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METABOLISM OF METRONIDAZOLE AND ANTIPYRINE IN ISOLATED RAT HEPATOCYTES

INFLUENCE OF SEX AND ENZYME INDUCTION AND INHIBITION

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Abstract—The metabolism of metronidazole and antipyrine was investigated in freshly isolated hepatocytes from 7 male and 6 female control Wistar rats, 8 males and 5 females pretreated with phenobarbital (PB) and 3 males pretreated with 3-methylcholanthrene (MC). Pretreatment with PB increased the intrinsic clearance ($CL_i = V_{max}/K_m$) of metronidazole to its acetic acid (MAA) and hydroxy metabolite (HM) 7- and 2.8-fold in the males and 3.2- and 3.0-fold in the females, whereas MC treatment increased the values 9- and 10-fold, respectively ($P < 0.05$). The CL_i of metronidazole to HM and its glucuronide conjugate was higher in the control and PB treated male than in the corresponding female groups, whereas the rank order was reversed for sulphate formation ($P < 0.05$). SKF 525A was a more potent inhibitor of MAA formation than of HM formation, except in the PB treated male group. Pretreatment with MC increased the inhibitory potency of α-naphthoflavone and antipyrine toward MAA and HM formation.

In male rats PB treatment increased the CL_i of antipyrine to 3-hydroxymethyl-(HMAP), nor-(NORAP) and 4-hydroxyantipyrine (OHAP) 2.5-, 2.1- and 4.5-fold, respectively ($P < 0.05$). Pretreatment with MC in male and with PB in female rats had no significant effect on antipyrine metabolism. SKF 525A was a more potent inhibitor of HMAP and OHAP formation than of NORAP formation. Treatment with MC increased the inhibitory potency of α-naphthoflavone toward the formation of all antipyrine metabolites. Metronidazole increased the formation rate of HMAP, but inhibited the formation of NORAP and OHAP, particularly the latter.

The results suggest that the formation of MAA, HM, HMAP, NORAP and OHAP from metronidazole and antipyrine is catalyzed by different cytochrome P-450 isozymes, which may be supplemented or substituted by PB or MC induced species. The involved P-450 isozymes have more or less overlapping substrate and product specificity. Metronidazole appears to be a sensitive probe for detection and identification of PB and MC type induction.

We have recently proposed the antimicrobial, metronidazole, as a probe for the study of drug metabolism capacity [1]. In man different cytochrome P-450 isozymes appear to be involved in the metabolism of metronidazole and the well-known probe, antipyrine [1, 2]. The two drugs have similar pharmacokinetic properties and their simultaneous use in a probe cocktail is right at hand. However, although the metabolism of metronidazole has been studied in man and some rodents [2–7], basal knowledge, such as kinetic constants and reactions to specific inducers and inhibitors of the involved enzymes, are not available. For the further evaluation of metronidazole as a probe, such information is warranted for comparison with antipyrine in appropriate model systems. Michaelis–Menten kinetics cannot be determined *in vivo* since sufficiently high steady-state substrate concentrations are unattainable. Isolated rat hepatocytes is a well-established *in vitro* system for the study of phase I and II drug metabolism and do not require reconstitution [8].

After administration of metronidazole three major metabolites are excreted into the urine of both man and rats, although some quantitative differences appear. Thus, in rats the predominant metabolism of metronidazole appears to be conjugation [4–6], whereas oxidative formation of the hydroxy (HM)§ and acetic acid (MAA) metabolite is the major route of elimination in man [2, 3, 7]. Antipyrine metabolism has previously been studied in isolated rat liver cells, but the basal kinetic constants are not available [9, 10].

So far, the rate of hydroxylation of metronidazole has been reported to be increased by phenobarbital (PB) treatment in man [3], whereas the rate of glucuronidation was increased in isolated perfused livers from rats and in intact mice by this pretreatment

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§ Abbreviations used: MAA, metronidazole acetic acid; HM, hydroxymetronidazole; GM, metronidazole glucuronide; SM, metronidazole sulphate; HMAP, 3-hydroxymethylantipyrine; NORAP, norantipyrine; OHAP, 4-hydroxyantipyrine; K_m, affinity constant; V_{max}, maximal velocity; CL_i, intrinsic clearance; K_i, competitive inhibition constant; PB, phenobarbital; 3-MC, 3-methylcholanthrene; NF, α-naphthoflavone; BSA, bovine serum albumin; EGTA, ethylenglycol-bis-(β-aminoethyl ether)-tetraacetic acid; BW, body weight.

[3, 6]. Whether these effects are due to changes in the maximal velocity (V_{max}) or affinity constant (K_m) of the involved enzymes are not known, neither is the effect of polyaromatic hydrocarbon-type inducers. The formation of metabolites from antipyrine is differentially affected by pretreatment with the inducers PB and 3-methylcholanthrene (3-MC) [11–14]. In man the rate of metabolite formation from metronidazole was not affected by concomitant treatment with cimetidine, a well-known inhibitor of the oxidative metabolism of many drugs, including antipyrine [3]. Moreover, single doses of metronidazole and antipyrine do not affect either's formation of metabolites and the clearances do not seem to be co-regulated [3, 15]. Further investigation of the substrate selectivity of the involved enzymes includes determination of mutual inhibition constants *in vitro*.

In the present study we investigated the effect of specific enzyme inducers and inhibitors on the metabolism of metronidazole and antipyrine and their mutual inhibition in freshly isolated hepatocytes from male and female rats.

MATERIALS AND METHODS

Chemicals. Collagenase was purchased from Worthington. Glucurase®, Glusulase®, bovine serum albumine (BSA) and ethylenglycol-bis-(β-aminoethyl ether)-tetraacetic acid (EGTA) were from Sigma (St. Louis, MO). Reference compounds for analysis of metronidazole and metabolites were kindly donated by Rhone-Poulenc Pharma (Vitry, France). Reference compounds for the analysis of antipyrine and metabolites were from EGA-Chemie (Steinheim, F.R.G.) or a kind gift from Drs. Danhof, Eichelbaum and Yoshimura (3-hydroxymethylantipyrine, HMAP). All solvents were of analytical or chromatographic grade and purchased from Merck (Darmstadt, F.R.G.). Probenecid and PB were obtained from the Danish Hospital Pharmacies, whereas 3-MC and α-naphthoflavone (NF) were purchased from Aldrich (Milwaukee, WI). Proadifen hydrochloride, SKF 525A, was a kind gift from Smith, Kline and French (Welwyn Garden, U.K.).

