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SEX DIFFERENCE IN ANTIPYRINE 3-HYDROXYLATION

AN *IN VIVO–IN VITRO* CORRELATION OF ANTIPYRINE METABOLISM IN TWO RAT STRAINS

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Abstract—Antipyrine metabolism depends on at least three isoenzymes of cytochrome P450 forming the main metabolites 3-OH-, 4-OH- and norantipyrine. We investigated to which extent antipyrine clearance and metabolite formation *in vivo* correlate with metabolite formation by microsomal fractions *in vitro*. The influence of sex was investigated in two rat strains. Antipyrine clearance in saliva was determined in 10-month-old Sprague–Dawley and Dark Agouti rats of either sex. Antipyrine and its metabolites in urine and microsomes were measured by a new HPLC method after solid phase or liquid extraction. Antipyrine clearance was 46% higher in males than in female rats. This was associated with a 40% higher urinary excretion of 3-OH-antipyrine in the male rats, the other metabolites being excreted to a similar extent. This higher production of 3-OH-antipyrine *in vivo* was paralleled by a higher intrinsic clearance *in vitro* while no sex difference in intrinsic clearance for the formation of the other metabolites was seen. The correlation between *in vivo* and *in vitro* metabolic clearance for 3-OH-antipyrine was good ($r = 0.75$) but unconvincing for 4-OH- ($r = 0.49$) and norantipyrine ($r = 0.01$). This could be due to further metabolism of 4-OH- and norantipyrine.

Clearance of low extraction xenobiotics is determined mainly by the so-called intrinsic clearance [1]. Although antipyrine was the first model low extraction drug [2], there is only little information about activity of the cytochrome P450 isoenzymes responsible for its metabolism [3, 4].

It has been postulated that antipyrine metabolism is subject to genetic control in man [5, 6]. However, environmental and dietary factors influence antipyrine metabolism and could easily obscure any genetic difference [7]. This polymorphism of antipyrine metabolism is apparently not linked with debrisoquine polymorphism [8] although Danhof *et al.* [9] found a 30% lower excretion of 3-OH-antipyrine in debrisoquine poor metabolizers.

Although sex affects drug metabolism in humans to some extent such effects are minor; influences of sex on drug metabolism are more readily recognized in the rat [10]. In particular, antipyrine metabolism is affected by neither sex [11] nor the menstrual cycle in man [12]. Information on sex dependence of antipyrine metabolism in the rat *in vivo* is scarce [13]; in rat hepatocytes a sex difference in antipyrine metabolite formation has recently been described [14]. Qualitative *in vitro* data show a sex difference in antipyrine cytochrome P450 isoenzyme activity [15]. No studies examine the influence of sex on antipyrine metabolism or the correlation between *in vivo* and *in vitro* metabolite formation in the rat.

We therefore investigated the influence of sex on antipyrine disposition in two strains of rats one of which exhibits deficiency of debrisoquine hydroxylation in females [16, 17]. Antipyrine salivary clearance and metabolite excretion into urine was measured *in vivo* and correlated with *in vitro* enzyme kinetics as measured by a newly developed HPLC method.

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MATERIALS AND METHODS

Chemicals. Antipyrine was from Fluka (Buchs, Switzerland), 4-hydroxy- and norantipyrine were from Aldrich (Milwaukee, IL, U.S.A.). 3-Hydroxymethylantipyrine was kindly supplied by Prof. Dr D. D. Breimer (Leiden, The Netherlands). Limpet acetone powder type I was from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Glucose-6-phosphate-dehydrogenase grade II and NADP were from Boehringer (Mannheim, F.R.G.). HPLC grade methanol, dichloromethane and acetonitril were from Rathburn (Walkerburn, U.K.). All other chemicals were of analytical grade.

Animals. Age matched Dark Agouti (DA) (Zentrales Versuchstier Institut, Hannover, F.R.G.) and Sprague–Dawley (SD) rats (Deutsche Versuchstierfarm Hartmutt-Voss, Tuttlingen, F.R.G.) of either sex were maintained on standard laboratory chow (Kliba-Futter, Basel, Switzerland) and tap water on a 12 hr light–dark cycle at 20–22° and 50–60% humidity for 10 months. One month prior to the experiments the animals were phenotyped with debrisoquine hemi-sulfate (5 mg/kg body wt free base); the result is reported as the metabolic ratio as previously described [17]. All experiments were started between 7.45 and 8.15 a.m.

