THE CONTRIBUTIONS OF VARIOUS CHLOROQUINE SALTS TO THE BILIARY AND URINARY EXECRETION OF HEPATIC PARACETAMOL CONJUGATION METABOLITES IN THE RAT

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ABSTRACT

As an approach to explain the possible *in vivo* interaction of paracetamol (acetaminophen) with various chloroquine salts that are often administered during malaria tropica, the effects of these salts (chloroquine sulphate, chloroquine phosphate, chloroquine hydrochloride and ferrous sulphate) were examined in male rats. The co-administration of chloroquine salts with paracetamol for 7 days showed varied effects on urinary and biliary excretion of paracetamol sulphate and paracetamol glucuronide conjugates - the major metabolites of paracetamol metabolism. These findings suggest that chloroquine sulphate and ferrous sulphate may enhance the sulphation pathway in paracetamol metabolism and influence detoxification of paracetamol in the liver and thus protect the liver. Chloroquine sulphate is therefore a better choice compared to other chloroquine salts in the treatment of malaria with paracetamol as an antipyretic and analgesic.

KEY WORDS

chloroquine salts, paracetamol (acetaminophen), biliary and hepatic conjugation metabolites, malaria

INTRODUCTION

Chloroquine is a 4-aminoquinoline chemical derivative, which has remained an effective treatment for malaria despite the increasing reports of resistant strains of *Plasmodium* /1-4/. It is administered as various salts, such as chloroquine sulphate, chloroquine phosphate, chloroquine hydrochloride, etc. Chloroquine has been reported to inhibit mixed function oxidases *in vitro* in rats /5-7/, but not *in vivo* in man /9/. In the rat, chloroquine did not alter pentobarbitone sleeping time nor zoxazolamine paralysis time, but reduced the clearance of antipyrine /7/. Thus the exact metabolic contributions of chloroquine to hepatic metabolism are ambivalent and the contributions of these various salts have not been reported.

Paracetamol (acetaminophen) is a widely used analgesic and antipyretic. It produces hepatotoxicity and death during untreated overdosing, in both animals and man /9,10/. Its biotransformation would normally skip phase I oxidative metabolism because of the ready availability of hydroxyl functional groups which would be geared preferentially to phase II conjugation reactions yielding predominantly paracetamol sulphate and paracetamol glucuronide metabolites. However, paracetamol conjugations often show Michaelis-Menten kinetics, i.e. non-linear, capacity limited and saturable during chronic dosage or excess overdose /11/. In diminished concentrations of sulphates or glucuronic acids, excess paracetamol will be diverted to phase I oxidation reactions mediated by the cytochrome P450 isozyme CYP2E1 (and to a lesser extent CYP3A4 and CYPIA2) whose oxidative product is the hepatotoxic metabolite N-acetyl-p-benzoquinonine imine (NABQI) /12-15/. The conjugation of NABQI by glutathione S-transferase (GST) is also capacity limited, as the major GST involved in the detoxification of paracetamol is the GST-pi in both rats and man /16,17/. Glutathione peroxidase overexpression diminishes replenishment of glutathione (GH) in liver and blood and influences the synthesis of oxidised paracetamol metabolites /18/. There are reports that inorganic sulphate or sulphate donors affect the metabolic clearance of paracetamol /19-21/.

As an approach to elucidate whether there is any possible *in vivo* interaction of chloroquine salts which are frequently used in combination with paracetamol during treatment for malarial fever, the effects of these various chloroquine salts on the overall biotransformation of

paracetamol in male rats were studied by analysis of excreted biliary and urinary paracetamol conjugated metabolites, hepatic function tests and pathohistology.

MATERIALS AND METHODS

Animals

Male albino Wistar rats weighing 180-200 g were procured from the Animal House Unit of the University of Uyo, Nigeria. They were housed in groups of six in plastic cages and had free access to pellet food and water. The animals were kept at 25°C in a 12 h light/12 h dark cycle for 7 days and were deprived of food for 12 h before experiments. This study complied with the University of Uyo Nigeria legislation governing experimentation with animals.