Cell preparation. Laboratory breed male (225–275 g) and female (200–250 g) Wistar rats were housed under constant temperature and humidity in a 12 hr light cycle with free access to food (Altromin®) and tap water. Seven male and 6 female rats served as controls, whereas 8 males and 5 females were pretreated with PB 1 mg ml^{-1} in the drinking water for 7 days and 3 males were pretreated with 3-MC 25 mg kg^{-1} i.p. for 3 days. Thiopental 9 mg per kg body weight (BW) i.p. was used for anesthesia. Heparin 250 IU per kg BW was injected in the inferior caval vein.

Liver perfusion was performed as described by Hogberg and Kristoferson [16] and Moldeus [8]. After cannulation of the portal vein the liver was excised and moved through a perfusion system consisting of 3 beakers with different buffers, a bubble oxygenator and a peristaltic pump delivering 100 ml/min. The flow was adjusted by changing gas flow and/or a shunt to a pressure of 10–15 cm H_2O. Carbogen gas (95% O_2; 5% CO_2) was used for bubbling of all solutions and incubations. The rat liver was perfused for 4 min with buffer A, a modified Hanks' solution, pH 7.4, with EGTA 0.5 mM, for 6 min with buffer B, a modified Hanks' solution with collagenase 0.8% and Ca^{2+} 4 mM, and for 2 min with buffer C, a Krebs–Henseleit buffer, pH 7.4.

Hepatocytes were liberated from the liver by blunt dissection and dispersed by shaking for 15 min in buffer C with 1% BSA in a carbogen atmosphere. After filtration through nylon gauze the cells were centrifuged at 15 G and washed twice with buffer C. The cell yield as counted in a Burker chamber was 200–500 × 10^6 per liver. The cell viability as assessed by Trypan Blue exclusion was 92 ± 2%, 93 ± 3%, 91 ± 1% in the three male groups as opposed to 85 ± 2% and 83 ± 2% in the two female groups (mean ± SD, $P < 0.05$). The significantly higher viability in the male rat liver cells irrespective of the pretreatment is not readily explained, but was regarded as being without importance, since a standardized number of viable hepatocytes was used and cells subjected to preincubation without carbogen gas until all took up Trypan Blue did not form detectable amounts of metabolites.

Incubations. Hepatocytes were incubated in buffer C with 1% BSA in 50 ml round-bottomed flasks, each containing 6 × 10^6 viable cells. Up to 12 flasks could be mounted on each of three rotary evaporator adapters with humidified carbogen gas introduced via plastic tubing through the vacuum exit and rotated at 20 rpm and a 45° angle through a bath (37°) [8, 16]. In some experiments with high cell yield a second series of incubations was run around 2 hr after isolation as the hepatocytes could be left in a carbogen gas atmosphere at room temperature without loss of viability or metabolic activity during the first series.

Metronidazole (0.05–10 mM) and/or antipyrine (0.05–20 mM) were added to a final volume of 3 ml in each flask. Flasks with both drugs contained combinations of 0.2, 1, 5 or 10 mM of each. The inhibitors SKF 525A and NF in concentrations of 25 and 500 μM were coincubated with flasks containing either metronidazole or antipyrine (1 and 5 mM), whereas probenecid (25 and 500 μM) was coincubated with metronidazole (1 and 5 mM only). The incubations were planned so that the Michaelis–Menten constants could be determined in each of all rats and the inhibition constants in each of 3–5 from each group with at least 8 available values for comparison between two relevant groups. Due to technical difficulties the inhibition constant of NF was determined in only 3 male control rats.

Samples (750 μl) were collected after 30, 60 and 120 min of incubation and immediately centrifuged at 4000 *g*. The supernatant was kept at −20° until analysis. The viability as assessed by Trypan Blue exclusion and pH was constant throughout the incubation. In pilot studies the formation of metabolites was linear with respect to cell number up to 4 × 10^6 cell ml^{-1}. Analysis of ultrasonicated cell pellets after centrifugation of samples showed similar intra- and extracellular concentrations of substrates and metabolites.

Analytical procedures. The concentrations of metronidazole and antipyrine and their metabolites

in the incubation medium were determined by modifications of previously-described HPLC procedures [2, 7].

The samples from the metronidazole experiments were extracted with 9 volumes of chloroform. Measuring samples and blind medium spiked with the reference compounds before and after extraction it was estimated that the procedure left 20% of the mother compound and 80% of the hydroxy metabolite in the nonorganic phase, whereas the acetic acid metabolite and conjugates were not detectably extracted. Before and after incubation for 16 hr with glucuronidase/arysulfatase 200/5 IU ml^{-1} (Glusulase® Type H-2 from *Helix pomatia*) the nonorganic phase was mixed with acetonitrile and the supernatant after centrifugation and part-evaporation was injected on a Spherisorb ODS 5 μm chromatographic column. The eluent was methanol (6% v/v) and acetonitrile (4% v/v) in phosphate buffer pH 4.0 monitored at 320 nm.

In the chromatograms of medium not subjected to enzymatic hydrolysis a peak eluted between the MAA and HM peaks. After incubation with pure bovine glucuronidase (Glucurase®) or glucuronidase/arylsulfatase this peak disappeared and the size of the metronidazole peak increased. The area of the unknown peak and the gain in the metronidazole correlated significantly (regression slope = 1.18; intercept = 3.3 μM; $r = 0.95$; $P < 0.000001$, $N = 152$). On this basis, it was assumed that the unknown peak represented a glucuronide conjugate of metronidazole with molar extinction coefficient identical to the mother compound. The concentration determined from the area of the supposed glucuronide peak and metronidazole as external reference, was used for the kinetic analysis.

In samples with high substrate concentrations another peak eluted close to the supposed glucuronide peak. In the chromatograms of medium from female rat hepatocytes incubated with metronidazole this peak was of a much larger size than that encountered in experiments with male rats. The peak was unaffected by enzymatic treatment, but disappeared after acid hydrolysis at 90° for 3 hr. Due to effects of this procedure on the chromatograms and the relatively small amount of the assumed metabolite its concentration could not be estimated as the difference in the concentration of mother compound with and without acid hydrolysis. It was assumed that the peak represented a sulphate conjugate, which was tentatively quantitated with metronidazole as external reference, just like the glucuronide, assuming identical molar extinction coefficients of mother compound and both conjugates. As a reference compound is not available the evidence of the identity of the peak as a sulphate conjugate rests on its disappearance only after acid hydrolysis and its dominating presence in medium from female hepatocytes. Thus, several studies have reported that sulphates of metronidazole are excreted into the urine in female rats to the same extent as glucuronides [4, 17], whereas this has not been demonstrated in male rats [5]. It has been reported for other drugs, such as paracetamol, that the sulphate and glucuronide conjugates have a molar extinction coefficient identical with their mother compound, which is used for their quantitation by HPLC with UV-detection [8]. The detection limit of the metronidazole metabolites was less than 0.2 μM and the inter-assay coefficient of variation less than 8%.

