graaff, M., Vermeulen, N. P. E., Hofman, P. H. & Breimer, D. D. (1987). Correlation of apparent intrinsic clearances of simultaneously administered S(+) and d₃R(–) hexobarbital in the rat. *Biochem. Pharmac.*, **36**, 1321–1323.
- Van der Graaff, M., Vermeulen, N. P. E., Joeres, R. P., Vlietstra, T. & Breimer, D. D. (1983a). Correlation between the *in vivo* metabolism of hexobarbital and antipyrine in rats. *J. Pharmac. exp. Ther.*, **227**, 459–465.
- Van der Graaff, M., Vermeulen, N. P. E., Langendijk, P. N. J. & Breimer, D. D. (1983b). Pharmacokinetics of simultaneously administered hexobarbital and heptabarbital in rats: an alternative approach to metabolic correlation studies. *J. Pharmac. exp. Ther.*, **225**, 747–751.
- Van der Velde, E. A. (1984). Orthogonal regression equation. In *Thromboplastin calibration and oral anticoagulant control*, eds Van den Besselaar, A. M. H. P., Gralnick, H. R. & Lewis, S. M., pp 25–39. Amsterdam: Martinus Nijhoff.
- Vesell, E. S. (1979). The antipyrine test in clinical pharmacology: conceptions and misconceptions. *Clin. Pharmac. Ther.*, **26**, 275–286.
- Vesell, E. S. (1984). New directions in pharmacogenetics. *Fed. Proc.*, **43**, 2319–2325.

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