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EFFECT OF MALARIA INFECTION AND ENDOTOXIN-INDUCED FEVER ON THE METABOLISM OF ANTIPYRINE AND METRONIDAZOLE IN THE RAT

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Abstract—Antipyrine and metronidazole were administered as a cocktail to young (4 weeks old) male Wistar rats (N = 12 for each treatment) to investigate the effect of malaria infection due to the rodent parasite *Plasmodium berghei* and *Escherichia coli* endotoxin-induced fever on the metabolism of the two compounds *in vivo*. Control rats received normal saline. Antipyrine and metronidazole clearances were estimated from a single saliva sample while the formation clearances of their metabolites (in malaria-infected and control rats) were estimated from the product of clearance of parent drug and the fraction of the administered dose excreted as metabolites in urine in 24 hr. Rats treated with endotoxin produced no urine during this period. Malaria infection had no effect on clearance of antipyrine or on formation clearance of any of its metabolites. However, the clearance of metronidazole was reduced by approximately 20% compared with controls as a result of decreased formation of hydroxymetronidazole. Fever decreased clearance of both antipyrine and metronidazole by approximately 36% and 23%, respectively. These results demonstrate that both malaria infection and fever can influence P450-dependent drug metabolism and the effects seen appear to be isozyme-selective.

Drug metabolism can be influenced by several factors including disease, age, other drugs administered concurrently, and genetic and environmental factors [1]. Disease may influence drug metabolism via both direct and indirect effects on drug-metabolizing enzyme activity. The consequences of altered drug metabolism can include potential toxicity or altered efficacy, depending on the drug in question. With disease-induced alterations in drug metabolism the consequences are greater the more prevalent a disease is in a given population.

The aim of the present study was to investigate the effect of fever and malaria infection on the *in vivo* metabolism of well-characterized probe compounds following administration as a cocktail to the rat. Administration of such a cocktail has been employed successfully in the rat [2, 3], and diminishes the influence of intra-individual variation in drug metabolism which may complicate the interpretation of the results of these studies. It allows also for a better characterization of the influence of specific factors on the activities of selected isozymes [4]. In the present study, we have used a cocktail of antipyrine and metronidazole, which has been used previously in the rat [3]. Formation of antipyrine metabolites is thought to be mediated by different (but as yet unidentified) isozymes [5, 6]. Formation of metronidazole metabolites is believed also to be catalysed by different isozymes which are distinct from those involved in antipyrine metabolism [3, 7–9]. Both metronidazole and antipyrine have similar pharmacokinetic properties, i.e. rapid and complete absorption after oral administration, distribution into total body water and first order elimination [10–12]. There is no pharmacokinetic interaction when both drugs are administered as a cocktail to the rat [3]. Finally, total clearance of antipyrine or metronidazole can be calculated from the drug concentration in a single saliva sample obtained at an accurately determined time after administration [3]. This avoids multiple blood sampling which may be stressful to rats, and may affect the activities of drug-metabolizing enzymes [13, 14].

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§ Abbreviations: UDPGA, UDP-glucuronic acid; LPS, lipopolysaccharide.

MATERIALS AND METHODS

Chemicals. Metronidazole injections (5 mg/mL) was obtained from the Pharmacy Department, Royal Liverpool University Teaching Hospital. Metronidazole, antipyrine, 4-hydroxyantipyrine, sulphathiazole, pilocarpine hydrochloride, glusulase® Type H-2 from *Helix pomatia* and *Escherichia coli* lipopolysaccharide (LPS§) were obtained from the Sigma Chemical Co. (Poole, U.K.). Hydroxymetronidazole and the potassium salt of metronidazole acetic acid were kind gifts from Dr Steffen Loft, University of Denmark. 3-Hydroxymethylantipyrine and norantipyrine were kind gifts from Dr D. Breimer, University of Leiden, The Netherlands. Phenacetin was purchased from the Aldrich Chemical Co. (Gillingham, U.K.). All organic solvents were of either analytical or chromatographic grade, and were purchased from Sigma or Fisons (Loughborough, U.K.).

Malaria and fever models in the rat. Malaria

infection and fever were produced in separate groups of male Wistar rats (N = 12 in each group) as described previously [15]. Control rats (N = 12) each received normal saline.