The samples from the antipyrine experiments were subjected to enzymatic hydrolysis and extracted with chloroform/ethanol after saturation with NaCl or mixed with acetonitrile and the organic solvents were evaporated under reduced pressure. The chromatographic column was a Waters Novapak® C18 radialpak and the eluent was methanol (35% v/v) in phosphate buffer pH 7.8 monitored at 254 nm. The limit of detection of the antipyrine metabolites was less than 1 μM and the coefficient of variation less than 8%.

Calculations. The rate of increase in the concentration of each metabolite was compared between the three sampling time points by three-factorial analysis of variance with substrate concentration and animal as the two other factors. Scheffe's test was used for *post hoc* comparison of means.

The V_{max} and K_m were determined for the formation of each metabolite in the hepatocytes from each rat by weighted derivative free nonlinear regression analysis (BMDP AR, Berkeley University, CA) to fit the equation:

$$v = V^*_{max}S/(K_m + S),$$

where v is the velocity of the reaction at substrate concentration S. At low concentrations significant amounts of substrate were metabolized during the incubation and the logarithmic average concentration was used for the calculations:

$$S = (Si - Se)/(\ln Si - \ln Se),$$

where Si and Se are the substrate concentrations initially and at the end of incubation, respectively. The velocity (v), expressed as product formed per min per 10^6 viable hepatocytes, was calculated from the metabolite concentrations measured after 120 and 30 min incubation with metronidazole and antipyrine, respectively. The intrinsic clearance (CL_i) for production of metabolites was calculated as V_{max}/K_m.

The inhibitory effect on metabolite formation of coincubating metronidazole and antipyrine with each other with one of the three known drug metabolism inhibitors was determined by weighted derivative free nonlinear regression to fit the equations:

$$v = V^*_{max}S/(K^*_m(1 + I/K_{is}) + S) \quad (1)$$

$$v = V^*_{max}S/(K_m + S^*(1 + I/K_{ii})) \quad (2)$$

$$v = V^*_{max}S/(K^*_m(1 + I/K_i) + S^*(1 + I/K_i)) \quad (3)$$

$$v = V^*_{max}S/(K^*_m(1 + I/K_{is}) + S^*(1 + I/K_{ii})) \quad (4)$$

where v is the velocity, S is the substrate concentration, I is the inhibitor concentration and K_{is}, K_{ii} and K_i are the incubation constants in competitive (1), uncompetitive (2), classical noncompetitive (3) and modern noncompetitive (4) inhibition, respectively.

The kinetic constants were compared between the groups by means of Kruskal–Wallis one-way analysis of variance. If this was significant differences between individual groups were tested by a two-sided Mann–Whitney U-test. The inhibition constants of NF were tested between the male control and the 3-MC pretreated group by one-sided test, according to the expectation of a decrease. Correlations were analyzed by the Spearman rank method. P values less than 0.05 were considered statistically significant.

RESULTS

In the suspensions of rat hepatocytes from all treatment groups the metabolites of metronidazole appeared at constant rate throughout the 120 min of incubation. The accumulation rate of antipyrine metabolites, in particular of norantipyrine (NORAP), decreased significantly in the last 60 min of incubation in agreement with observations in cultured hepatocytes [10]. In contrast to the *in vivo* situation almost all the NORAP and about half of the 4-hydroxyantipyrine (OHAP) appeared in their free form, which are known to be unstable [18, 19]. Accordingly, the values obtained after incubation for 120 and 30 min were used for kinetic analysis of metronidazole and antipyrine metabolism, respectively. The formation rates of metabolites from both drugs followed Michaelis–Menten kinetics (Figs 1 and 2). For the description of the effect of the inhibitors the fit to the competitive model was in all cases as good as, or better than, the other investigated models, i.e. gave the smallest regressional residual sums of squares. Thus, K_{is} is the inhibition constant reported here. The error risk introduced by estimating the K_{is}s for SKF 525A NF and probenicid from combinations of only two substrate and two inhibitor concentrations was much less than the inter-individual variation, which caused a considerable type 2 error risk, when comparing the K_{is} between the groups.

Metronidazole metabolism

In the hepatocytes from PB pretreated male rats the median oxidative CL_i of metronidazole to MAA and HM was 7- and 3-fold increased compared to control values, whereas in those from the male rats pretreated with 3-MC it was 9- and 10-fold increased, respectively (Figs 1 and 3). The effect of PB treatment on the CL_i appeared as increases in V_{max} for formation of both MAA and HM together with a smaller increase in K_m for HM formation (Table 1, Figs 1 and 3). Pretreatment with 3-MC increased V_{max} for HM formation without a significant change in K_m, which tended to decrease and was significantly lower than after PB treatment (Table 1). The V_{max} and K_m values of MAA formation showed a similar pattern in the 3-MC treated rats although the changes did not reach statistical significance (Table 1). Moreover, as shown in Table 3 the inhibitory potency of SKF 525A toward HM formation was 50-fold increased in the hepatocytes from PB treated male rats in accordance with its preference for PB induced P-450 isozymes [20]. In the male control group the inhibitory potency of SKF 525A toward MAA for-