Drugs and chemicals

Chloroquine sulphate (Nivaquine®; M&B, Nigeria), chloroquine phosphate (Resochin®; Bayer, Nigeria), chloroquine hydrochloride (Lek, Slovenia), ferrous sulphate (Ranbaxy, India) and paracetamol (Panadol®; Sterling Products, Nigeria) were all commercially available and of finest pharmaceutical grade. Paracetamol metabolites were purchased from Sigma Chemical Co., MO, USA. Kits B8120 and B8110 for serum glutamine pyruvate transaminase assay were from MIFRD Diagnostics, Italy. Other chemicals or reagents were of analytical grade.

Drug administration

Paracetamol (300 mg/kg p.o.) was given daily for 7 days to rats which were co-administered with chloroquine sulphate (10 mg/kg p.o.), chloroquine hydrochloride (10 mg/kg p.o.), chloroquine phosphate (10 mg/kg p.o.) or ferrous sulphate (200 mg/kg p.o.). The dose of chloroquine salts used was calculated from the base. One group of rats for each treatment was studied and sacrificed on day 1 and a matching group was carried through to day 7 before sacrifice.

Urine analysis

Urine was collected for 4 hours after drug administration on day 1 and for 4 hours after drug administration on day 7. Urine samples which were not analysed immediately for paracetamol and its conjugated metabolites were stored in the dark at -20°C until assay. The concentrations of paracetamol and its conjugated metabolites were determined by high performance liquid chromatography (HPLC) using a C18-μ Bondpak reverse column (Waters Associates Inc., USA). The conjugated metabolites and unchanged paracetamol were eluted with water:methanol:acetic acid in the ratio of 900:80:8% v/v/v at the flow rate of 1.5 $ml.min^{-1}$. The eluted metabolites and paracetamol were monitored at 254 nm; the detection limit was 0.02 $\mu g.ml^{-1}$. Each sample was analysed using a calibration curve spiked with known amounts of paracetamol and its conjugated metabolites. The intraday variation was 3% and interday variation was 4.7%. Recovery of paracetamol of known amounts (10 μg/ml and 50 μg/ml) was 98% with coefficients of variation of 1.89% and 3.0% respectively (n = 6).

Biliary analysis

Rats on day 1 and day 7 were anesthetized with urethane ($6mg.kg^{-1}$) (2.5% w/v). The abdomen was opened at the left side and the common bile duct was exposed and cannulated with 20 cm long silastic medical grade tubing. Body temperature was maintained at 37°C by means of a heating pad placed under the animals. Equilibrium was allowed for 20 min after preparation and initiation of the experiment. Bile was collected in a graduated tube for 4 hours on days 1 and 7, and analysed for paracetamol and its conjugated metabolites (using HPLC, as described for urine analysis). Volume flow rate of the bile which was collected for the 4 hour period was expressed as $ml.h^{-1}.kg^{-1}$.

Assessment of liver function

Assay of serum glutamine pyruvic transaminase (GPT) activity

GPT is a specific liver enzyme and thus is an assay for liver cell integrity. GPT was determined from serum, frozen at -20°C for not

more than 7 days, with standardised optimal reagent kits B8120 and B8110 of MIFRD Diagnostics, Italy using the method of Bergmeyer *et al.* /22/.

Assay of glutathione S-transferase (GST)

The method of assay was a slightly modified Ellman principle /23/ using the reagent 5,5-dithiobis-(2-nitrobenzoic acid). 1 g of liver was homogenised in 1 ml cold 0.1 M sodium phosphate buffer (pH 7.4). Two ml of homogenate were thoroughly mixed with 2 ml 4% 5-sulphosalicylic acid and centrifuged at 4000 *g* for 5 min at 5°C. Aliquots of the supernatant (0.5 ml) were added to 4.5 ml 0.1 mM Ellman reagent dissolved in 0.1 M sodium phosphate buffer, pH 8.0. All solutions, mixtures and reagent tubes were kept ice-cold except for the centrifugation period. Absorbance was determined using Unicam UV/VIS (model 8625) at 412 nm.

Histology

The liver was fixed in 10% neutral formalin for 7 days. Tissues were dehydrated with graded ethanol, 50-100%, embedded in paraffin, then cut into 5 μm slices with a microtome (model 5030; Bright, UK). The cut tissues were stained with haematoxylin-eosin and observed under a photomicroscope. The morphological changes investigated were focal necrosis around the central vein, necrosis surrounding and extending to the central vein and confluent necrosis extending from the central vein.