Administration of antipyrine and metronidazole cocktail to rats. Rats were dosed with antipyrine (14 mg/kg i.p., as a solution in sterile normal saline) and metronidazole (10 mg/kg i.p. as metronidazole injection) [8], and placed in metabolic cages to facilitate the collection of urine. Endotoxin-treated rats were dosed with antipyrine and metronidazole 1 hr following endotoxin administration. Animals had free access to food and water. The containers for urine collection were kept at 0° and each contained sodium pyrosulphite (50 mg) as an antioxidant to prevent degradation of drug metabolites. Four hours post drug administration, a saliva sample (approximately 80 μL) was collected following stimulation of saliva production with pilocarpine (0.2 mg s.c.) [8]. For fever studies, rectal temperature was recorded immediately before the collection of the saliva sample (i.e. 5 hr post endotoxin administration). Urine was collected from malaria-infected and control rats over a 24 hr period. Endotoxin-treated rats failed to produce any urine over this period. Urine and saliva samples were stored at −80° until assayed for antipyrine, metronidazole and their respective metabolites as described below.

Analytical procedures. For the analysis of antipyrine, saliva (50 μL) was mixed with an equal volume of ice-cold zinc sulphate solution (0.1 M) and the internal standard phenacetin (10 μL containing 200 ng) in methanol was added. Following further mixing and centrifugation (650 *g*; 10 min), an aliquot (50 μL) of the supernatant was analysed by HPLC [8]. Calibration curves were constructed daily by spiking zinc sulphate solution (0.1 M) with phenacetin (200 ng; 20 μL of a solution in methanol) and antipyrine (50 μL of appropriate dilutions of a 1 mg/mL solution in distilled water).

Urine samples (50 μL) were assayed for antipyridine and metabolites before and after incubation (37°; 3 hr) in acetate buffer (0.5 mL; pH 4.8) containing glucuronidase : arylsulphatase; 6000 : 150 IU/mL [8]. After addition of the internal standard (phenacetin; 200 ng as above), the mixture was processed according to Witkamp *et al.* [16]. The residue was reconstituted in mobile phase (methanol : phosphate buffer, pH 7.8; 35 : 65% v/v; 100 μL) and analysed by the method of Loft *et al.* [8]. Standard curves were constructed by spiking urine from non-treated rats with increasing amounts (1–50 μg; 50 μL of appropriate dilutions of stock solutions) of antipyrine and the metabolites, followed by extraction as described above.

For the analysis of metronidazole in saliva and metronidazole and metabolites in urine the procedure of Loft *et al.* [8] was followed, except that sulphathiazole (20 μg; 20 μL of a 1 mg/mL solution in methanol) was used as the internal standard. Conjugated metronidazole in urine was quantitated following enzymatic hydrolysis according to the procedure of Loft *et al.* [8]. For analysis of saliva samples standard curves were constructed daily by spiking zinc sulphate solution (0.1 M) with the internal standard (20 μg as above) and increasing amounts (1–30 μg; 50 μL of appropriate dilutions of a 1 mg/mL stock solution in methanol) of metronidazole. For the analysis of urine samples, urine from non-treated rats was spiked with the internal standard (20 μg as above) and increasing amounts (1–3 μg; 50 μL of appropriately diluted stock solutions) of metronidazole and the metabolites (1–40 μg).

Calculation of pharmacokinetic parameters. The one-sample clearance (CL) for antipyrine or metronidazole was estimated from the concentration (C) of antipyrine or metronidazole determined in the 4 hr saliva sample, the dose (D) administered and the assumed volume of distribution (V), thus [17]:

$$CL = [\ln(D/V) - \ln C] - V/t$$

where V for antipyrine and metronidazole was assumed to be equivalent to the volume of total body water in the rat (0.67 L/kg body weight) [17] and t is the time of sampling (i.e. 4 hr). Clearance attributable to metabolism (CL_m) was estimated from CL for antipyrine and metronidazole after correcting for the fraction of the dose administered which was excreted as unchanged drug in the urine. Biliary excretion of unchanged drug was assumed to be negligible. The partial clearance for the production of each metabolite (i.e. formation clearance, CL_f) was estimated from the product of CL_m and the fraction of the dose (expressed in parent drug equivalents) excreted as the metabolite in urine in 24 hr (f_m). The conjugated metronidazole was assumed to be the glucuronide [8]. The bioavailability of antipyrine following intraperitoneal administration to the Wistar rat has been shown to be complete [10] and this was assumed in the present study. The bioavailability of metronidazole was also assumed to be unity [7].

