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Role of hepatic metabolism in the bioactivation and detoxication of amodiaquine

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1. The hepatic metabolism of the antimalarial drug amodiaquine was investigated in order to gain further insight into the postulated metabolic causation of the hepatotoxicity, which restricts the use of the drug. After intraportal (i.p.) administration (54 μmol/kg) to the anaesthetized rat, the drug was excreted in bile (23 ± 3% dose over 5 h; mean ± SD, $n = 6$) primarily as thioether conjugates.

2. After i.p. administration, 20% of the dose was excreted into urine over 24 h as parent compound and products of N-dealkylation and oxidative deamination. Desethylamodiaquine accumulated in liver, but was not a substrate for bioactivation as measured by biliary elimination of a glutathione adduct.

3. Prior administration of ketoconazole, an inhibitor of P450, reduced biliary excretion by 50% and effected a corresponding decrease in the amount of drug irreversibly bound to liver proteins. This indicated a role for P450 in the bioactivation of amodiaquine to a reactive metabolite that conjugates with glutathione and protein.

4. De-ethylation and irreversible binding were observed *in vitro* using male rat liver microsomes, and were again inhibited by ketoconazole. However, no such binding was observed with human (six individuals) hepatic microsomes despite extensive turnover of amodiaquine to desethylamodiaquine.

5. Amodiaquine quinoneimine underwent rapid reduction in the presence of either human or rat liver microsomes. Therefore *in vitro* studies may underestimate the bioactivation of amodiaquine *in vivo*. These data indicate that the extent of protein adduct formation in the liver will depend on the relative rates of oxidation of amodiaquine and reduction of its quinoneimine. This in turn may be a predisposing factor in the idiosyncratic hepatotoxicity associated with amodiaquine.

6. Substitution of a fluorine for the phenolic hydroxyl group in amodiaquine blocked bioactivation of the drug *in vivo*. Insertion of an N-hydroxyethyl function enabled partial clearance of amodiaquine and its deshydroxyfluoro analogue via O-glucuronidation and altered the balance between phase I oxidation and direct phase II conjugation of amodiaquine.

Introduction

Amodiaquine (7-chloro-4-(3′-diethylamino-4′-hydroxylanilino)quinoline) (AQ) is a 4-aminoquinoline antimalarial drug, which is effective against strains of chloroquine-resistant *Plasmodium falciparum* and has been used in malaria prophylaxis and treatment (Watkins *et al.* 1984). However, because of an unacceptable prevalence of serious adverse reactions (*ca.* 1 in 2000), including fatal hepatotoxicity and agranulocytosis (Hatton *et al.* 1986, Neftel *et al.* 1986, Raymond *et al.* 1989), the drug has been withdrawn from prophylactic use and its treatment of malaria has been restricted.

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The mechanisms involved in these toxicities have not been fully defined, but early studies indicated an immunological mechanism consistent with the clinical characteristics of the reactions (Christie *et al.* 1989, Rouveix *et al.* 1989). Further work has shown that administration of the drug to rat induces a specific anti-drug immunoglobin G (IgG) response (Clarke *et al.* 1990). In a retrospective study, IgG anti-AQ antibodies were detected in sera from patients with serious adverse drug reactions (Clarke *et al.* 1991). However, AQ has too low a molecular weight to be immunogenic *per se*, and is therefore likely to function as an immunogen *in vivo* only when conjugated to a macromolecular carrier. *In vitro*, the 4-hydroxyanilino side chain of AQ is readily autoxidized to a chemically reactive quinoneimine (Maggs *et al.* 1988). The quinoneimine can conjugate directly with sulphydryl groups in glutathione and proteins, and the identification of the C-5′ glutathione conjugate of AQ as the major metabolite of the drug in rat bile demonstrated that this process occurs *in vivo* (Harrison *et al.* 1992). Furthermore, radiometric and immunochemical analyses revealed a drug-related antigen in the rat liver. These data indicate a role for reactive metabolites in the various toxicities associated with AQ.

The principal aims were, first, to investigate the ability of hepatic enzymes to bioactivate AQ, and second, to identify simple chemical modifications of AQ that either limit bioactivation or enhance detoxication.

Materials and methods

Reagents

AQ, desethylamodiaquine (DEAQ) and bisdesethylamodiaquine (BisDEAQ) were gifts from Parke-Davis (Ann Arbor, MI, USA). [Quinoline-2-^{14}C]AQ (sp. act. 9·4 μCi/μmol, radiochemical purity > 95%) and 4,7-dichloro-[G-^{3}H]quinoline (213 Ci/mol; radiochemical purity > 98%) were prepared by Amersham Int. (Amersham, UK). [Quinoline-G-^{3}H]AQ (specific activity 106·5 μCi/μmol) was synthesized according to the method of Burckhalter *et al.* 1948). Glutathione and cysteine conjugates of AQ were synthesized as described by Harrison *et al.* (1992) (chemical purity > 95%). Ketoconazole, $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, Glucurase (5×10^3 units/ml β-glucuronidase) and H-2 β-glucuronidase-arylsulphohydrolase were purchased from Sigma Chemical Co. (Poole, UK). All other reagents were from Aldrich Chemical Co. (Gillingham, UK).

Synthesis of 7-chloro-4-[4′-hydroxy-3′-[2″-(ethylamino)ethanol]methylanilino]-quinoline (hydroxyamodiaquine; HAQ)

4-Hydroxyacetanilide (33 mmol) was refluxed with 2-(ethylamino)ethanol (50 mmol) and aqueous formaldehyde (147 mmol; 33%, w/v) in ethanol (250 ml) for 48 h. The solvent was evaporated, and the product purified by flash column (silica) chromatography using methanol (1–15%)-dichloromethane as eluent. 4-Hydroxy-3-[2′-(ethylamino)ethanol methyl]acetanilide (20 mmol) was obtained as a viscous oil: EIMS *m/z* 252 (M^+,8) 221 (57), 164 (54), 122 (63), 58 (100); $C_{13}H_{20}N_2O_3$ requires 252·1474, found 252·1479.

The acetanilide (4 mmol) was heated under reflux in 40% HCl (5 ml) for 1·5 h. Solvent was removed, and the residue dissolved in ethanol (20 ml). The pH was adjusted to 5·5 with aqueous ammonia. 4,7-Dichloroquinoline (3·8 mmol) was added, and the mixture refluxed for 24 h. Solvent was evaporated under reduced pressure. Flash column (silica) chromatography using methanol–dichloromethane (1 : 4, v/v) as eluent gave HAQ (3·1 mmol; 47% overall yield) as a yellow foam: ^{1}H nmr (DMSO, 200 MHz) δ8·30 (1H, d, $J_{H\text{-}H}$ = 5 Hz, Ar-quin. H2), 8·18 (1H, d, $J_{H\text{-}H}$ = 8·8 Hz, Ar-quin. H5), 7·7 (1H, d, $J_{H\text{-}H}$ = 2·1 Hz, Ar-H), 7·58 (1H, dd, $J_{H\text{-}H}$ = 8·8 Hz, $J_{H\text{-}H}$ 2·2 Hz, Ar-quin. H6), 7·40–7·49 (2H, m, Ar-quin. H8, Ar-H), 7·18 (1H, d, $J_{H\text{-}H}$ = 8 Hz, Ar-H), 6·9 (1H, d, $J_{H\text{-}H}$ = 5 Hz, Ar-quin. H3), 4·5 (2H, s, CH_2N), 4·0 (2H, t, CH_2CH_2OH), 3·5 (4H, m, CH_2CH_2OH, NCH_2CH_3), 1·5 (3H, t, NCH_2CH_3); CIMS *m/z* 372 (M + 1, 67), 324 (15), 283 (60), 171 (100), 136 (58), 107 (66), 91 (80); $C_{20}H_{23}N_3O_2Cl$ requires 372·14788, found 372·14689.