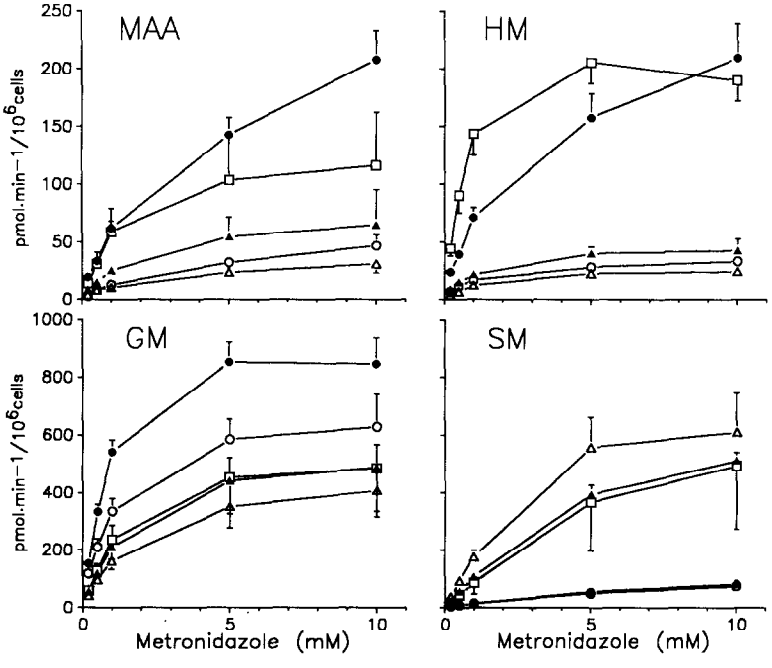

Fig. 1. Relationships between concentration of metronidazole and formation of its acetic acid (MAA) and hydroxy (HM) metabolite and glucuronide (GM) and sulphate (SM) conjugate in isolated hepatocytes from male control (○); phenobarbital pretreated male (●); 3-methylcholanthrene pretreated male (□); control female (△); and phenobarbital pretreated female (▲) Wistar rats. Values are mean with SEM of 3–8 rats.

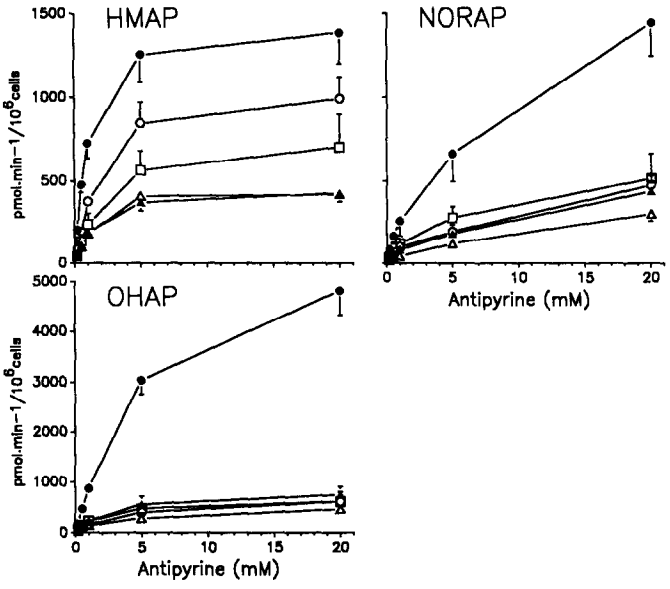

Fig. 2. Relationship between concentration of antipyrine and formation of 3-methylhydroxy- (HMAP), nor- (NORAP) and 4-hydroxyantipyrine (OHAP) in isolated hepatocytes from male control (○); phenobarbital pretreated male (●); 3-methylcholanthrene pretreated male (□); control female (△); and phenobarbital pretreated female (▲) Wistar rats. Values are mean with SEM of 3–8 rats.

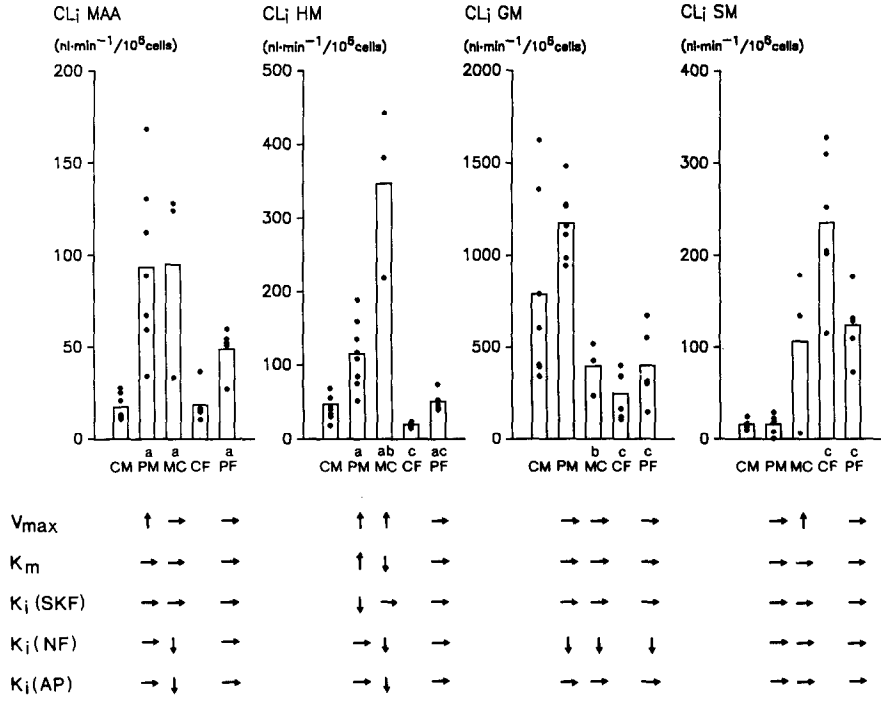

Fig. 3. Individual and average (boxes) values of intrinsic clearance (CL_i) of metronidazole to its acetic acid (MAA) and hydroxy (HM) metabolite and glucuronide (GM) and sulphate (SM) conjugate in isolated hepatocytes from male control (CM); phenobarbital pretreated male (PM); 3-methylcholanthrene pretreated male (MC); control female (CF); and phenobarbital pretreated female (PF) Wistar rats: a, b and c indicate significant differences from control values, between the PB and MC group and between the sexes, respectively. The vertical arrows indicate significant changes of Michaelis–Menten constants and competitive inhibition constants (K_i) of SKF 525A (SK), α-naphthoflavone (NF) and antipyrine (AP), the horizontal arrows indicate lack of significant changes.