Experimental design. On day 1 antipyrine 40 mg/kg was given i.p., the animals were placed in metabolic cages and urine was collected for 24 hr while allowing free access to food and water. The collecting

vial contained 0.5 g sodium azide. On day 3 antipyrine was given again (40 mg/mL, i.p.) and saliva samples were obtained at 3, 5 and 7 hr each, 5 min after pilocarpine nitrate stimulation as described by Wilson *et al.* [18].

On day 4 or 5 the microsomal fraction was prepared by differential centrifugation [19]. Cytochrome P450 content [20], NADPH dependent cytochrome *c* reductase activity [21] and protein content [22] were determined by standard techniques. Antipyrine metabolism was measured within 4 hr in a mixture containing 1 mg/mL microsomal protein, antipyrine (0–16 mM) and a NADPH generating system consisting of 5 mM glucose-6-phosphate, 1 mM NADP, 5 mM $MgCl_2$ and 2 units glucose-6-phosphate dehydrogenase in a total volume of 1 mL (pH 7.4).

The NADPH generating system containing the antipyrine and the microsomes were preincubated separately for 5 min. The microsomes were added and the mixture was incubated for 10 min at 37°. Antipyrine 3- and 4-hydroxylation were linear with time and protein from 5 to 20 min and 0.5 to 2.0 mg/mL, respectively. Nor-antipyrine formation was linear with protein but with time only up to 10 min. The metabolites were stable in the absence of NADP up to 20 min with a slight decline in 4-OH-antipyrine concentration after 10 min as reported by others [23].

The reaction was stopped with 333 μL 15% trichloracetic acid. Sodium pyrosulfite (40 mg) and 4-methylbenzoic acid (0.1 μg) were added as antioxidant and internal standard, respectively. The mixture was stored on ice and analysed within 15 min.

Assay of antipyrine in saliva. Antipyrine was extracted from saliva with dichloromethane after addition of butalbital (0.02 mg/mL) as internal standard. The organic phase was evaporated under reduced pressure and the residue dissolved in 20 μL methanol and diluted with 60 μL water. Of this solution 20 μL were injected on a 100 × 3 mm MOS Hypersil column (Shandon, Southern Instruments Inc, Astmoor, U.K.).

The HPLC-system consisted of a Waters M45 pumping device and a WISP 712 automatic sample injector, a Kratos Spectroflow 757 variable UV detector set at 244 nm and a Merck-Hitachi D-2000 chromato-integrator. To prevent peak tailing diethylamine was included in the mobile phase (acetonitril, 0.02 M phosphate buffer and diethylamine 18:82:0.5 pH 9.1). The flow was 2.0 mL/min requiring 2.5 min for a chromatogram.

The recovery averaged 104% and the coefficient of variation in samples extracted from saliva was 2.4 and 0.7% at 5 and 80 μg/mL, respectively (N = 10).

Assay of 3-OH-, *nor-*, 4-OH- *and antipyrine in urine.* The method developed by Teunissen [24] was used with some modifications. Duplicate samples were hydrolysed with 10 mg limpet acetone powder at 37° in the presence of 100 mg sodium pyrosulfite added [25]. After 3 hr incubation, samples were centrifuged. To 0.5 mL of the supernatant 0.5 mL 0.5 M acetate buffer pH 4.5, 20 μg coffein as internal standard and 200 mg NaCl were added. Samples were extracted with 5 mL chloroform/ethanol (9:1). The organic phase was evaporated under reduced pressure; the residue was redissolved in 100 μL methanol and diluted with 200 μL 0.02 M acetate buffer pH 4.5 containing 20 mg/mL sodium pyrosulfite. In this medium the metabolites were stable for at least 18 hr. Fifteen μL were injected on a Macherey-Nagel phenyl end-capped column (125 × 4 mm, 5 μm). The instrumentation as described above was used. The mobile phase was a mixture of 0.02 M phosphate buffer pH 6.5 and methanol (80:20) adjusted to pH 7.1. Flow rate was 1.8 mL/min resulting in 14 min chromatograms.

The recoveries of 3-OH-, nor-, 4-OH- and antipyrine were 103, 102, 103 and 104%, respectively. The interday coefficients of variation for 3-OH-, nor-, 4-OH- and antipyrine were 5.4% (N = 7), 3.4% (N = 4), 4.6% (N = 7) and 5.7% (N = 7), respectively.