Synthesis of 7-chloro-4-[4′-fluoro-5′-[(diethylamino)methylanilino]-[G-^{3}H]-quinoline ([^{3}H]DFAQ)

4,7-Dichloro-[G-^{3}H]-quinoline (253 μmol, 1 mCi) and 4-fluoro-3-diethylaminomethylaniline (383 μmol) in 5 ml acidified ethanol were refluxed for 16 h. Excess amine was removed by silica-column chromatography (methanol–dichlormethane, 1 : 9 v/v) to give [^{3}H]DFAQ (radiochemical purity, 87% by hplc) in 42% yield. Final purification was effected by elution from a C_{18} hplc column with acetonitile (10–20%) over 18 min) in 0·1 M ammonium acetate, pH 3·8, at 1·5 ml/min. [^{3}H]DFAQ (radiochemical

purity > 99%) was recovered by ether extraction (1 : 1, v/v) of concentrated eluate adjusted to pH 8·0 with 5 M NaOH. It was characterized by EI mass spectrometry (O'Neill *et al.* 1994).

Unlabelled DFAQ was prepared according to O'Neill *et al.* (1994).

Synthesis of 7-chloro-4-[4'-fluoro-5'-[2"-(ethylamino)ethanol]methylanilino]-[G-^{3}H]-quinoline ([^{3}H]HDFAQ)

4,7-Dichloro-[G-^{3}H]-quinoline (253 μmol, 1 mCi) and 4-fluoro-3-[2'-(ethylamino)ethanol]aniline (383 μmol) in 5 ml acidified ethanol were refluxed for 16 h. [^{3}H]HDFAQ (radiochemical purity 95%) was isolated in 20% yield by silica-column chromatography (methanol–dichloromethane, 1 : 9, v/v). It was characterized by CI mass spectrometry: *m/z* 374 (M + 1, 100), 340 (39), 330 (11), 296 (10), 287 (12), 253 (15).

Unlabelled HDFAQ was prepared by condensation of 4,7-dichloroquinoline (3·0 mmol) and 4-fluoro-3-[2'-(ethylamino)ethanol]aniline (3·0 mmol) in 15 ml acidic ethanol (pH 5·5). EIMS *m/z* 373 (M^+, 2), 342 (100), 285 (45), 270 (30), 250 (58).

Administration of drugs to the anaesthetized rat

Male (n = 5, 200–260 g) and female (n = 5, 195–210 g) Wistar rats were anaesthetized with urethane (7% w/v in 0·9% saline, 20 ml/kg), and bile duct cannulae inserted. [^{14}C]AQ (54 μmol/kg, 10 μCi/kg) in polyethylene glycol (PEG) 200–saline (1 : 1, v/v), HAQ (54 μmol/kg) in PEG 200–saline–ethanol (12 : 5 : 2, v/v) and [^{3}H]HDFAQ (54 μmol/kg, 20 μCi/kg) in PEG 200–saline–ethanol (3 : 3 : 1, v/v) were injected via the hepatic portal vein. Bile fractions were collected every 30 min up to 5 h after dosing. After 5 h, urine was aspirated from the bladders of the animals dosed with [^{3}H]DFAQ and [^{3}H]HDFAQ. Rats were killed by exsanguination and their livers removed; additional organs were removed from the animals dosed with [^{3}H]HDFAQ. Bile was stored at − 20°C and the organs at − 80°C.

To examine the effect of inhibition of P450, male (n = 6, 225–280 g) and female (n = 5, 190–200 g) Wistar rats were administered ketoconazole (50 mg/kg, i.p.). Rats were anaesthetized, and [^{14}C]AQ administered as described above exactly 1 h after ketoconazole pretreatment.

Administration of DEAQ to rat and analysis of biliary metabolites

DEAQ (54 μmol/kg) was administered to male rat (n = 2, 250 g) following the procedure used for [^{14}C]AQ. Bile fractions were collected every 30 min for 5 h. Pooled bile samples were analysed by hplc using acetonitrile (10–25% over 30 min) in ammonium dihydrogen orthophosphate (10 mM, pH 4·6) at 1·5 ml/min; analytes were detected at 254 nm.

Administration of [^{3}H]AQ to rat

Male Wistar rats (n = 4, 230–250 g) were dosed i.p. with [^{3}H]AQ (54 μmol/kg, 20 μCi/kg) in PEG–saline–ethanol (3 : 3 : 1, v/v). They were housed in metabolism cages for 24 h with water available *ad libitum* but without food. Urine was collected over 24 h.

Measurement of radioactivity

Bile (20 μl) and urine (10–200 μl) were mixed with scintillant (4 ml), and assayed for radioactivity with external quench correction. Total radioactivity in tissues was measured as described by Harrison *et al.* (1992). Irreversibly bound radiolabelled material in liver was determined by exhaustive solvent extraction as described by Harrison *et al.* (1992).

High performance liquid chromatography (hplc)

A μBondapak C_{18} (300 × 3·9 mm i.d.; 10 μm; Waters, Watford, UK) was connected to either a Kontron 325 or a Spectra Physics SP8800 pump. Compounds were detected with a Spectrasystem UV1000 spectrophotometer set at 254 nm; integration was performed using the analogue input of a Radiomatic A250 Flo-one/β radioactive flow detector (Canberra-Packard, Pangbourne, UK). Radiolabelled analytes were determined on-line with the flow detector. The recovery of chromatographed radiolabelled material was 85–90%. Except as indicated, compounds were eluted with a linear gradient of acetonitrile (10–20% over 18 min) in 0·1 M ammonium acetate, pH 3·8, at a flow rate of 1·5 ml/min.

Quantification of the biliary metabolites of HAQ

Portions (1–25 nmol) of the C-5' glutathione adduct of AQ (Harrison *et al.* 1992) dissolved in water were eluted from a C_{18} column with a linear gradient of acetonitrile (10–25% over 30 min) in ammonium dihydrogen orthophosphate (10 mM, pH 4·6) at a flow rate of 1·5 ml/min. A calibration graph of peak area versus mass of standard was constructed by the least-squares method. Aliquots (50 μl) of the bile collected during the 15 min prior to drug administration and of the bile collected for 5 h thereafter were analysed.

Chromatographic analysis of radiolabelled biliary and urinary metabolites

Aliquots (*ca.* 100 μl) of pooled bile fractions (0–5 h) were analysed by hplc. The metabolites of [^{14}C]AQ were eluted with acetonitrile (10–25% over 30 min) in ammonium dihydrogen orthophosphate buffer,

pH 4·6, at a flow rate of 1·5 ml/min. The metabolites of [^{3}H]DFAQ and [^{3}H]HDFAQ were analysed using the acetonitrile–ammonium acetate mobile phase.