Statistical analysis. Between group mean pharmacokinetic parameters were compared by the non-parametric Mann–Whitney one-way test and the Kruskal–Wallis test. $P < 0.05$ was taken as significant.

RESULTS

Analysis of antipyrine, metronidazole and their metabolites

Mean recoveries of antipyrine and its metabolites from spiked urine were as follows: antipyrine, 79 ± 3% at 40 μg/mL (N = 6); norantipyrine, 82 ± 5% at 30 μg/mL (N = 6); 3-hydroxymethylantipyrine, 70 ± 6% at 30 μg/mL (N = 6) and 4-hydroxyantipyrine, 78 ± 4% at 30 μg/mL (N = 6). Calibration curves were linear ($r = 0.95 \pm 0.03$) within the following concentration ranges: antipyrine, 1–50 μg/mL; norantipyrine, 1–50 μg/mL; 3-hydroxymethylantipyrine, 1–50 μg/mL and 4-hydroxyantipyrine, 5–50 μg/mL. The intra-assay coefficients of variation were 3.2, 4.3, 6.6 and 5.4% for antipyrine, norantipyrine, 3-hydroxymethylantipyrine and 4-hydroxantipyrine, respectively. The inter-assay coefficients of variation were 5.8, 5.4, 7.3 and 7.1% for antipyrine, 3-hydroxymethylantipyrine, norantipyrine and 4-hydroxyantipyrine, respectively.

The mean recoveries of metronidazole and its

Table 1. Clearance (mL/min/kg) of antipyrine to the various metabolites in control (C) and malaria-infected (MI) rats

	C	MI	P
CL (AP)	5.5 ± 1.1	5.0 ± 0.4	>0.05
CL_f (HA)	0.38 ± 0.1	0.36 ± 0.17	>0.05
CL_f (HMA)	0.24 ± 0.14	0.29 ± 0.22	>0.05
CL_f (NORA)	0.66 ± 0.29	0.61 ± 0.25	>0.05
Recovery in urine (% dose)	36.4 ± 1.6	37.7 ± 3.1	>0.05

Values are means ± SD; N = 12.

CL, total clearance of antipyrine (AP); CL_f, formation clearance of metabolites: 4-hydroxyantipyrine (HA), 3-hydroxymethylantipyrine (HMA) and norantipyrine (NORA).

Renal clearance of AP was negligible.

metabolites from spiked urine were as follows: metronidazole, 83 ± 6% at 20 μg/mL (N = 6); hydroxymetronidazole, 75 ± 3% at 30 μg/mL (N = 6) and metronidazole acetic acid, 70 ± 5% at 30 μg/mL (N = 6). Calibration curves were linear ($r = 0.96 \pm 0.02$) within the following concentration ranges: metronidazole, 1–30 μg/mL; metronidazole acetic acid, 1–40 μg/mL; and hydroxymetronidazole, 1–30 μg/mL. The intra-assay coefficients of variation were 4.6, 6.8 and 5.2% for metronidazole, metronidazole acetic acid and hydroxymetronidazole, respectively. The inter-assay coefficients of variation were 6.3, 7.2 and 6.6% for metronidazole, metronidazole acetic acid and hydroxymetronidazole, respectively.

Mean rectal temperature determined immediately before saliva sample collection (i.e. 5 hr post endotoxin administration) was 39.1 ± 0.2°. This was approximately 2° higher than mean temperature determined both at zero time (i.e. immediately prior to) and 1 hr post endotoxin administration (36.9 ± 0.3 and 37.3 ± 0.1°, respectively). This difference was significant ($P < 0.005$).

Effect of malaria infection on antipyrine and metronidazole clearance

Malaria infection had no significant effect on the clearance of antipyrine (Table 1) whereas the clearance of metronidazole was significantly ($P < 0.005$) reduced (by approximately 20%) compared to controls (Table 2). Malaria infection did not alter the formation clearance (CL_f) of antipyrine metabolites norantipyrine, 3-hydroxymethylantipyrine and 4-hydroxyantipyrine (Table 1) and the renal clearance of antipyrine was negligible both in control and malaria-infected rats. Malaria infection significantly ($P < 0.005$) reduced (by approximately 60%) CL_f of hydroxymetronidazole but not metronidazole acetic acid. CL_R and the extent of conjugation of metronidazole were also unaffected by malaria infection (Table 2).