Assay of 3-OH-, *nor- and* 4-OH-*antipyrine in microsomes.* Due to instability of 4-OH-antipyrine all subsequent steps were performed at 4°. The samples were centrifuged for 5 min at 10,000 *g*. After addition of 400 μL of 2.5 N NaOH, the 1000-fold excess of antipyrine (needed to reach V_{max} of metabolite formation) was removed with 5 mL toluol–dichloromethane (7:3). The organic layer was discarded and 1 mL of 2 M acetate buffer (pH 4.5) was added at room temperature since cooling was no longer needed after this step. Solid phase columns (J. T. Baker SPE 500 mg phenyl with stainless steel valves) were preconditioned consecutively with 2 mL methanol, 3 mL water and 2 mL 1 M acetate buffer (pH 4.5) the latter two containing 30 mg/mL sodium pyrosulfite. Then the sample was applied and the column was washed consecutively with 1 mL acetate buffer, 3 mL water, and 2 mL hexane. Metabolites were eluted with 2 × 2 mL methanol. Before evaporation under reduced pressure 100 μL of a mixture of glycerine–methanol (1:1) was added.

The residue was dissolved as described above for urine and 15 μL were injected onto a Macherey-Nagel phenyl column (250 × 4 mm, 7 μm) heated at 35° and flow set at 1.8 mL/min. A typical chromatogram of a microsomal sample and a standard curve is shown in Figs 1 and 2, respectively.

The recoveries of 3-OH-, nor- and 4-OH-antipyrine from spiked microsomes were 109, 98 and 83%, respectively. The coefficients of variation of a 0.5 μg/mL sample of 3-OH-, nor- and 4-OH-antipyrine were 8.6, 8.4 and 7.7%, respectively. The corresponding values of a 7 μg/mL sample were 2.4, 5.5 and 4.7%, respectively.

Calculations. The disappearance of antipyrine from saliva could adequately be described by a one-compartment open model. Consequently half-life ($T_{1/2}$) was determined for each animal by least square regression analysis of the log–linear saliva concentration versus time curve. The volume of distribution was determined by dividing the dose through the extrapolated antipyrine concentration at time 0. The clearance (*Cl*) was calculated as:

$$Cl = \frac{\ln 2 \,.\, V_d}{T_{1/2}}. \qquad (1)$$

The amount of metabolite excreted in urine was expressed as molar per cent of the dose. Partial

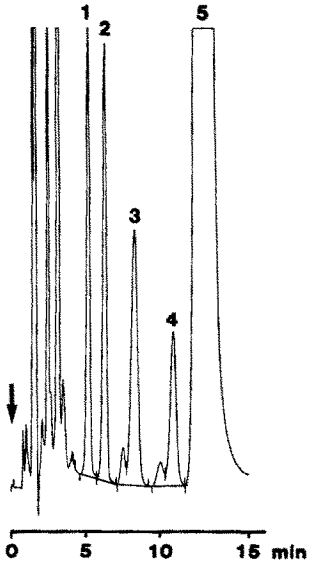

Fig. 1. Chromatogram obtained on a microsomal extract after incubation with antipyrine. (1) 3-OH-antipyrine; (2) internal standard (4-methylbenzoic acid); (3) norantipyrine; (4) 4-OH-antipyrine; (5) antipyrine.

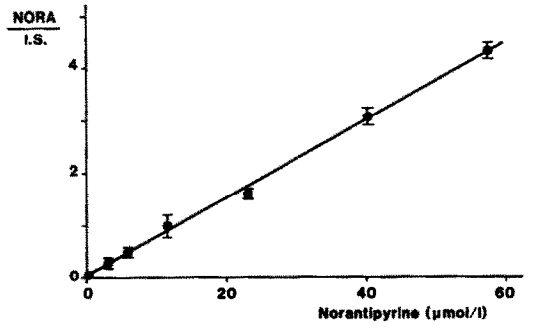

Fig. 2. Standard curve of norantipyrine (NORA) expressed as a function of the ratio of the area of NORA to internal standard (N = 5). Mean ± SD are given.

clearance for production of the metabolites (Cl_m) was calculated as the product of the total clearance (Cl) and the fraction of the dose excreted as that metabolite (f_m) in 24 hr by the equation:

$$Cl_m = Cl \cdot f_m. \quad (2)$$

Clearance values were normalized per kg of body weight.

The apparent V_{max} and K_m of microsomal enzyme kinetics were calculated by non-linear fitting to the Michaelis–Menten equation [26].