Urine from rat administered [^{3}H]AQ was filtered (Puradisc 25 AS, 0·45 μm; Whatman, UK), and concentrated via adsorption onto a Sep-Pak C_{18} cartridge conditioned by washing with methanol (20 ml) and water (10 ml) in turn. The cartridge was washed with water (20 ml) and methanol (4 ml). The recovery of radiolabel in the methanolic effluent was 80%. Reconstituted effluent (80 μl methanol) was chromatographed using acetonitrile (5–25% over 30 min) in 0·1 M ammonium acetate, pH 3·8, at a flow rate of 1·5 ml/min.

The urinary metabolites of [^{3}H]DFAQ were likewise chromatographed but without prior concentration.

Identification and quantification of hepatic residues of [^{14}C] AQ and DEAQ

Liver from rat dosed with either [^{14}C]AQ or DEAQ was homogenized (25%, w/v) with 0·1 M sodium phosphate buffer, pH 7·4, homogenate (0·5 ml) was mixed with methanol (5 ml) and left overnight at 5°C. The precipitated protein was exhaustively extracted with methanol and 70% methanol (Harrison *et al.* 1992). Supernatants from the precipitation and extractions were combined, evaporated to dryness under nitrogen, and reconstituted in methanol (200 μl). DEAQ was quantified using a calibration graph of peak area versus mass (3–25 nmol; $r^2 = 0{\cdot}998$). [^{14}C]AQ and [^{14}C]DEAQ were quantified by radiometric hplc. [^{14}C]DEAQ and DEAQ were isolated by hplc. The eluent was concentrated under nitrogen at 50°C, and loaded onto conditioned Sep-Pak C_{18} cartridges. The cartridges were washed with water (20 ml) and the absorbed aminoquinolines eluted with methanol (4 ml). The metabolites were identified by co-chromatography and EI mass spectrometry.

Half-life of AQ and DEAQ in rat liver

Male Wistar rat ($n = 3$ per time point, 230–250 g) received AQ (54 μmol/kg) in PEG 200–saline (1:1, v/v, 4 ml/kg, i.p.). They were killed by cervical dislocation after 2, 4, 6, 12, and 24 h. Precipitation and exhaustive extraction of protein in liver homogenates were carried out as before. The washes were combined, evaporated to dryness and reconstituted in methanol (200 μl). Aliquots (2×25 μl) were analysed by hplc. AQ and DEAQ were quantified using a calibration graph of peak area versus mass: aliquots (5, 10, 20, 40, 80 μl; 0·5–8 nmol) of a 0·98-μM aqueous solution of AQ were chromatographed in triplicate.

Enzymic hydrolysis of metabolites

Bile (50 or 100 μl) was incubated at 37°C for 16 h with either Glucurase (0·75–1·5 ml) alone or H-2 preparation (42 units of arylsulphohydrolase activity) in 0·1 M sodium acetate (pH 5·0, 240 μl) containing D-saccharic acid-1,4-lactone (10 mM). Hydrolysis of glutathione adducts was achieved by treating aliquots (50 μl) of bile with γ-glutamyl transpeptidase (1 unit) in 0·125 M Tris buffer (pH 8·0, 350 μl) and 0·1 M $MgCl_2$ (110 μl) at 37°C for 90 min. Control incubations were carried out in the absence of the enzymes. Samples of all incubations were analysed by hplc; those taken from incubations of HAQ metabolites with γ-glutamyl transpeptidase were eluted with the acetonitrile–phosphate buffer eluent.

Isolation and characterization of biliary and unity metabolites

[^{14}C]AQ: bile. The major biliary metabolite was isolated as previously described (Harrison *et al.* 1992).

[^{3}H]AQ: urine. The metabolites concentrated by solid-phase extraction were dissolved in methanol and fractionated by elution from a C_{18} column with acetonitrile (5–25% over 30 min)–ammonium acetate. The eluate constituting each of the metabolites was concentrated under nitrogen and loaded onto a conditioned Sep-Pak C_{18} cartridge; from which the metabolite was eluted with methanol (5 ml). The fractions were analysed by EI mass spectrometry.

HAQ: bile. Two biliary metabolites were resolved with the acetonitrile (10–25%, 30 min)–phosphate buffer eluent. Bile (50 μl aliquots) collected up to 90 min after administration of HAQ was chromatographed. The metabolites were recovered by solid-phase extraction, and characterized by fast ion bombardment (FIB), atmospheric pressure chemical ionization (ACPI) and electrospray (ESP) mass spectrometry.

[^{3}H]DFAQ: Urine. [^{3}H]DFAQ was recovered from urine (1 ml) by ether (2 ml) extraction. It was analysed by EI mass spectrometry without purification.

[^{3}HDFAQ: bile. Parent compound was recovered by ether extraction (4 vol. × 2) of bile (0·7–1·5 ml; 0·9–1·8 mg equivalent radiolabelled material) mixed with 75 mM sodium phosphate, pH 7·2; a number of minor radiolabelled components, which were less polar than [^{3}H]HDFAQ, were also extracted. The pooled extracts were evaporated to a dry residue, reconstituted in methanol (200–300 μl), and chromatographed (50 μl aliquots) to isolate the [^{3}H]HDFAQ.

HDFAQ glucuronide, the principal metabolite in the bile of male rat with a high biliary output of total radioactivity, was isolated by hplc; bile collected during the first 90 min was chromatographed in 50 μl aliquots. The purified glucuronide was characterized by APCI mass spectrometry. It was hydrolyzed with β-glucuronidase, the hydrolysate diluted with water (5:2, v/v), and the aglycone isolated by hplc.

Hepatic metabolites

Liver from rat administered either [^{3}H]DFAQ or [^{3}H]HDFAQ was homogenized in phosphate buffer (Harrison *et al.* 1992). Aliquots (0·5 ml) of each homogenate were mixed with methanol (3 ml), and the protein thus precipitated was extracted with methanol (3 ml). The combined supernatant and extract was evaporated to dryness under nitrogen, and reconstituted in methanol (200 μl) for analysis and isolation of radiolabelled components by hplc.

Preparation of human and rat liver microsomes

Microsomes were prepared from histologically normal human livers as described previously (Purba *et al.* 1987). Rats were killed by cervical dislocation and microsomes prepared by the same method. Microsomal samples were stored at −80°C. Protein concentrations were measured by the method of Lowry *et al.* (1951).

Incubation of liver microsomes with [^{14}C]AQ

[^{14}C]AQ (10 μM, 0·1 μCi) was incubated in duplicate with human and rat liver microsomes (2 mg protein/incubation) in sodium phosphate buffer (0·1 M, pH 7·4) at 37°C for up to 60 min. Microsomes were preincubated with an NADPH-regenerating system (glucose 6-phosphate, 10 mM; $NADP^+$, 0·5 mM; glucose 6-phosphate dehydrogenase, 2 units/incubation) for 2 min. Glutathione (1 mM) and ketoconazole (50 μM) were included as required. Final incubation volume was 1 ml. The incubation was initiated upon addition of AQ, and terminated by adding acetonitrile (3 ml). The protein precipitated at 5°C overnight. Irreversibly bound radiolabelled material was determined as described previously (Harrison *et al.* 1992). Supernatant from the precipitation was analysed by radiometric hplc. The major metabolites were isolated by hplc. They were recovered from the eluate via concentration on Sep-Pak C_{18} cartridges, and characterized by EI mass spectrometry.