Data evaluation

Data were analysed using analysis of variance followed by Tukey multiple rank tests. Histological samples were analysed by Logrank test (χ). $P < 0.05$ was regarded as statistically significant.

RESULTS

Urinary excretion

The effects of the various chloroquine salts and ferrous sulphate on the urinary excretion of paracetamol and its conjugate metabolites on

days 1 and 7 are shown in Tables 1 and 2. There were differences in the amount of excreted paracetamol and its metabolites depending on the chloroquine salt administered. For paracetamol sulphate on Day 1, excretion was increased by about 19%, decreased by 11.5%, decreased by 4% and increased by 49% with chloroquine sulphate, chloroquine phosphate, chloroquine hydrochloride and ferrous sulphate, respectively.

On Day 7, in the control group, the amount of excreted paracetamol was higher than on Day 1, and the excretion of paracetamol sulphate and paracetamol glucuronide were decreased. However, there was ~40% increase in excretion of the mercaptourate conjugate. Administration of chloroquine sulphate increased the excretion of paracetamol sulphate compared to control ($P<0.05$). The excretion of paracetamol mercaptourate was significantly reduced in all experimental groups, but paracetamol glucuronide excretion was unchanged. Chloroquine phosphate administration did not significantly change excretion of paracetamol, its sulphate or glucuronide. Ferrous sulphate administration increased the excretion of paracetamol sulphate, reduced excretion of paracetamol mercaptourate, and decreased excretion of paracetamol glucuronide by ~30%. However, the total amount of paracetamol excreted, i.e. the amount of unchanged paracetamol and its metabolites, was not significantly different in any group on Day 1 or Day 7.

Biliary excretion

The effects of chloroquine salts on biliary excretion of paracetamol and its metabolites on days 1 and 7 are shown in Tables 3 and 4. On Day 1, administration of chloroquine salts did not significantly affect the biliary excretion of paracetamol or its metabolites. Administration of ferrous sulphate caused a significant increase in the excretion of paracetamol sulphate.

On Day 7, co-administration of chloroquine sulphate increased the excretion of paracetamol, increased paracetamol sulphate, significantly increased paracetamol glucuronide, and decreased the mercaptourate conjugate. Chloroquine phosphate caused decreased biliary excretion of paracetamol ($p<0.05$), increased paracetamol sulphate, and significantly increased paracetamol glucuronide. Ferrous sulphate caused a significant decrease in excreted paracetamol, increased

TABLE 1

Urinary excretion of paracetamol (P) and its conjugated metabolites ($\mu mole.kg^{-1}$) on Day 1

Treatment	P	P sulphate	P glucuronide	P mercapto-urate	Total
Control (P)	2.7±0.5	26.8±9.2	20.4±1.5	3.61±0.20	53.7
Chloroquine sulphate + P	2.40±0.08	30.9±4.6	14.5±0.3	1.5±0.03*	49.3
Chloroquine phosphate + P	2.0±0.04	23.7±6.3	22.6±3.4	1.90±0.07*	49.2
Chloroquine hydro-chloride + P	2.4±1.2	25.8±1.7	17.0±1.8	3.36±0.06	48.5
Ferrous sulphate + P	1.84±0.08	39.8±1.7	8.0±1.4*	1.20±0.02*	50.8

Values are means ± SEM (n = 6).
*P < 0.05 compared to control.

TABLE 2

Urinary excretion of paracetamol (P) and its metabolites ($\mu mole.kg^{-1}$) on Day 7

Treatment	P	P sulphate	P glucuronide	P mer-captourate	Total
Control (P)	5.9±2.6	16.8±1.5	13.1±1.5	13.1±1.5	48.9
Chloroquine sulphate + P	0.90±0.08*	40.5±3.7*	11.2±1.7	2.0±0.2*	53.6
Chloroquine phosphate + P	4.3±0.8	15.5±1.8	16.2±1.8	4.20±0.04*	41.2
Chloroquine hydro-chloride + P	6.8±1.5	17.7±2.2	10±0.7	6.9±0.1*	41.4
Ferrous sulphate + P	0.6±0.2*	44.0±7.1*	9.1±0.3*	1.80±0.05*	54.8

Values are means ± SEM (n = 6).
*P < 0.05 compared to control.