Table 1. Michaelis–Menten constants for the production of metabolites from metronidazole in suspensions of freshly isolated hepatocytes from male and female rats in control experiments (CO) and after pretreatment with phenobarbital (PB) or 3-methylcholanthrene (MC)

	MAA		HM		GM		SM§	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Male (CO)	67	2.8	41	1.1	841	1.3	138	8.4
N = 7	(19–108)	(1.4–8.4)	(23–52)	(0.3–1.5)	(511–1140)	(0.4–2.6)	(116–221)	(7.0–16)
Male (PB)	191*	2.8	203*	2.2*	860	0.7	116	5.2
N = 8	(127–350)	(0.8–5.7)	(131–439)	(1.1–3.2)	(701–1210)	(0.6–0.9)	(44–1144)	(3.9–22)
Male (MC)	150	1.2	198*	0.6†	526	1.6	1486*	11.1
N = 3	(37–212)	(1.1–1.7)	(187–253)	(0.5–0.9)	(407–672)	(1.0–1.7)	(330–1508)	(8.5–48)
Female (CO)	47	2.1	31	1.6	511	2.4	855‡	4.7
N = 6	(12–60)	(0.9–3.6)	(23–56)	(1.0–2.3)	(284–888)	(1.5–4.2)	(592–2772)	(2.5–13)
Female (PB)	41‡	1.3	34‡	0.9	499	1.7	854	6.5
N = 5	(34–181)	(0.6–3.3)	(31–72)	(0.5–1.5)	(272–1071)	(0.8–1.9)	(532–1932)	(3.0–27)

V_{max} values are expressed as pmol product per min per 10^6 hepatocytes and K_m values as mM.
* $P < 0.05$ compared with the control experiment.
† $P < 0.05$ between male PB and MC group.
‡ $P < 0.05$ compared with the opposite sex.
§ N = 5 in the male CO and PB group.
Values are median range in parenthesis.

mation was higher than toward HM formation and PB treatment did not increase it further (Table 3). In agreement with its specificity toward 3-MC induced P-450 isozymes [20], NF inhibited the formation of MAA and HM with an at least 50-fold increased potency in the hepatocytes from 3-MC treated rats (Table 4). In the hepatocytes from female PB pretreated rats the CL_i of metronidazole to MAA and HM was increased to about the same relative extent as in the males (Figs 1 and 3). The increases in the CL_i values were largely due to decreases in their K_m values although these did not reach statistical significance (Fig. 1 and Table 1). In control and PB treated female rats the CL_i to HM, but not to MAA, was significantly lower than in the corresponding male groups (Figs 1 and 3). Moreover, the inhibitory potency of SKF 525A toward HM formation was not altered by PB treatment of the female rats (Table 3). The ratio between the CL_i of metronidazole by oxidative processes (to MAA and HM) and by glucuronide conjugation was 1.06 (0.97–1.35; median with range) in the 3-MC pretreated group compared to 0.09 (0.04–0.15) and 0.16 (0.08–0.30) in the male control and PB pretreated group, respectively ($P < 0.05$). The metronidazole metabolite pattern is in agreement with *in vivo* and the liver perfusion studies in male rats [5, 6]. Assuming a hepatocyte number of 2.5×10^9 per kg BW their CL_i of metronidazole amounts to around 2 ml min^{-1} per kg BW compared to 1.5–3 ml min^{-1} per kg BW reported for male rats *in vivo* and corrected for renal excretion of unchanged compound [17, 21]. Thus, no major differences in metronidazole metabolism appear between isolated rat hepatocytes and the *in vivo* situation.

The ratio between the CL_i of metronidazole by glucuronide and sulphate conjugation was 30–50 in the male opposed to 1–3 in the female hepatocytes, irrespective of PB pretreatment (Figs 1 and 3). In agreement, only glucuronide and oxidated metabolites of metronidazole have been reported to be excreted into the urine of male rats [5], whereas excretion of equivalent amounts of sulphate and glucuronide conjugates have been demonstrated in female rats [4, 17]. The sulphotransferase(s) involved in the metabolism of metronidazole had low affinity reflected by median K_m values from 5 to 11 mM as opposed to 0.7 to 2.4 mM for the glucuronosyltransferase(s) (Table 1). The increase in V_{max} of metronidazole sulphation in the 3-MC pretreated group is not readily explained, but it must be noted that the determinations were uncertain since the K_m values were just below, or higher than, the maximal substrate concentration (Table 1). The formation of glucuronide conjugates was inhibited by SKF 525A, NF, as well as antipyrine at low K_{is} values, whereas the formation of sulphates was much less affected (Tables 3–5). The K_{is} values of probenecid, an inhibitor of conjugation reactions [22], toward glucuronidation ranged from 62 to 450 μM, irrespective of sex or pretreatment, whereas the sulphation was unaffected by probenecid. The mechanisms of these potent effects on glucuronidation remain to be determined, but may be related to inhibition of the involved glucuronosyltransferases or reduction in supply of energy or cofactors.

Table 2. Michaelis–Menten constants for the production of metabolites from antipyrine in suspensions of freshly isolated hepatocytes from male and female rats in control experiments (CO) and after pretreatment with phenobarbital (PB) or 3-methylcholanthrene (MC)

	HMAP		NORAP§		OHAP	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Male (CO)	1060	2.1	588	7.7	672	3.6
N = 7	(587–1655)	(1.6–2.6)	(332–882)	(2.8–10.6)	(524–917)	(1.4–5.7)
Male (PB)	1425	1.2*	2175*	15.4*	6700*	5.6*
N = 8	(934–2279)	(0.4–1.8)	(1300–7550)	(4.9–47.1)	(4800–10,200)	(4.7–17.5)
Male (MC)	991	2.6†	415†	5.3	593†	2.3†
N = 3	(400–1064)	(2.3–2.8)	(402–1038)	(2.1–6.3)	(397–1059)	(1.0–3.7)
Female (CO)	523‡	2.0	583	14.1	437	2.3
N = 6	(384–789)	(1.4–2.1)	(286–814)	(2.9–55.2)	(200–1129)	(1.6–9.9)
Female (PB)	421‡	1.5	616‡	13.5	1075‡	2.5‡
N = 5	(396–645)	(1.2–1.9)	(403–799)	(2.1–17.2)	(256–1382)	(1.5–4.5)

V_{max} values are expressed as pmol product per min per 10^6 hepatocytes and K_m values as mM.
* $P < 0.05$ compared with the corresponding value from the control experiment.
† $P < 0.05$ between PB and MC group.
‡ $P < 0.05$ compared with the opposite sex.
§ N = 6 in male CO group.
Values are median with range in parentheses.