Incubation of human liver microsomes with P450 inhibitors

[^{14}C]AQ (10 μM, 0·1 μCi) was incubated with pooled human liver microsomes (n = 6, 0·4 mg) in sodium phosphate buffer (0·1 M, pH 7·4) at 37°C for 10 min in the presence of an NADPH-regenerating system as before. Ketoconazole (20 μM), proguanil (20 μM), quinine (20 μM), quinidine (10 μM), enoxacin (50 μM), cyclosporin (20 μM) and sulphaphenazole (5 μM) were included in the incubation mixtures. Gestodene (20 μM) was preincubated with microsomes and NADPH for 12 min.

Incubation of liver microsomes with [^{14}C]amodiaquine quinoneimine

[^{14}C]amodiaquine quinoneimine (AQQI) (10 μM, 0·1 μCi), synthesized as described previously (Harrison *et al.* 1992), was incubated with pooled human (n = 6, 2 mg protein/incubation) and pooled male rat (n = 6, 2 mg/incubation) liver microsomes in sodium phosphate buffer (0·1 M, pH 7·4) in quadruplicate at 37°C for up to 60 min. Microsomes were preincubated with the NADPH-regenerating system for 2 min prior to addition of the quinoneimine. Irreversibly bound radiolabelled material was determined as before. Supernatants from the precipitation were analysed by hplc.

Analysis of glutathione conjugate of AQ formed in vitro

Supernatants from the precipitation and first extraction of rat and human liver microsomes incubated with [^{14}C]AQ (10 μM, 0·1 μCi) and glutathione (1 mM) were combined. The supernatants were evaporated to dryness, reconstituted in methanol and analysed by hplc; the eluent was acetonitrile (10–25% over 25 min, 25–40% over the next 15 min) in ammonium dihydrogen orthophosphate (10 mM, pH 4·6) at a flow rate of 1·5 ml/min.

Statistical analysis

All values are given as mean ± SD of the mean. Statistical analysis were carried out using an unpaired t-test. Differences were deemed significant at the 5% level.

Mass spectrometry

Electron impact (EI), chemical ionization (CI) and FIB spectra were obtained with a Fisons Instruments TS-250. Direct-probe samples were analysed with probe heating: source temperature, 180°C, electron energy, 70 eV; accelerating voltage, 4×10^3V. Full-scan spectra were recorded over m/z 50–800 (1 scan/s) at a resolution of 800. Ammonia at a souce pressure of 2×10^{-4} mBar was used as reagent gas. Samples were dissolved in α-thioglycerol for caesium-beam FIB: gun voltage, $14{\cdot}5 \times 10^3$ V; beam energy, $10{\cdot}5 \times 10^3$ V. Spectra were acquired over m/z 100–800 (1 scan/10s) via multichannel analysis.

ACPI analyses were performed on a Sciex API III fitted with a heated (500°C) nebulizer interface (Thornhill, Ontario, Canada). Isolated metabolites redissolved in mobile phase were introduced by direct

injection in methanol–4 mM ammonium acetate (1:1, v/v) flowing at 50 μl/min. Full-scan spectra were acquired over *m/z* 100–700 at 1 scan/s. Daughter spectra were generated using argon as collision gas.

ESP spectra were obtained with a Quattro tandem quadrupole instrument (Fisons). Solutions of isolated metabolites (methanol–water, 1:1, v/v) were injected into a mobile phase of acetonitrile–water (1:1, v/v) flowing through the ESP interface at 10 μl/min. Data were acquired over *m/z* 100–600, and the extent of fragmentation was controlled by varying the cone voltage. Daughter spectra were recorded at a collision gas (argon) pressure of 4×10^{-3} mBar and at an energy of 16 eV. For hplc-ESP mass spectrometry, whole bile was eluted from a Supelcosil LC-18-DB column (3·3 × 0·46 cm i.d., 3 μm; Supelco, Bellefone, PA, USA) with acetonitrile–50 mM ammonium acetate (2:3, v/v) at 200 μl/min; the entire eluate was admitted to the ESP interface.

Results

Metabolism of [^{14}C]*AQ* in vivo

Metabolites of [^{14}C]AQ were eliminated in bile after intraportal administration of the drug to male rat (table 1). Pretreatment with ketoconazole significantly ($p < 0{\cdot}001$) reduced biliary excretion (table 1). The principal metabolites were C-5′-glutathionyl AQ (15% of dose) and C-5′-cysteinyl AQ (3%) (figure 1); they were identified by co-chromatography with unlabelled standards (Harrison *et al.* 1992). FIB mass spectrometry gave unambiguous characterization of the major biliary metabolite as a glutathione adduct of AQ (*m/z* 661 $[M+1]^+$, 588, 315); the metabolite's spectrum matched that of a synthetic standard (Harrison *et al.* 1992). Biliary excretion of radioactivity in female rat (14·0 ± 2·0%) was significantly less than in males ($p < 0{\cdot}01$) but was also reduced by pretreatment with ketoconazole (4·8 ± 1·4%). The major metabolite was C-5′-glutathionyl AQ.

Table 1. Effect of ketoconazole on the hepatic disposition of [^{14}C]AQ in the male rat.

	Control ($n = 5$)	Test ($n = 6$)
Total radioactivity in the bile (0–5 h)	22·9 ± 2·9	11·0 ± 2·0
Total radioactivity in the liver	24·4 ± 3·9	29·9 ± 5·4
Covalent binding to liver protein	1·3 ± 0·6	0·9 ± 0·2

Data are mean % ± SD of dose. [^{14}C]AQ was administered intraportally (54 μmol/kg, i.p.v.) to the anaesthetized male rat cannulated via the bile duct. Rats were killed after 5 h. Ketoconazole was administered i.p. 60 min before [^{14}C]AQ in test animals.

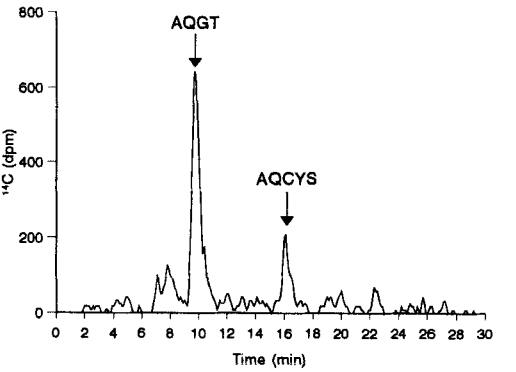


Figure 1. Radiometric hplc chromatogram of bile (0–5 h) from the male Wistar administered [^{14}C]AQ (54 μmol/kg, i.p.v.). Arrows indicate the retention times of authentic standards of the C-5′ glutathione adduct of amodiaquine (AQGT) and the C-5′ cysteine adduct of amodiaquine (AQCYS).