The intrinsic clearance (Cl_{int}) of the whole liver was calculated as the ratio of V_{max}, expressed per mole cytochrome P450 and K_m multiplied by the total amount of cytochrome P450 in liver by the equation:

$$Cl_{int} = \frac{V_{max}}{K_m} \cdot \text{cytochrome P450}. \quad (3)$$

Statistical analysis. All results are expressed as mean ± SD. Differences between the four groups were evaluated by Peritz' F-test [27]. Linear regression analysis was carried out by the method of least squares [28]. $P < 0.05$ was considered statistically significant.

RESULTS

Since the groups were age-matched (10 months) they varied considerably in body weight: it averaged 758 ± 58, 331 ± 9, 429 ± 40 and 199 ± 9 in SD males, DA males, SD females and DA females, respectively. The corresponding liver weights were 24.6 ± 2.9, 8.7 ± 0.9, 12.8 ± 1.7 and 5.5 ± 0.5 g. As previously described by others and our own group [16, 17], female DA rats were poor metabolizers of debrisoquine; the metabolic ratio (debrisoquine/4-OH-debrisoquine in 24 hr urine) was 1.0 ± 0.1, 0.3 ± 0.1, 0.3 ± 0.1 and 2.5 ± 0.6 in the four groups.

Antipyrine metabolism in vivo

The results of antipyrine elimination in saliva are shown in Table 1. There was a clear-cut sex difference in salivary antipyrine clearance, the clearance of females being about half that of males ($P < 0.02$). The half-life of antipyrine was similar in all groups except for the shorter $T_{1/2}$ in DA males ($P < 0.01$).

Clearance for production of the antipyrine metabolites is reported in Table 2. No effect of debrisoquine polymorphism on metabolism formation *in vivo* could be detected.

Males of either strain excreted significantly more

Table 1. Pharmacokinetics of salivary antipyrine elimination

Rats	$T_{1/2}$ (min)	V_d (L/kg)	Cl (mL/min/kg)
SD male	177 ± 26‡	1.38 ± 0.61	5.4 ± 2.0*
DA male	128 ± 14†	1.12 ± 0.20	6.1 ± 1.3†
SD female	206 ± 63	0.93 ± 0.45	3.0 ± 0.6
DA female	184 ± 20	0.87 ± 0.07	3.3 ± 0.2

Mean ± SD are given.
Male versus female: * $P < 0.05$, SD male versus SD female; † $P < 0.05$, DA male versus DA female.
SD versus DA: ‡ $P < 0.05$, SD male versus DA male.

Table 2. Clearance for production (mL/min/kg) in the different groups

Groups	3-OH	NORA	4-OH
SD male	24.8 ± 3.4*	5.1 ± 1.1	14.7 ± 1.9
DA male	23.8 ± 0.3†	8.1 ± 2.7†	15.9 ± 2.5†
SD female	14.8 ± 3.2	7.0 ± 2.5	18.1 ± 2.6
DA female	12.9 ± 1.3	5.8 ± 1.4	17.9 ± 1.2

Mean ± SD are given.
* $P < 0.05$, SD male versus SD female.
† $P < 0.05$, DA male versus DA female.

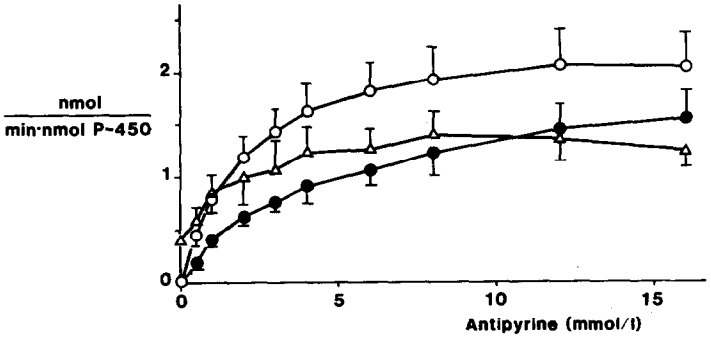

Fig. 3. Antipyrine metabolite formation by hepatic microsomes of male SD rats (N = 4). 3-OH-antipyrine (○), norantipyrine (●), 4-OH-antipyrine (△). Mean ± SD are given.

3-OH-antipyrine into urine than the females ($P < 0.001$) while nor- and 4-OH-antipyrine showed no sex difference. Males excreted significantly less unchanged antipyrine than did the females ($P < 0.02$, data not shown).

Microsomal metabolism of antipyrine

The characteristics of the microsomal preparations were comparable in the four groups: the relative specific activity of the microsomal marker, cytochrome *c* reductase, was 4.1 ± 0.8, 4.1 ± 0.5, 4.9 ± 3.6 and 5.0 ± 1.3 in male SD, DA, female SD and DA rats, respectively. The corresponding cytochrome P450 content was 0.93 ± 0.21, 0.67 ± 0.16, 0.65 ± 0.13 and 0.48 ± 0.09 nmol/mg (not significant).