Formation of metronidazole acetic acid was the main route of elimination of metronidazole in both malaria-infected and control rats (accounting for approximately 39% and 36% of the dose administered which was recovered in urine in control and malaria-infected rats, respectively), while formation of norantipyrine was the main route of elimination of antipyrine in both malaria-infected and control rats (accounting for approximately 16% of the dose administered which was recovered in urine in both malaria-infected and control rats; Fig. 1). Malaria infection had no significant effect on the percentage of the administered dose of antipyrine or metronidazole recovered in urine (Tables 1 and 2).

Effect of fever on antipyrine and metronidazole clearance

Fever significantly ($P < 0.05$) decreased the total clearance of antipyrine and metronidazole in treated rats compared with controls. Fever decreased the total clearance of metronidazole by approximately 23% (6.9 ± 0.5 mL/min/kg in control; 5.2 ± 0.6 mL/min/kg in fever) and that of antipyrine by approximately 36% (5.1 ± 1.1 mL/min/kg in control; 3.5 ± 0.5 mL/min/kg in fever). Hence fever and malaria infection reduced the clearance of metronidazole by a similar magnitude.

DISCUSSION

The results of the present study show that malaria infection and fever have different effects on the metabolism of antipyrine and metronidazole when the two compounds are administered simultaneously to the rat. Malaria infection had no effect on antipyrine metabolism. This is contrary to a previous report [18] in which it was shown that malaria infection selectively impaired the formation of 3-hydroxymethylantipyrine and 4-hydroxyantipyrine, and increased the urinary excretion of antipyrine. However, the two studies differ in several respects. First, a different strain of rats (male Sprague–Dawley) was used in the previous study [18]. Second, in the previous study [18] the formation clearances (CL_f) of metabolites of antipyrine, and the total clearance of antipyrine were not determined, with the conclusions being based solely on differences in the relative urinary recovery of antipyrine and its metabolites. Whether there is a true strain difference in the clearance of antipyrine between young male Wistar and Sprague–Dawley rats is not known, but clearance of antipyrine in control rats determined in the present study was lower compared to the values

Table 2. Clearance (mL/min/kg) of metronidazole to the various metabolites in control (C) and malaria-infected (MI)

	C	MI	P
CL (MZ)	6.9 ± 0.5	5.4 ± 0.8	<0.05
CL_R	0.5 ± 0.3	0.3 ± 0.1	>0.05
CL_f (HM)	1.2 ± 0.5	0.4 ± 0.3	>0.05
CL_f (MG)	0.16 ± 0.1	0.15 ± 0.13	>0.05
CL_f (MAA)	2.5 ± 1.6	1.6 ± 1.2	>0.05
Recovery in urine (% dose)	46 ± 2	48.6 ± 6	>0.05

Values are means ± SD; N = 12. Parasitaemia in MI rats = 38.5 ± 9.6%.
CL, total clearance of metronidazole (MZ); CL_f formation clearance of metabolites: metronidazole acetic acid (MAA), hydroxymetronidazole (HM) and metronidazole glucuronide (MG); CL_R renal clearance of unchanged MZ.

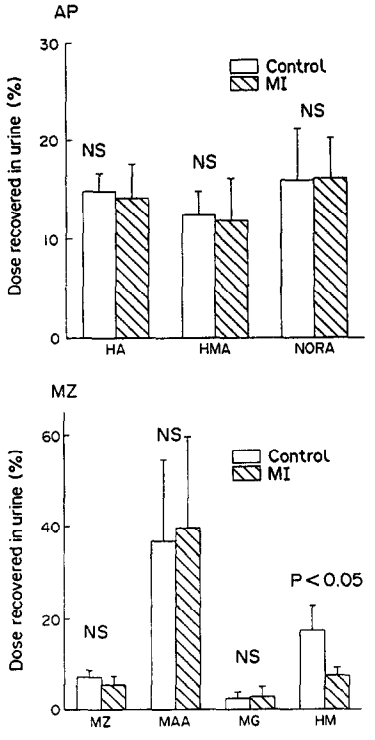

Fig. 1. Effect of malaria infection (MI) on the urinary excretion of antipyrine (AP) and metronidazole (MZ) and their respective metabolites: HA, 4-hydroxyantipyrine; HMA, 3-hydroxymethylantipyrine; NORA, norantipyrine; MAA, metronidazole acetic acid; MG, metronidazole glucuronide; HM, hydroxymetronidazole. NS, no statistically significant difference between treatments.

reported in adult male Sprague–Dawley rats [19, 20] and in adult male Wistar rats [16]. In this case, the difference in the age of rats used (4 weeks and approximately 100 g in the present study, and approximately 250 g in the other studies) could in part offer an explanation for the observed differences.