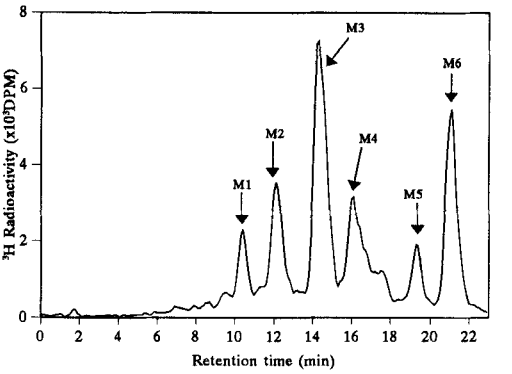


Figure 2. Radiometric hplc chromatogram of urine (0–24 h) from the male rat dosed with [^{3}H]AQ (54 μmol/kg, 20μCi/kg, i.p.). Arrows represent the retention times of authentic BisDEAQ (**1**), DEAQ (**2**), and AQ (**3**). Metabolites **4** and **5** were not identified. Metabolite **6** was a carboxylic acid metabolite of AQ.

Table 2. Urinary metabolites of [^{3}H]AQ in the male rat.

Metabolite	% Dose (mean ± SD, $n = 4$)
1	1·3 ± 0·5
2	2·9 ± 0·8
3 (AQ)	5·3 ± 1·1
4	4·3 ± 0·4
5	1·0 ± 0·3
6	3·7 ± 1·3

[^{3}H]AQ (54 μmol/kg, i.p.) was administered i.p. and urine collected over 24 h. Metabolites were analyzed by hplc and characterized by EI mass spectrometry/co-chromatography. Metabolite **1**, bisDEAQ; **2**, DEAQ; **4** and **5**, not identified; and **6**, carboxylic acid metabolite of AQ (Figure 2).

A small percentage (0·9%) of the dose became irreversibly bound to liver tissue in the control male rat (table 1).

The major radiolabelled compound recovered from the liver of male rat was DEAQ (EI mass spectrum: m/z 327[M^+], 282, 253, 218), representing 14·4 ± 2·5% of the dose. The ratio of [^{14}C]AQ to [^{14}C]DEAQ in liver extracts increased from 0·27 ± 0·22 to 3·32 ± 3·26 as a consequence of pretreatment with ketoconazole. The liver half-life of AQ and DEAQ was 4·1 and 8·4 h respectively.

After i.p. administration of [^{3}H]AQ to male rat, 19·2 ± 1·8% of the dose was eliminated in urine over 24 h. Six major radiolabelled compounds (figure 2) were found in urine (table 2). Metabolite **1** ($R_t = 10$ min) was preliminarily characterized as BisDEAQ by co-chromatography with authentic standard. Metabolite **2** ($R_t = 12$ min) co-chromatographed with authentic DEAQ and yielded an identical EI mass spectrum: m/z 329 (M^+, 12%), 327 (34), 284 (50), 282 (100), 253 (41), 218 (25). Metabolite **3** ($R_t = 14$ min) co-chromatographed with authentic AQ and yielded an identical mass spectrum: m/z 357 (M^+, 17%), 355 (45), 284 (72), 282 (100), 253 (26), 218 (18). Metabolites **4** and **5** ($R_t = 16$ and 21 min respectively) were not identified. Metabolite **6** ($R_t = 21$ min) was the carboxylic acid product of oxidation of the C-3′ methylene group: m/z 316 (M^+, 26%), 314 (81), 298 (35), 296 (100), 270 (26), 239 (51), 205 (39).

Metabolism of desethylamodiaquine in vivo

Analysis of liver extracts 5 h after dosing showed the presence of one major peak (19·7 ± 0·6% of dose), which co-chromatographed with DEAQ (R_t = 9 min). EI mass spectrometry gave unambiguous characterization of this metabolite as DEAQ (*m/z* 327 [M^+], 282, 253, 218).

Comparative hplc analyses of bile collected before and after the administration of DEAQ did not reveal the presence of any metabolites.

Metabolism of [^{14}C]AQ by rat and human liver microsomes

[^{14}C]AQ was metabolized to DEAQ (R_t = 8 min) and BisDEAQ (R_t = 6 min) in hepatic microsomes (figure 3). Rat microsomes were also found to generate a non-polar metabolite (R_t = 17 min). Both the dealkylated products were identified by co-chromatography. The major metabolite of both rat and human microsomal incubations, DEAQ, was characterized by EI mass spectrometry: *m/z* 327 (M^+, 41%), 282 (100), 253 (38), 218 (26). The non-polar metabolite was tentatively characterized as AQ N-oxide: *m/z* 371 (M^+, 4%), 355 (55), 282 (100), 253 (30), 218 (19); facile loss of 16 amu (oxygen) is frequently observed during EI analysis of N-oxides (Bryce and Maxwell 1965). DEAQ production by rat microsomes was significantly inhibited by 50 μM ketoconazole (table 3). However over 60 min, ketoconazole did not significantly decrease metabolite production by human liver microsomes (table 3). It had no significant effect on the production of the putative N-oxide of AQ by rat liver microsomes.

Hepatic microsomes from male rat were able to metabolize a small fraction of the [^{14}C]AQ to irreversibly bound material in the presence of the NADPH regenerating system (table 4); glutathione and ketoconazole significantly ($p < 0·05$) reduced the binding.

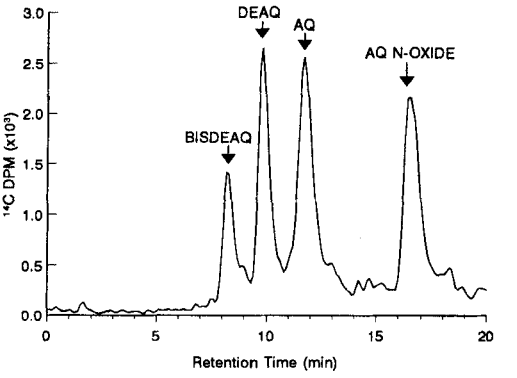
The C-5′ glutathione adduct of AQ, identified by co-chromatography with the appropriate standard, was formed with the drug was incubated with male rat and human liver microsomes in the presence of glutathione (1 mM) (5 and 1·8% of incubated radiolabelled material respectively; figure 4).

Metabolism of [^{14}C]AQ quinoneimine by human and rat liver microsomes

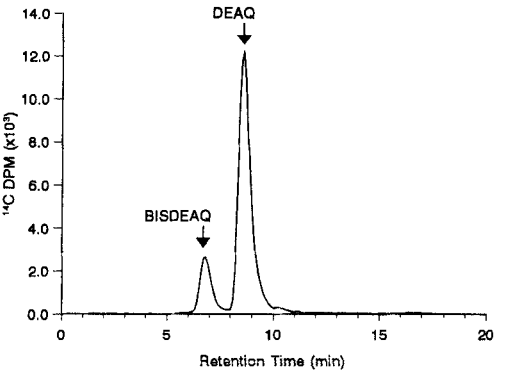
In the absence of NADPH, approximately 50% of the [^{14}C]AQ quinoneimine incubated with rat and human microsomes became irreversibly bound (table 5). Binding was significantly inhibited by NADPH. AQ (18·0 ± 1·5% incubated radioactivity) was recovered from incubations containing rat microsomes but not from those containing human microsomes. DEAQ, identified by co-chromatography, was the major stable metabolite of AQ quinoneimine in the presence of NADPH after a 60 min incubation. It represented 50·1 ± 1·3 and 78·3 ± 4·0% of the radioactivity in rat and human incubations respectively. BisDEAQ, also identified by co-chromatography, comprised 6·2 ± 0·5 and 6·3 ± 0·9% of the radioactivity in rat and human incubations respectively. In the absence of NADPH, all of the unbound radiolabelled material co-chromatographed with AQ.