The kinetics of the microsomal production of the three metabolites from male SD rats are shown in Fig. 3. The apparent V_{max} and K_m for all groups are reported in Table 3; there was no major difference apparent when expressed per nmole cytochrome P450 (Table 3). The intrinsic clearance of 3-OH-antipyrine, in contrast, showed a clear-cut sex difference (Table 4), the males having a higher intrinsic clearance than the females ($P < 0.01$).

Clearance for production of 3-OH-antipyrine *in vivo* was closely correlated with intrinsic clearance *in vitro* ($r = 0.748$; see Fig. 4). The correlation was not evident for norantipyrine ($r = 0.493$) or 4-OH-antipyrine ($r = 0.013$; data not shown).

Table 4. Intrinsic clearance (mean ± SD) of *in vitro* antipyrine metabolite formation (μL/min/liver)

Rats	3-OH	NORA	4-OH
SD male	182.0 ± 63.3*‡	63.9 ± 24.8*‡	296 ± 80
DA male	42.1 ± 12.9†	9.9 ± 5.1	79 ± 7
SD female	36.4 ± 18.6	14.0 ± 3.3	211 ± 78
DA female	8.4 ± 3.8	4.6 ± 1.3	67 ± 26

Male versus female: * $P < 0.05$, SD male versus SD female; † $P < 0.05$, DA male versus DA female.
SD versus DA: ‡ $P < 0.05$, SD male versus DA male.

DISCUSSION

We investigated to what extent sex and debrisoquine polymorphism affect antipyrine metabolite formation *in vivo* and *in vitro*. We found a clear-cut sex difference in salivary antipyrine clearance: this was mostly due to a sex-linked difference in 3-hydroxylation both, *in vivo* and *in vitro*, the males exhibiting more active metabolism. Debrisoquine polymorphism did not affect antipyrine metabolism similar to studies in man [9].

The clearance of antipyrine was estimated in saliva, a well established method in both man [7, 29] and rat [18, 30, 21] allowing estimation of antipyrine disposition without the stress of surgery or anesthesia. The clearances and urinary metabolite

Table 3. Kinetic parameters of antipyrine metabolite formation by rat liver microsomes *in vitro* (mean ± SD)

	3-OH		NORA		4-OH	
Group	V_{max}*	K_m†	V_{max}	K_m	V_{max}	K_m
SD male	2.49 ± 0.29	2.65 ± 1.4	1.95 ± 0.35	4.7 ± 0.7‡	1.4 ± 0.19‡	0.7 ± 0.26‡
DA male	1.84 ± 0.62	1.73 ± 0.29	2.35 ± 0.79	10.9 ± 4.8	3.0 ± 1.17	1.4 ± 0.45
SD female	1.59 ± 0.38	2.00 ± 0.28	2.00 ± 0.70	8.0 ± 3.0	1.4 ± 0.28	0.3 ± 0.28
DA female	1.36 ± 0.44	2.05 ± 0.52	3.79 ± 1.22	10.2 ± 2.8	2.1 ± 0.75	0.4 ± 0.20

* Values in nmole/min/nmole cytochrome P450.
† Values in mM.
‡ $P < 0.05$, SD male versus DA male.

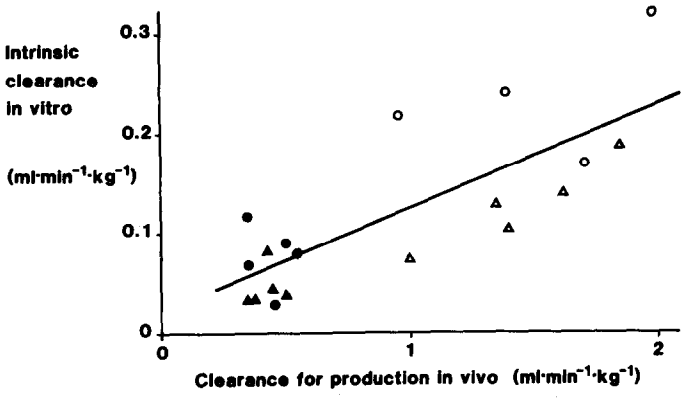

Fig. 4. *In vitro* intrinsic clearance (Cl_{int}) versus clearance for production of 3-OH-antipyrine *in vivo*. Male SD (○), male DA (△), female SD (●), female DA (▲). The regression equation was $y = 0.0205 + 0.104\ x$ ($r = 0.748$; $P < 0.001$).

pattern in male rats in our study are in agreement with most studies [32–35]; some authors reported a higher clearance in male [36] or female [37] rats. It is unlikely that this is due to the older age (10 months) of our animals. The rats were studied at this age since the debrisoquine hydroxylation deficiency is not manifest in young DA rats [16, 17]. Although some sex-linked differences in cytochrome P450 disappear in old rats [38, 39] this occurs only in rats of 12 months and older [39, 40].