Antipyrine metabolism

As anticipated, the CL_i of antipyrine to its major metabolites was increased in the hepatocytes from PB treated male rats (Figs 2 and 4) [10–14, 24–26]. The 2.5-fold increased CL_i to HMAP was mainly related to a decrease in K_m, whereas the V_{max} values for NORAP and 4-hydroxyantipyrine (OHAP) formation increased 4- and 10-fold, respectively, together with a two-fold increase in K_m (Table 2). This pattern, including the rank order of V_{max} values and K_m range, resembles observations in rat liver microsomes, with the exception that K_m for NORAP and OHAP formation was unchanged after PB treatment [13]. Assuming a hepatocyte number of 2.5×10^9 per kg BW the CL_i to each metabolite was also in the range of microsomal preparations and in the lower end of the range of reported *in vivo* values [11–14, 23–26]. In the hepatocytes from 3-MC pretreated rats the CL_i, K_m and V_{max} for antipyrine metabolite formation were not significantly altered (Figs 2 and 4, Table 2) partly in agreement with studies on rat liver microsomes, in which unchanged NORAP and OHAP, but considerably decreased HMAP, formation have been observed [13]. By contrast, the *in vivo* clearance of antipyrine to NORAP and OHAP has been reported to be 5–10-fold increased and to HMAP 0.5-fold decreased after 3-MC administration [11, 14]. The increased inhibitory potency of NF toward formation of all three metabolites from antipyrine after 3-MC administration

Table 3. Competitive inhibition constants of SKF-525A for the formation of metabolites from metronidazole or antipyrine in suspensions of freshly isolated hepatocytes from male and female rats in control experiments (CO) and after pretreatment with phenobarbital (PB) or 3-methylcholanthrene (MC)

Substrate	Metronidazole				Antipyrine		
Metabolite	MAA	HM‡	GM	SM	HMAP	NORAP§	OHAP
Male (CO)	14	235§	32	>1000	6.2	>1000	3.3
N = 4	(11–15)	(74–725)	(7.5–97)	(163–>1000)	(1.5–13)	(15–>1000)	(0.8–10.5)
Male (PB)	11	4.7*	147	129	11.2	25	54
N = 5	(3.1–23)	(2.8–116)	(0.1–785)	(12–>1000)	(0.6–43)	(7.2–74)	(2.0–92)
Male (MC)	18	102	8.9	206	1.1	16	5.1
N = 3	(13–57)	(24–136)	(1.7–14)	(169–210)	(1.0–1.8)	(7.3–79)	(2.9–5.2)
Female (CO)	20	200	24	40	0.9	<1000	1.5
N = 4	(16–248)	(39–>1000)	(4.6–73)	(9.1–>1000)	(0.4–2.2)	(2.0–>100)	(1.3–2.1)
Female (PB)	7.2	302†‡	57	>1000	2.4	5.1	2.9
N = 5	(0.3–93)	(115–>1000)	(36–70)	(>1000)	(0.4–5.4)	(0.03–303)	(0.7–5.3)

Values are median (range) expressed in μM.
* $P < 0.05$ vs CO group.
† $P < 0.05$ vs group of opposite sex.
‡ $P < 0.05$ vs K_{is} values for formation of MAA and GM.
§ $P < 0.05$ vs K_{is} values for formation of HMAP and OHAP.

Table 4. Competitive inhibition constants of α-naphthoflavone for the formation of metabolites from metronidazole or antipyrine in suspensions of freshly isolated hepatocytes from male and female rats in control experiments (CO) and after pretreatment with phenobarbital (PB) or 3-methylcholanthrene (MC)

Substrate	Metronidazole				Antipyrine		
Metabolite	MAA	HM	GM	SM	HMAP	NORAP	OHAP
Male (CO)	>1000	355	198	>1000	113	406	72
N = 3	(>1000)	(52–>1000)	(114–302)	(>1000)	(89–143)	(10–>1000)	(25–673)
Male (PB)	>1000	>1000	33*	553	47*	150	163
N = 5	(680–>1000)	(>1000)	(12–46)	(438–>1000)	(23–86)	(15–195)	(120–192)
Male (MC)	17†	1.0†	51†	925	49†	8.1†	5.1†
N = 3	(3.3–26)	(1.0–4.8)	(20–88)	(660–>1000)	(44–72)	(7.5–9.5)	(2.9–5.2)
Female (CO)	>1000	330	212	>1000	131	>1000	84
N = 4	(11–>1000)	(6.1–527)	(171–>1000)	(>1000)	(20–269)	(4.7–>1000)	(7.7–188)
Female (PB)	140‡§	64‡§	97*	>1000	54	9.5‡	26‡
N = 4	(1.3–341)	(1.5–189)	(75–141)	(>1000)	(34–64)	(4.0–14)	(14–44)

Values are median (range) expressed in μM.
* $P < 0.05$ vs CO group.
† $P < 0.05$; one-sided test vs CO group.
‡ $P < 0.05$ vs other treatment group.
§ $P < 0.05$ vs group of opposite sex.

indicates that 3-MC induced P-450 isozymes substituted some of the involved constitutive enzymes (Table 4). By the same token, monoclonal antibodies raised against 3-MC induced P-450 species have been reported to inhibit the formation of antipyrine metabolites in rat liver microsomes only after 3-MC pretreatment [15]. By contrast, NF had little effect on antipyrine metabolism *in vivo* after pretreatment with β-naphthoflavone, a 3-MC type inducer, but the dose of NF may have been insufficient [26]. The inhibition constants of SKF 525A and metronidazole towards NORAP formation were higher than towards formation of HMAP and OHAP lending further support to differential involvement by constitutive P-450 isozymes (Tables 3 and 5). In contrast to our results SKF 525A had minimal effect on the *in vivo* formation of OHAP in Sprague–Dawley rats, whether PB treated or not [26, 27]. As shown in Figs 2 and 4 and Table 2 the formation rate of HMAP was lower in hepatocytes from female than from male rats and the effect of PB treatment on the formation of all antipyrine metabolites was smaller and did not attain statistical significance, in agreement with studies *in vivo* and *in vitro* [12, 25].

Cointeraction of metronidazole and antipyrine

The inhibition constants of antipyrine towards the formation of MAA and HM from metronidazole were in the range of and correlated with the K_m values of OHAP formation, although 3-MC decreased the K_{is} values (Tables 2, 5 and 6). By contrast, the K_{is} values for OHAP formation were much lower than the K_m values for formation of the metronidazole metabolites, particularly in hepatocytes from female rats, and without significant correlations (Tables 1, 5 and 6). The V_{max}, CL_i and inhibitory potency of NF for formation of MAA and HM on the one hand and of NORAP and OHAP on the other were significantly correlated, whereas the K_{is} values of SKF 525A were not (Table 6).