While malaria infection had no effect on the total clearance of antipyrine, the metabolism of antipyrine in both control and malaria-infected rats was quantitatively different from that reported in the literature. In the present study, formation of norantipyrine was quantitatively the most important elimination pathway, with approximately 16% of the administered dose of antipyrine recovered in urine as norantipyrine in both control and malaria-infected rats. However, in other studies using adult Wistar rats, formation of 3-hydroxymethylantipyrine has been shown to be the most important elimination pathway [16]. Again, the differences in the age of rats used in the present study and those used in earlier work [16] could account for these differences. CL_f for 3-hydroxymethylantipyrine observed in control and malaria-infected rats used in the present study was approximately 7-fold lower, while CL_f for norantipyrine was approximately 2-fold higher than reported previously in Wistar rats [16]. CL_f for 4-hydroxyantipyrine estimated in the present study was, however, comparable with values reported previously in adult Wistar rats [16].

Fever decreased the clearance of antipyrine by approximately 36% compared with controls. This effect of fever on antipyrine clearance is also contrary to that reported in the literature. For example, Aarbakke *et al.* [10] reported that fever had no effect on antipyrine clearance in adult Wistar rats. In addition, it was reported [10] that fever increased the volume of distribution (V) of antipyrine in the rat by approximately 13%. Apart from differences in the age of the rats used (older rats were used by Aarbakke *et al.* [10]) the other important difference is that *E. coli* LPS was used to induce fever in the present study whereas prostaglandin was used in the previous study [10]. Whether fever produced by LPS and prostaglandin has different effects on antipyrine clearance is not known. In addition, V for antipyrine was assumed in the present study to be unaffected by fever. Antipyrine distributes into total body water, and the fraction unbound in rat plasma is approximately 0.96 [21]. Since antipyrine is negligibly bound to tissue, the mechanism for the reported increase in V [10] is not clear. Although some intracellular binding of antipyrine in rat perfused liver has been reported [21], the liver constitutes only about 4% of total body weight and even if fever affected intracellular binding of antipyrine in rat

liver, it is unlikely to affect V. Endotoxin-treated rats used in the present study produced no urine during the 24 hr collection period. This may have altered V in these rats. However, at the time when saliva samples were collected (i.e. 4 hr after drug administration) the control rats also had produced little urine. Therefore, the assumption of a constant value for V for both groups of rats up to the time for saliva collection is reasonable, and the effect of fever on hepatic elimination of antipyrine is consistent with reports on the effect of fever on the elimination of other compounds in the rat [22] and on the elimination of antipyrine in the rabbit [23].

Malaria infection and fever caused a decrease in the clearance of metronidazole by 20% and 23%, respectively. Malaria infection appeared mainly to affect the formation of hydroxymetronidazole with no effect on the formation of metronidazole acetic acid, although metronidazole acetic acid was quantitatively the major metabolite in rats used in the present study (accounting for approximately 39% and 36% of the administered dose of metronidazole recovered in urine from control and malaria-infected rats, respectively; Fig. 1). There was also a distinct difference in the clearance of metronidazole in the rats used in the present study compared with the clearance of metronidazole in rats as reported in the literature. For example, clearance of metronidazole in the control rats was approximately 20% higher than the value reported in adult male Wistar rats [8]. In addition, CL_f for metronidazole acetic acid was 3-fold higher, while CL_f for hydroxymetronidazole was 10-fold higher in the control rats compared with reported values in adult Wistar rats [8]. Formation of metronidazole glucuronide and excretion of unchanged metronidazole in urine have been reported to be major routes of elimination of metronidazole in the rats [8]. However, in the present study it was found that CL_f for metronidazole glucuronide was approximately 9-fold lower compared with previously reported values [8], and less than 8% of the administered dose was recovered as the glucuronide in control and malaria-infected rats (Fig. 1). Moreover, malaria infection had no effect on the glucuronidation of metronidazole. The renal clearance of unchanged metronidazole in control rats (0.5 mL/min/kg) was, however, close to a previously reported value of 0.75 mL/min/kg [8]. As has been mentioned, these discrepancies may be due in part to differences in the age of rats used in the present study and those used in the previous study [8]. Age-related differences in P450-dependent metabolic activity in the rat is thought to be due to changes in isozymic composition of cytochrome P450 [24]. In man, the pharmacokinetics of metronidazole has also been reported to vary with chronological age [25]. Whether this is also the case in the rat is unknown. The percentage of the dose recovered in urine as metabolites and unchanged drug was low for both metronidazole and antipyrine (Tables 1 and 2). It has been suggested that further metabolism of norantipyrine and 4-hydroxyantipyrine may occur *in vivo* [19]. The same may be true for some of the metabolites of metronidazole, but this remains to be confirmed.