The method of Wilson for measurement of antipyrine in saliva [18] applies saliva directly onto the column; this procedure led to an increased broadening of the antipyrine peak and very short column life in our hands. Our new method is accurate (coefficient of variation 2.4% at 5 μg/mL), selective, rapid and easy.

Antipyrine metabolites in urine were measured using a modification of the method of Teunissen *et al.* [24]; slightly different HPLC conditions gave better separation without tailing at the expense of somewhat longer chromatograms. To improve the recovery of norantipyrine more sodium pyrosulfite during hydrolysis was added as described by Nakagawa and Nakomura [25]. Under these conditions the metabolites were stable for at least 18 hr in solution permitting use of an automatic sample injector.

To measure metabolite formation by microsomes *in vitro* a new method was developed. We had to overcome the problems of removing the 1000-fold excess of antipyrine and of the notorious instability of 4-OH-antipyrine. The method of Kahn *et al.* [3] requires triple extraction of excess antipyrine at pH 13 and fractional collection of radioactivity. The elegant method of Nakagawa *et al.* [15] requires double labelled antipyrine and a GC/MS. The method described in the present investigation permits estimation of enzyme kinetics without labeled compounds using a simple, isocratic HPLC system.

Male rats of both strains had a 46% higher salivary clearance than the females (Table 1); this is partly due to the higher excretion of 3-OH-antipyrine by males. Similar sex specific urinary excretion rates were also found by Inabe *et al.* [13]. These investigators found an increase in 4-OH-antipyrine excretion in the female rat: in our study this was demonstrable in the SD, but not in the DA rat. In contrast, norantipyrine excretion is unaffected by sex.

The kinetic parameters for the formation of 3-OH- and norantipyrine *in vitro* for male rats are in close agreement with the literature [41].

The intrinsic clearances determined *in vitro* (Table 4) are different between the strains; this is mainly due to the differences in body and liver size. Only the intrinsic clearance of 3-OH-antipyrine showed a sex difference in both strains. This is in agreement with Nakagawa *et al.* [15] who purified hepatic cytochrome P450 and found that males have an isoenzyme producing 3-OH-antipyrine that is absent in the female. Similarly, Loft and Poulson [14] found a sex-dependence of 3-OH-antipyrine formation in isolated hepatocytes. Sex-dependent phase I drug metabolism in the rat is well known; this topic has recently been reviewed by Skett [10].

The *in vivo* clearance for production of 3-OH-antipyrine correlates well with the intrinsic clearance measured *in vitro* ($r = 0.75$). A similar *in vivo–in vitro* correlation was shown in humans by Boobis *et al.* [4]. They argue that 3-OH-antipyrine is metabolized further to 3-carboxyantipyrine thereby obscuring a better *in vivo–in vitro* correlation. 3-Carboxyantipyrine is only a minor metabolite making up for 1.1% of the dose of antipyrine in the rat and is probably formed from 3-OH-antipyrine [2]. For norantipyrine and 4-OH-antipyrine our *in vivo–in vitro* correlations are not very convincing suggesting that factors other than hepatic isoenzyme activity determine nor- and 4-OH-antipyrine formation.

In rats only about 50–65% of the dose is excreted as 3-OH-, nor- and 4-OH-antipyrine [24, 41]. Double oxidized metabolites of antipyrine have been described: 4,4′-Dihydroxy-antipyrine makes up for 15% of the total dose excreted in urine of rats. This results in part from oxidation of 4-OH-antipyrine [24]. Norantipyrine is rapidly metabolized by microsomes *in vivo*; this could also take place *in vivo* [7]. Further oxidation of 4-OH- and norantipyrine could explain the poor *in vivo–in vitro* correlation [42].

In conclusion, we report a sex-linked difference in

salivary antipyrine clearance, urinary excretion and *in vitro* metabolism of 3-OH-antipyrine in two rat strains the males being the more active metabolizers.

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