Effect of P450 inhibitors on the metabolism of [^{14}C]AQ by human liver microsomes

[^{14}C]AQ and a number of relatively selective P450 inhibitors (Halpert *et al.* 1994) were incubated with pooled human liver microsomes. Gestodene (20 μM) and ketoconazole (20 μM) significantly ($p < 0·05$) decreased DEAQ formation to 39 and



(*a*)



(*b*)

Figure 3. Radiometric hplc chromatogram of the metabolites extracted from 60-min incubations of [^{14}C]AQ (0·1 μCi, 10 μM) with (*a*) male rat, and (*b*) human hepatic microsomes.

Table 3. Stable metabolites of [^{14}C]AQ formed by liver microsomes.

Species	Control	Glutathione (1 mM)	Ketoconazole (50 μM)
Rat ($n = 6$)			
AQ	23·3 ± 4·9	30·9 ± 10·4	45·3 ± 9·4*
DEAQ	16·5 ± 4·3	13·9 ± 4·0	8·2 ± 5·9*
BisDEAQ	16·7 ± 6·3	9·4 ± 5·4	1·6 ± 0·4
AQ N-oxide	37·0 ± 4·9	31·4 ± 12·7	40·2 ± 9·8
Man ($n = 6$)			
AQ	1·1 ± 2·7	2·7 ± 4·2	7·5 ± 6·3
DEAQ	74·6 ± 9·1	70·0 ± 6·8	76·4 ± 7·6
BisDEAQ	21·5 ± 5·7	21·6 ± 5·7	14·8 ± 2·9
AQ N-oxide	—	—	—

Male rat and human liver microsomes were incubated with [^{14}C]AQ (10 μM, 0·1 μCi) in the presence of either ketoconazole (50 μM) or glutathione (1 mM) at 37°C for 60 min. Metabolites were determined by radiometric hplc. Data are mean ± SD% of chromatographed radioactivity for individual livers.

*$p < 0·05$ compared with control.

Table 4. Effect of ketoconazole and glutathione on the irreversible binding of [^{14}C]AQ to liver microsomes.

	Male rat ($n = 6$)	Man ($n = 6$)
Control	12·6 ± 5·4	2·4 ± 1·7
− NADPH	3·7 ± 0·9*	3·8 ± 1·7
+ Glutathione (1 mM)	1·7 ± 0·5*	1·2 ± 0·6
+ Ketoconazole (50 μM)	4·7 ± 1·1*	1·4 ± 0·3

Microsomal protein (2 mg) was incubated with [^{14}C]AQ (10 μM, 0·1 μCi) at 37°C for 60 min in the presence of an NADPH-regenerating system. Irreversibly bound radioactivity was determined by exhaustive solvent extraction of precipitated microsomes. Data are mean % ± SD of radioactivity incubated with microsomes from individual livers.

*$p < 0.05$ compared with control.

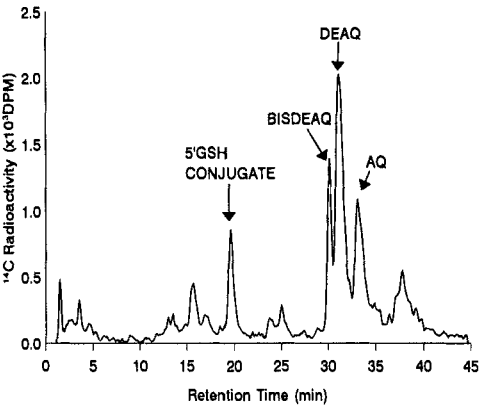


Figure 4. Radiometric hplc chromatogram of the metabolites extracted from 60-min incubations of [^{14}C]AQ (0·1 μCi, 10 μM) and GSH (1 mM) with hepatic microsomes from the male rat.

Table 5. Irreversible binding of [^{14}C]AQ quinoneimine to human and male rat liver microsomes.

	Irreversible binding			
	0 min		60 min	
	− R.SYST	+ R.SYST	− R.SYST	+ R.SYST
Rat	59·3 ± 2·3	15·5 ± 2·3*	91·5 ± 13·0	18·8 ± 1·7*
Man	50·0 ± 7·3	9·1 ± 0·8*	68·0 ± 3·2	13·9 ± 2·8*

Pooled ($n = 6$) human and male rat microsomes (2 mg/protein) were incubated with [^{14}C]AQ quinoneimine (10 μM, 0·1 μCi) at 37°C for 60 min. Irreversibly bound radioactivity was determined by exhaustive solvent extraction of precipitated proteins. Data are mean % ± SD of incubated radioactivity from quadruplicate incubations.

+/− R.SYST = NADPH regenerating system.

*$p < 0.001$.

21% of control values respectively. The other inhibitors appeared to have no significant effect on deethylation (data not shown).

Metabolism of HAQ

Two metabolites were found in the bile of male rat administered HAQ (figure 5). The first peak ($R_t = 8·6$ min; 7·0 ± 2·6% of dose) was completely

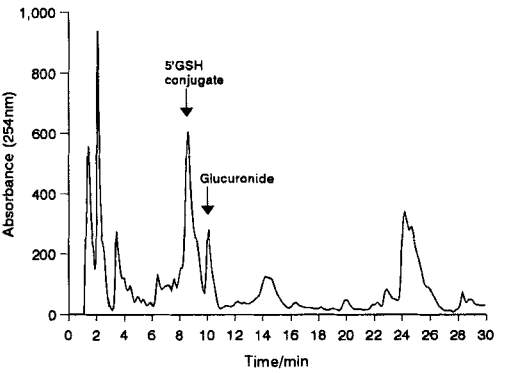


Figure 5. Biliary metabolites of HAQ in the male rat. Metabolites were detected at 254 nm.

hydrolysed by γ-glutamyl transpeptidase. The FIB spectrum of the isolated metabolite contained ions at *m/z* 677 ($[M+1]^+$, 15%) and 588 ($[M+1-NH(C_5H_5)C_2H_4OH]^+$, 8%); the fragment, arising from loss of the side chain, is seen in the spectrum of the C − 5′ glutathione adduct of AQ (Harrison *et al.* 1992). This metabolite was characterized as a glutathione conjugate of HAQ. The second peak (R_t = 10 min; 3·0 ± 0·6% of dose) underwent complete hydrolysis by β-glucuronidase; the liberated aglycone co-eluted with authentic HAQ. The APCI spectrum of the isolated fraction contained the pseudomolecular ion ($[M-1]^-$) of HAQ glucuronide at *m/z* 546 (16%) and the anion of the sodium salt at *m/z* 568 (2%). MS–MS analysis of *m/z* 546 revealed fragments at *m/z* 279 and 264, seemingly derived from the glucuronidated side-chain moiety via scission either side of the nitrogen, which indicated that conjugation had occurred through the alkylic rather than phenolic hydroxyl group. The same pseudomolecular ion (41%) and sodium salt (3%) were formed when the fraction was analysed by direct-introduction ESP mass spectrometry. Higher cone voltages (*m/z* 546, 14%) generated fragments, at *m/z* 281 (45%) and 264 (50%), arising from breakage of the proximal C–N bond with charge retention by the 4′-hydroxy-3-methylanilinoquinoline and glucuronidated side-chain moiety respectively. Daughter-ion scans of *m/z* 546 only detected the *m/z* 281 and 264 fragments.