TABLE 3

Biliary excretion of paracetamol (P) and its metabolites ($\mu mole.kg^{-1}$) on Day 1

Treatment	P	P sulphate	P glucuronide	P mercapto-urate	Total
Control (P)	0.90±0.01	0.19±0.04	1.58±0.06	1.76±0.06	4.33
Chloroquine sulphate + P	0.74±0.07	0.24±0.03	1.48±0.12	1.94±0.02	4.41
Chloroquine phosphate + P	0.74±0.04	0.20±0.09	1.68±0.04	1.46±0.03	4.08
Chloroquine hydro-chloride + P	0.85±0.06	0.19±0.04	1.70±0.12	1.67±0.34	4.50
Ferrous sulphate + P	0.65±0.11	0.34±0.06*	1.37±0.12	1.60±0.07	3.93

Values are means ± SEM (n = 6).
*P < 0.05 compared to control.

TABLE 4

Biliary excretion of paracetamol (P) and its metabolites ($\mu mole.kg^{-1}$) on Day 7

Treatment	P	P sulphate	P glucuronide	P mercapto-urate	Total
Control (P)	0.68±0.14	0.08±0.02	0.11±0.04	1.10±0.09	1.97
Chloroquine sulphate + P	0.35±0.01	1.40±0.16	0.86±0.03*	0.65±0.14	2.60
Chloroquine phosphate + P	0.34±0.07*	0.64±0.07*	0.94±0.09*	0.93±0.16	3.05
Chloroquine hydrochloride + P	0.05±0.11*	0.24±0.90	0.09±0.12	0.97±0.17	2.61
Ferrous sulphate + P	0.21±0.09*	1.96±0.25*	0.45±0.15*	0.42±0.11*	2.82

Values are means ± SEM (n = 6).
*P < 0.05 compared to control.

paracetamol sulphate and paracetamol glucuronide, and decreased paracetamol mercaptourate.

The total amount of paracetamol, i.e. the amount of unchanged paracetamol and its metabolites, excreted in the bile, was lower on Day 7 than on Day 1 in all groups, but the results did not reach significance.

There were no significant effects on bile flow in any of the experimental groups, but bile flow was significantly decreased in the paracetamol only group on Day 7 (Table 5).

Effect on hepatic function

The activities of GPT and GST on Day 1 were affected by paracetamol ($p<0.05$); the effects of co-administration of paracetamol with chloroquine salts or ferrous sulphate were not significantly different from the Control I (normal saline) group. On Day 7 the values of the hepatic enzyme markers were markedly affected in the paracetamol group and less affected but still significantly by chloroquine phosphate and chloroquine hydrochloride (Table 6).

On Day 7, there was general necrosis and other hepatic lesions with paracetamol (see Table 7). These effects were least with chloroquine sulphate and ferrous sulphate compared to the other salts tested.

DISCUSSION

At the 300 mg/kg dose employed, paracetamol affected hepatic functions somewhat on Day 1, and enough to cause marked hepatic dysfunction after continuous administration for 7 days. On Day 7, decreased biliary excretion rate, high serum GPT, low hepatic GST level and histological necrosis were evident. Paracetamol at lower doses is metabolised via the conjugating phase II reaction because of readily available hydroxyl groups which conjugate with the sulphates mediated by 3-phosphoadenosine-5-phosphosulphate (PAPS) which is the sole sulphuryl group donor in metabolism and uridyl diphosphate glucuronic acid (UDP) which donates the glucuronic acid. The other lesser conjugation is with mercaptouric acid. At high doses or under chronic dosing, the conjugating systems - sulphates, glucuronic acid or mercaptouric acid - can be depleted. Of the chloroquine salts used in this study, chloroquine sulphate increased the excretion of paracet-

TABLE 5

Bile flow (ml.kg ^{1}.h^{-1}) in rats after drug treatments

Treatment	Day 1	Day 7
Control I (normal saline)	5.45±0.17	5.53±0.14
Control II (P)	4.12±0.35	2.50±0.44*
Chloroquine sulphate + P	5.57±0.26	4.70±0.16
Chloroquine phosphate + P	4.47±0.86	4.30±0.72
Chloroquine hydrochloride + P	4.60±1.20	3.84±0.68
Ferrous sulphate + P	5.04±0.34	5.12±1.02

P = paracetamol.
Values are means ± SEM (n = 6).
*p<0.05compared to control I (normal saline).