The increased formation of HMAP from antipyrine in the presence of metronidazole, irrespective of the sex or pretreatment (Table 5), is not readily explained, but supports the theory that the involved P-450 isozyme(s) is(are) different from those catalyzing the formation of the other major antipyrine metabolites. The effects of metronidazole appeared mainly to be a decrease in the K_m for HMAP formation with a V_{max} increase only after PB pretreatment (Fig. 5).

DISCUSSION

The formation of metabolites from metronidazole and antipyrine by freshly isolated rat hepatocytes adhered to Michaelis–Menten kinetics and was differentially influenced by sex and specific enzyme induction and inhibition. The metabolites of the two drugs were identical to those formed by humans [2, 3, 7, 28].

It is well-established that PB and 3-MC induce two distinct families of cytochrome P-450 isozymes [11–14, 29, 30]. The present results indicate that constitutive as well as PB and 3-MC induced P-450 isozymes, catalyze the oxidative formation of metabolites from metronidazole, the 3-MC induced with a

Table 5. Competitive mutual inhibition constants of metronidazole and antipyrine for metabolite formation when coincubated in suspensions of freshly isolated hepatocytes from male and female rats in control experiments (CO) and after pretreatment with phenobarbital (PB) or 3-methylcholanthrene (MC)

Inhibitor	Antipyrine			Metronidazole	Antipyrine	
Substrate	Metronidazole					
Metabolite	MAA	HM	GM	SM	HMAP	NORAP§	OHAP
Male (CO)	5.9	1.9	0.4	10	−1.2	2.0	0.2
N = 5	(1.5–13)	(1.1–18)	(0.02–0.8)	(0.02–11)	(−1.3–−0.6)	(0.5–20)	(0.1–0.3)
Male (PB)	9.5	4.7	0.2	1.9	−1.0	0.6	0.3
N = 5	(3.2–33)	(2.0–29)	(0.1–0.4)	(1.1–2.0)	(−1.1–−0.5)	(0.1–1.6)	(0.1–0.6)
Male (MC)	1.1*	0.3*†	0.5	16†	−0.8	2.2	0.3
N = 3	(0.9–1.1)	(0.1–0.4)	(0.3–0.6)	(2.3–19)	(−1.4–−0.7)	(0.4–6.4)	(0.2–0.9)
Female (CO)	3.4	3.1	0.6	8.2	−1.1	6.4	0.05‡
N = 4	(1.6–10)	(0.9–8.1)	(0.1–0.7)	(4.7–>50)	(−1.3–−1.0)	(0.5–>50)	(0.04–0.08)
Female (PB)	1.4	2.3	0.6	76	−1.1	0.2	0.06‡
N = 5	(0.1–8.4)	(1.1–3.5)	(0.3–0.7)	(15–>50)	(−1.1–−1.0)	(0.002–14)	(0.01–0.1)

Values are median (range) expressed in mM.
* $P < 0.05$ vs CO group.
† $P < 0.05$ vs other treatment group.
‡ $P < 0.05$ vs group of opposite sex.
§ $P < 0.05$ vs K_{is} of HMAP and OHAP formation.

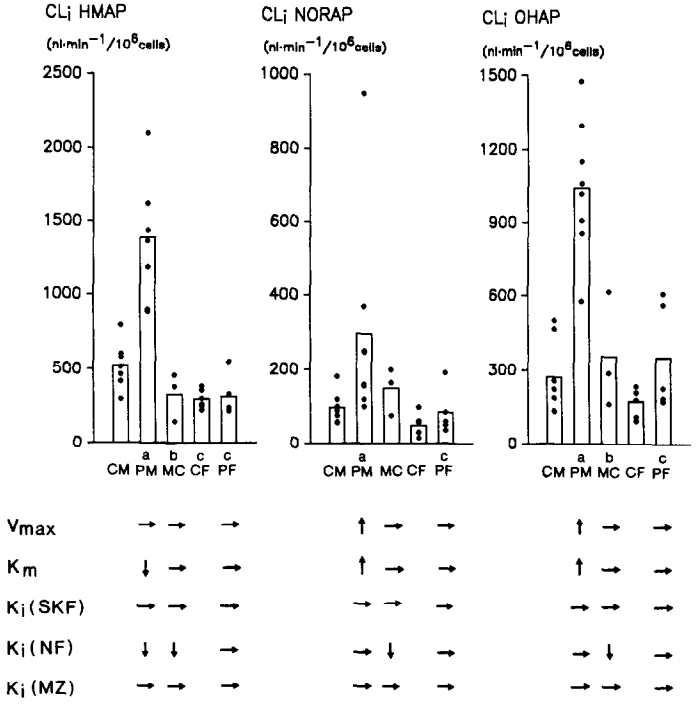

Fig. 4. Individual and average (boxes) values of intrinsic clearance (CL_i) of antipyrine to 3-methylhydroxy (HMAP), nor- (NORAP) and 4-hydroxyantipyrine (OHAP) in isolated hepatocytes from male control (CM), phenobarbital pretreated male (PM), 3-methylcholanthrene pretreated male (MC), control female (CF) and phenobarbital pretreated female (PF) Wistar rats. a, b and c indicate significant differences from control values, between the PB and MC group and between the sexes, respectively. The vertical arrows indicate significant changes of Michaelis–Menten constants and competitive inhibition constants (K_i) of SKF 525A (SK), α-naphthoflavone (NF) and antipyrine (MZ), the horizontal arrows indicate lack of significant changes.

Table 6. Spearman correlation coefficients between Michaelis-Menten constants for the oxidative formation of metabolites from metronidazole (MZ) or antipyrine (AP) in suspensions of freshly isolated hepatocytes, between related competitive inhibition constants (K_{is}) of SKF-525A (SK), α-naphthoflavone (NF) and between the mutual K_{is} values of MZ and AP and their K_m values

	MAA					HM					
MZ:	V_{max}	Cli	K_{is}(SK)	K_{is}(NF)	K_{is}(AP)	K_m	V_{max}	Cli	K_{is}(SK)	K_{is}(NF)	K_{is}(AP)	K_m
AP:	V_{max}	Cli	K_{is}(SK)	K_{is}(NF)	K_m	K_{is}(MZ)	V_{max}	Cli	K_{is}(SK)	K_{is}(NF)	K_m	K_{is}(MZ)
N:	29	29	21	21	22	22	29	29	21	21	22	22
HMAP	0.77*	0.32	−0.36	0.27	−0.42	−0.26	0.53*	0.41*	0.30	0.13	−0.57*	0.22
NORAP	0.55*	0.66*	0.27	0.53*	0.34	0.07	0.57*	0.62*	−0.08	0.47*	0.58*	−0.25
OHAP	0.76*	0.66*	−0.30	0.54*	0.77*	0.30	0.74*	0.55*	0.07	0.69*	0.71*	0.05

* $P < 0.05$.