[^{3}H]DFAQ

[^{3}H]DFAQ administered to male rat was eliminated in bile (6·7 ± 1·2% of dose) and urine (4·6 ± 1·6%) over 5 h. Radiolabelled material was located in liver (19·0 ± 2·0%), kidney (6·5 ± 0·5%), lung (2·1 ± 0·3%), spleen (0·8 ± 0·2%), and brain (0·6 ± 0·1%). Negligible quantities of radiolabel were irreversibly associated with hepatic protein.

DFAQ (R_t = 8·1 min; 5·3 ± 1·9% of dose) and one metabolite (R_t = 6·7 min; 11·7 ± 1·6%) were recovered from the liver. The former was identified as unchanged drug by co-chromatography with authentic standard and by CI mass spectrometry: *m/z* 358 ($M^{+\cdot}$, 88%) 343 (59), 329 (43), 324 (33), 286 (20), 250 (13). The metabolite was identified as desethyl-DFAQ from its CI spectrum: 330 ($M^{+\cdot}$, 100%), 314 (6), 286 (19), 270 (5), 250 (9).

Parent compound (2·7 ± 1·0%) and desethyl-DFAQ (0·8 ± 0·1%) were eliminated in urine. DFAQ was characterized by co-chromatography and its EI spectrum. The metabolite had the same retention time as desethyl-DFAQ recovered from liver

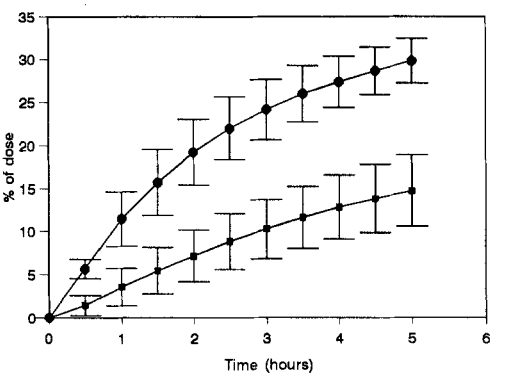


Figure 6. Cumulative excretion of radiolabelled material into the bile of the male rat administered [^{3}H]HDFAQ (54 μmol/kg, i.p.v.). ● High elimination group ($n = 3$); and ■, low elimination group ($n = 6$). Data are mean ± SD.

Table 6. Metabolism of [^{3}H]HDFAQ in rat: distribution and elimination of total radioactivity in high and low biliary elimination groups.

	Biliary elimination group	
	High ($n = 3$)	Low ($n = 6$)
Liver	16·7 ± 7·1	16·4 ± 3·3
Kidney	4·8 ± 0·5	4·1 ± 0·9
Lung	1·5 ± 0·4	1·4 ± 0·3
Spleen	1·0 ± 0·1	0·9 ± 0·4
Bile	29·8 ± 2·6*	14·7 ± 4·2*
Urine	10·9 ± 5·0	6·3 ± 3·6

[^{3}H]HDFAQ (54 μmol/kg, 20 μCi/kg) was administered to the anaesthetized male rat via the hepatic portal vein. Bile was collected for 5 h. Urine was removed from the bladder after 5 h. Data are mean % ± SD of dose.

*$p < 0·01$.

and was refractory to the actions of γ-glutamyl transpeptidase, β-glucuronidase and arylsulphohydrolase.

Chromatographic analysis of bile revealed only one discernible radiolabelled component ($R_t = 9·3$ min), which was not hydrolysed by any of the above enzymes.

[^{3}H]HDFAQ

Elimination and distribution of total radioactivity. Male rats studied appeared to consist of two groups in respect of the cumulative elimination of total radioactivity in bile (figure 6, table 6). However, neither urinary excretion nor tissue distribution of total radioactivity was subject to discontinuous variation. Only negligible amounts of radiolabelled material were irreversibly bound to hepatic protein.

Two radiolabelled components, resolved by hplc, were recovered from the livers of both high and low elimination groups of male rat. The second peak (8·6 ± 3·5 and 9·7 ± 3·2% of dose) co-eluted with HDFAQ. The first ($R_t = 6·4$ min; 6·2 ± 4·4 and 3·7 ± 0·8%) was an unidentified metabolite.

Biliary metabolites. Two distinct metabolite profiles, each exclusively associated with one of the biliary elimination groups, were observed (figure 7).

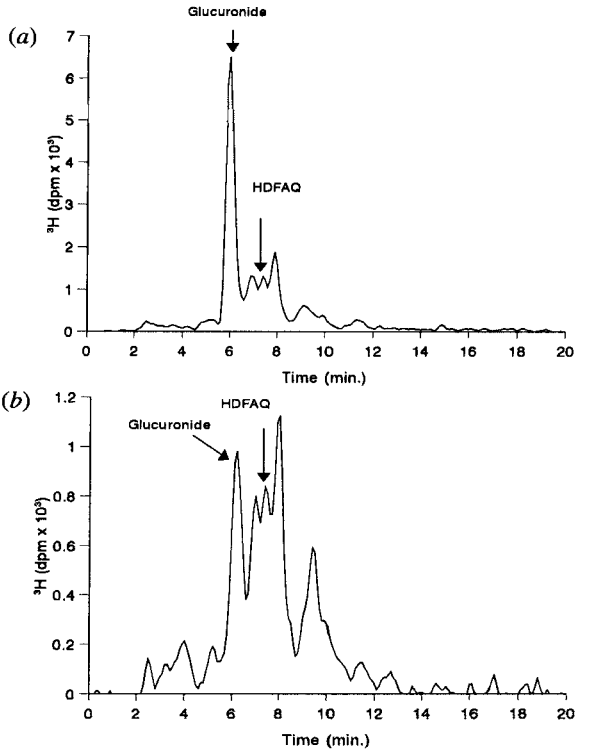


Figure 7. Biliary metabolites of [^{3}H]HDFAQ (54 μmol/kg, i.p.v.) in high (*a*), and low (*b*) elimination groups in the male rat.

The polar metabolite concurrent with extensive biliary elimination was hydrolysed by β-glucuronidase irrespective of elimination phenotype. The liberated aglycone co-eluted with authentic HDFAQ and yielded matching EI and CI spectra: EI *m/z* 373 (M^+, 1%), 342 (100), 285 (51), 270 (34), 250 (74); CI *m/z* 374 ($[M + 1]^+$, 100%), 340 (61), 330 (15), 296 (10), 287 (22), 253 (18).

APCI analysis of the isolated metabolite yielded the $[M + 1]^+$ ion for HDFAQ glucuronide at *m/z* 550 and a sodium adduct at *m/z* 572. A daughter-ion spectrum of *m/z* 550 consisted of the expected aglycone moiety at *m/z* 374; neutral loss of the glucuronyl fragment (176 amu) is typical of *O*-glucuronides (Straub *et al.* 1987). When whole bile was subjected to hplc-ESP mass spectrometry in negative-ion mode, with a run time of 50 min, the anions of HDFAQ glucuronide, i.e. *m./z* 548 (100%) and 550 (37%), were observed as single coincidental peaks at 9·4 min. The glucuronide, irrespective of elimination phenotype, gave a weak CI spectrum of HDFAQ and, surprisingly, a strong EI spectrum; repeated hplc analyses confirmed the absence of unconjugated HDFAQ.