TABLE 6

The effect of chloroquine salts and ferrous sulphate on serum glutamine pyruvate transaminase (GPT) and glutathione S-transferase (GST).

Treatment	Day 1		Day 7	
	GPT	GST	GPT	GST
Control I (normal saline)	11.5±0.4	3.12±0.20	12.8±3.5	2.96±1.40
Control II (P)	33.67±8.46*	1.15±0.61*	56.76±11.8*	0.77±0.05*
Chloroquinesulphate + P	14.7±2.90·	3.80±0.11·	16.5±1.6·	2.1±0.07·
Chloroquine phosphate + P	23.5±1.45*	1.45±0.73*	24.5±8.4	1.5±0.01*
Chloroquine hydrochloride + P	23.5±10.8*	1.25±1.2*	38.5±14.3*	1.86±0.21*
Ferrous sulphate + P	12.90±1.20·	3.17±0.21*	18.7±4.6*	2.06±0.42*

P = paracetamol.
Values are means ± SEM of triplicate assays.
*p<0.05 compared to control I (normal saline).
GSH (mmol/g liver); serum GPT (μmol/l).

TABLE 7

Histological analysis of hepatic tissue by photomicroscopy after drug treatments

Treatment	Hepatic necrosis score	
	Day 1	Day7
Control (normal saline)	6/6 (0)	6/6 (0)
Paracetamol (P)	4/4 (1)	6/6 (3)
Chloroquine sulphate + P	6/6 (0)*	1/6 (1)*
Chloroquine phosphate + P	6/6 (0)	3/6 (2)
Chloroquine hydrochloride + P	1/6 (1)*	4/6 (2)*
Ferrous sulphate +P	6/6 (0)*	6/6 (0)*

*$P < 0.05$, compared to administration of paracetamol alone.
The severity score of the lesion is shown in parentheses.
Severity score: 0 = normal, 1 = focal necrosis around the central vein, 2 = circumferential necrosis around the central vein, 3 = confluent necrosis extending from the central vein. Severity scores are averages based on the number of animals with lesions from groups of 6. Each tissue slide had three samples from each animal.

amol sulphate and reduced paracetamol glucuronide, indicating that the sulphate ion was donated to the depleted sulphate pool for the enzymatic catalysis of the reaction. Ferrous sulphate also increased the excretion of paracetamol sulphate. This agrees with the replenishment of depleted sulphate ion by the administration of sodium sulphate /19,20/. Chloroquine phosphate co-administration showed increased glucuronidation, by both the urinary and biliary routes, probably by the donation of phosphate ions to increase ATP production.

The extent of sulphation based on the excreted amount of paracetamol sulphate was greatest with ferrous sulphate co-administration. This is because most enzyme catalysed transfer reactions involving nucleotides require divalent cations. In addition, the biliary excretion route showed increased sulfation with chloroquine salts. It is noteworthy that organic anion biliary excretion is mediated by the canalicular multispecific organic transporter which is also capacity limited. Chloroquine sulphate and ferrous sulphate could enable

sulphate replenishment for the canalicular sulphate transporters and so increase paracetamol sulphate excretion compared to other conjugation systems. These results show that chloroquine sulphate and ferrous sulphate are sulphate donors, like sodium sulphate in paracetamol treated rats at various doses /18,20/. Paracetamol on Day 1 and Day 7 affected the activity of the enzymes GST and GPT, as evident by the hepatic and serum levels. These effects were minimised on Day 7 by chloroquine sulphate or ferrous sulphate compared to the other chloroquine salts. Chloroquine sulphate and ferrous sulphate may have enhanced the activities of GST and GPT by donating sulphate ions.

As malaria is often accompanied by anemia, and chloroquine is widely prescribed for the treatment of malaria, the choice of which chloroquine salt and which ferrous salt should be used in therapy becomes important. Although the results of this study still have to be verified in humans, chloroquine sulphate may protect against some of the hepatic damage caused by chronic administration of paracetamol. The use of ferrous sulphate, a drug that is administered in malaria to treat the accompanying anemia, and chloroquine sulphate might attenuate the depletion of sulphate ions caused by paracetamol conjugation. We advise that in malaria therapy, if paracetamol is to be given with a chloroquine drug, that the sulphate salt of chloroquine be preferred with the inclusion of ferrous sulphate.

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