Malaria infection has been reported to be associated with a decrease in the glucuronidation of paracetamol [26] and phenol [27] in the rat. In the present study malaria infection was shown to have no effect on the glucuronidation of metronidazole. There are two possible explanations for the apparent lack of effect of malaria infection on the glucuronidation of metronidazole. First, both paracetamol and phenol have phenolic hydroxyl groups whereas metronidazole has a non-phenolic hydroxyl group available for glucuronidation. Therefore, different isozymes may mediate the glucuronidation of phenol, paracetamol and metronidazole. Malaria infection may differentially affect these isoenzymes. Second, the availability of the co-factor UDP-glucuronic acid (UDPGA) is important for the glucuronidation reaction. Malaria infection is thought to result in a decrease in the synthesis of UDPGA [27]. When the levels of UDPGA are low, decreased glucuronidation can be observed at high substrate concentrations, whereas at low substrate concentrations glucuronidation may be normal despite the low levels of UDPGA. This has been confirmed with phenol [27]. In the present study, it is possible that the dose used (10 mg/kg) resulted in systemic levels of metronidazole which were too low to exhaust the UDPGA reserves. Hence, malaria infection had no effect on the glucuronidation of metronidazole.

The present study has highlighted differential effects of malaria infection and fever on the hepatic elimination of the two probe compounds antipyrine and metronidazole in the rat. It is known that different, although as yet undefined, isozymes of P450 are involved in the production of the respective phase I metabolites of antipyrine and metronidazole [3, 7–9], and our findings suggest that malaria infection affects selectively the isozymes involved in the metabolism of metronidazole but not those involved in the metabolism of antipyrine. In separate experiments [15] we have demonstrated that both malaria infection and fever appear to inhibit phenacetin O-deethylation by rat liver microsomes. However, metronidazole is not a substrate for cytochrome P4501A2 [28]. Thus, malaria infection and fever appear to affect isozymes involved in metronidazole metabolism and also cytochrome P4501A2 which mediates phenacetin O-deethylation.

Several factors may be responsible for the decreased clearance of certain compounds during malaria infection and under febrile conditions. These include the presence of cytokines (e.g. tumour necrosis factor and interleukin-6) released in response to malaria infection and fever, and a possible decrease in cytochrome P450 levels. In separate experiments [15] we have found that cytochrome P450 levels are decreased by almost 50% in livers from malaria-infected rats when parasitaemia is high. However, decreased cytochrome P450 levels in malaria infection cannot explain the marked effect of malaria infection and fever on metronidazole metabolism and a lack of effect on antipyrine metabolism. This apparent selectivity in the metabolism of the two compounds

is probably due to other factors. At present we do not know what these factors are.

The differential effects of malaria infection and fever on the metabolism of antipyrine and metronidazole lend support to the approach of using a cocktail of probe compounds rather than a single substance. This approach using metronidazole and antipyrine is applicable also to humans [29]. In future studies we aim to compare the findings of the present study with results obtained in man and, if possible, in patients with malaria. Antipyrine clearance is decreased in children with fever [30] and we specifically aim to assess whether a similar decrease in clearance of antipyrine occurs in fever associated with malaria. It may also be possible to assess the effect of malaria infection and fever on the clearance of other probe compounds in man, using the single sample approach, since several suitable probe compounds have been identified [31].

In conclusion, we have shown, using a rat model for malaria infection and fever, and a cocktail of antipyrine and metronidazole, that differential effects on the metabolism of the two compounds occur. This approach can form part of preliminary metabolic studies in animals before confirmatory studies in man, where possible, are performed. Alternatively, where such studies are not possible in man, the cocktail approach can be used in a suitable animal model as a predictive tool to possible alterations in drug metabolism in man, provided that the limitations of such extrapolations are fully appreciated.

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