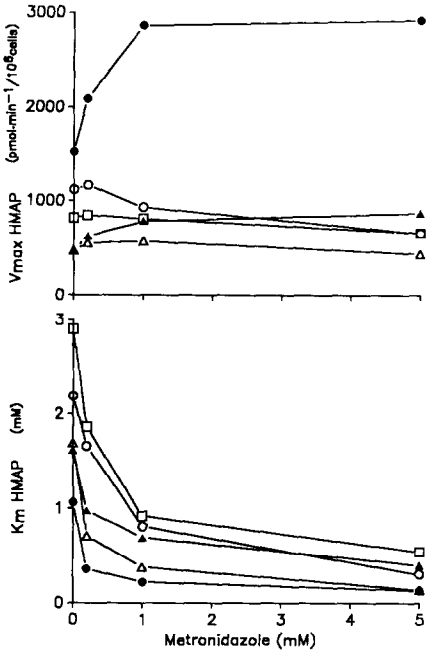

Fig. 5. Effect of increasing concentration of metronidazole on mean V_{max} and K_m for formation of 3-methylhydroxyantipyrine (HMAP) in isolated hepatocytes from male control (○, N = 5); phenobarbital pretreated male (●, N = 5); 3-methylcholanthrene pretreated male (□, N = 3); control female (△, N = 4); and phenobarbital pretreated female (▲, N = 4) Wistar rats.

preference for HM formation. The differential effects of sex, PB pretreatment and addition of SKF 525A suggest that different P-450 isozymes catalyze the formation of MAA and HM.

If the formation of two metabolites from different drugs is catalyzed by a common P-450 isozyme one would expect that the K_m for the metabolite formation from one drug covaried with its inhibition constant toward formation of metabolite from the other drug and that the kinetic constants and effects of inducers and inhibitors covaried between the drugs. Thus, the present results are not clear-cut as to whether the same P-450 isozyme(s) catalyze the formation of MAA and/or HM from metronidazole and one or more metabolite(s) from antipyrine.

In humans the clearance of metronidazole to HM and of antipyrine to NORAP was selectively increased by pretreatment with phenobarbital-type inducers, but the formation rates of MAA and HM were unaffected by coadministration of cimetidine, an inhibitor of antipyrine metabolism, and did not correlate with the clearance of antipyrine to any of its metabolites [2]. In the present study the only K_{is} value between metronidazole and antipyrine comparable to plasma concentrations attainable in humans was that toward OHAP formation in female rat hepatocytes (Table 5). In fact, the clearance of antipyrine to OHAP was decreased by 10% after administration of metronidazole 800 mg/day to volunteers for 6 days [31], whereas single doses of metronidazole and antipyrine have no effect on the

metabolism of either [2, 15]. Thus, there is some support to formation of MAA and/or HM from metronidazole and NORAP and/or OHAP from antipyrine by shared isozyme(s). The low K_{is} values of metronidazole for OHAP formation and the lack of covariation with the K_m values for MAA and HM formation should thus be explained by another mechanism than simple competition for the enzyme, whereas the lack of covariation between the inhibitory effects of SKF 525A remains unexplained. Considering all data, it appears most likely that the formation of each of the two oxidative metronidazole and three antipyrine metabolites is catalyzed by different P-450 isozymes, which may be supplemented with or substituted by PB or 3-MC induced forms. Each isozyme preferentially forms a particular metabolite with more or less overlapping substrate and/or product specificity.

The average K_m of the enzymes involved in metronidazole metabolism ranged from 1 to 3 mM. These values are in agreement with our previous report of a 9% decrease in total clearance in humans when an intravenous dose of metronidazole was increased from 500 to 2000 mg, which yielded maximal plasma concentrations of about 60 and 250 μM, respectively [7]. However, in agreement with previous *in vivo* results the predominant metabolism of metronidazole in rat hepatocytes was conjugation [4–6], whereas oxidative formation of HM and MAA is the major route of elimination in man [2, 3, 7].

In humans pretreated with PB or antipyrine, which are thought to have similar enzyme inducing properties, the clearance of metronidazole to HM was increased, whereas the clearance to MAA and glucuronide conjugates was unchanged [2]. In studies of intact mice and isolated perfused rat liver PB treatment increased the formation rate of metronidazole glucuronide, although quantitative data were not reported [3, 6]. In the present PB treated male and female groups the CL_i to metronidazole glucuronide was 1.9 times (0.7–2.5) and 1.7 times (0.6–2.7) the values in the control groups, respectively (median ratios with 95% confidence interval, Table 1). Thus, the data do not exclude a two-fold increase in CL_i for glucuronide formation. A more intriguing divergence between our previous results in humans and the present hepatocyte experiment is the lack of correlation between tobacco smoking and the total metronidazole clearance in the former [1]. Tobacco smoke contains polyaromatic hydrocarbons resembling 3-MC. However, compared to 3-MC tobacco smoke is a rather poor inducer of the liver enzymes as assessed by the *in vivo* formation of antipyrine metabolites [32]. Moreover, in the cross-sectional design used in our human study other factors, whether related to smoking or not, could have counteracted an effect on the oxidative metabolism of metronidazole or affected other elimination pathways.

In conclusion, the present data demonstrate that the oxidative metabolism of metronidazole in isolated rat hepatocytes can be induced by pretreatment with PB and 3-MC. The formation of HM is particularly induced by the latter treatment and with inclusion of the formation of glucuronide conjugate the metronidazole metabolite profiles allows detection of, and distinction between, prototype inducers of cytochrome P-450 isozymes. In this respect metronidazole is superior to antipyrine, since the formation of metabolites from the latter in rat hepatocytes allowed detection of only PB and not 3-MC type induction. The main bulk of evidence, including previous studies, appears compatible with the hypothesis that different constitutive isozymes, which may be supplemented or substituted by PB or 3-MC induced species, are involved in the formation of the five different metabolites from the two drugs.

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