HDFAQ recovered by ether extraction of bile was identified from its EI and CI spectra.

The quantity of HDFAQ glucuronide eliminated in bile—14·4 ± 1·7% ($n = 3$) and 2·6 ± 0·8% ($n = 6$) of the dose in high and low elimination groups respectively—was seemingly the principal determinant of the extent of biliary elimination (table 6). Only small amounts of HDFAQ were excreted in bile. In the absence of

extensive glucuronylation, HDFAQ was metabolized to several uncharacterized non-conjugated metabolites.

Discussion

The liver plays a central role in the disposition of AQ in the rat (figure 8). Five hours after the administration of [^{14}C]AQ via the hepatic portal vein, 25–30% of the radiolabelled material remained in the liver while up to 25% had been excreted into the bile. In keeping with a previous study in the male rat (Harrison *et al.* 1992), the biliary metabolites were almost exclusively thioether adducts, principally C-5′ glutathionyl AQ in both the male and female rat. The significantly greater rate of formation of thioether conjugates in the male rat may indicate a role for the sex-specific P450s 3A2 and 2C11 (Vage and Svensson 1994) in the bioactivation of

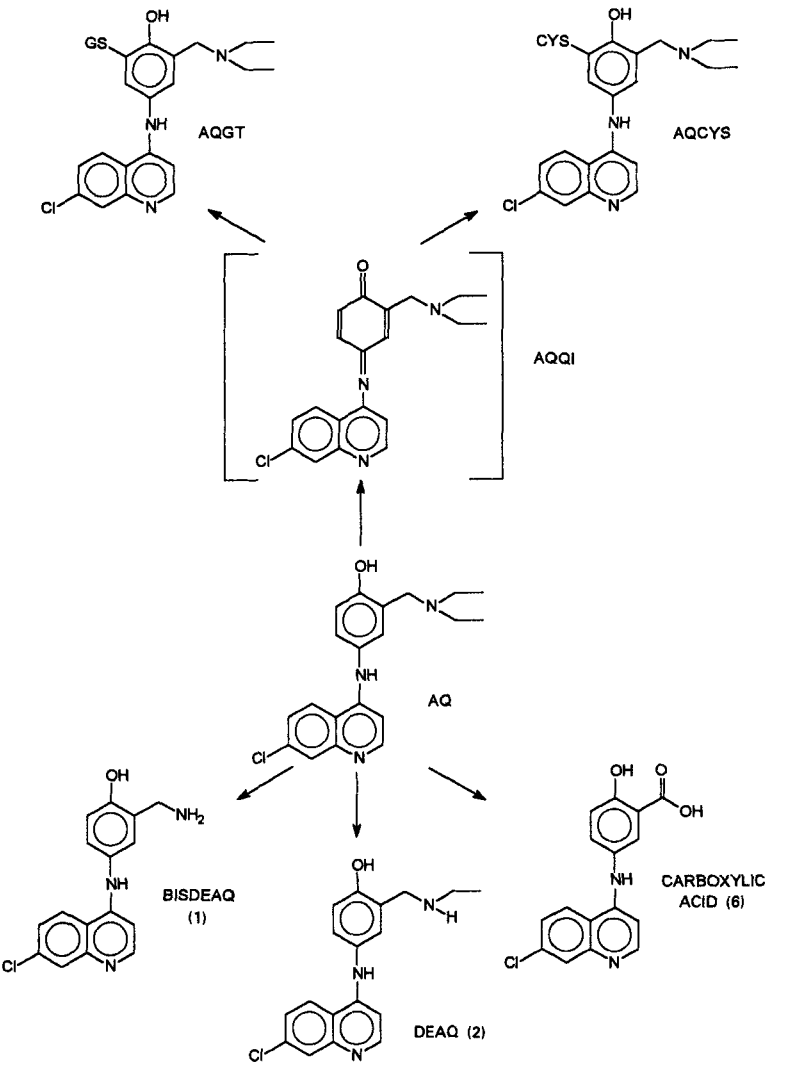


Figure 8. Metabolic fate of AQ in rat. Glutathione (AQGT) and cysteine (AQCYS; male rat only) adducts are extreted into the bile; AQ, **1**, **2**, and **6** are excreted into the urine.

AQ. The excretion of a single regioisomer suggests that the addition of glutathione to the putative oxidation product of AQ *in vivo*—AQ quinoneimine—is catalysed by a transferase since the direct chemical reaction furnishes three regioisomeric conjugates (Harrison *et al.* 1992). The rate of excretion of biliary metabolites was significantly reduced by prior dosing with ketoconazole in both the male and female rat, in accordance with the oxidation of AQ being catalysed predominantly by P450 (Meredith *et al.* 1985).

DEAQ, which accumulated in the liver, did not appear to undergo bioactivation as no glutathione conjugates of the metabolite could be found in bile after administration of either AQ or DEAQ. DEAQ, the major circulating metabolite in man, has a mean plasma half-life 20-fold greater than that of AQ (Pussard *et al.* 1987, Laurent *et al.* 1993), but a similar antimalarial activity *in vitro*, and is therefore probably responsible for most of the activity *in vivo* (Churchill *et al.* 1984).

Approximately 20% of an i.p. dose of AQ was excreted into urine over 24 h: 5% as unchanged drug and 5–10% as de-ethylated metabolites. No thioether conjugates of AQ were detected in urine. Since approximately 20% of the intraportal dose was eliminated in bile over 5 h, the preponderance of the i.p. dose must have been excreted in faeces. Barrow (1974) and Winstanley *et al.* (1988) found that [^{14}C]AQ-derived radioactivity was principally recovered in faeces following oral administration of the drug to rat. There was no evidence for conjugation—either glucuronidation or sulphation—of the phenol group of either AQ or DEAQ. It is conceivable that the (di)ethylaminomethyl side chain sterically hinders these reactions. Clearance of the drug from rat is therefore pre-eminently dependent on bioactivation coupled with glutathione conjugation.

To gain further insight into the possible relevance of AQ's bioactivation in rat to the postulated metabolic causation of the hepatotoxicity of the drug in man, we undertook parallel experiments with human and rat hepatic microsomes. The asessment of AQ's metabolic activation *in vitro*—via measurement of irreversible binding—was complicated by autoxidation of the aminophenol side-chain at physiological pH (Maggs *et al.* 1988). Nevertheless, it was possible to demonstrate binding at three-times the background level upon addition of a NADPH regenerating mixture to male rat liver microsomes. The existence of a quinoneimine intermediate was implied by the formation of C-5′ glutathionyl AQ when AQ was co-incubated with glutathione. The inhibition by ketoconazole of both irreversible protein binding *in vitro* and formation of glutathionyl AQ *in vivo* implicates an hepatic P450-mediated pathway in the generation of AQ-related antigen (Harrison *et al.* 1992). Bioactivation was not observed in either female rat or human liver microsomes, though a glutathione conjugate of AQ was found in female rat bile. The explanation for this apparent contradiction probably lies in the present observation that AQ quinoneimine is readily reduced *in vitro*; quinoneimines are known to undergo spontaneous reduction by NADPH in aqueous solutions (Potter and Hinson 1986). The extensive binding to microsomes in the absence of NADPH was suppressed, and the AQ formed from its quinoneimine was further metabolized by dealkylation. It is evident that the relative rates of quinoneimine formation and reduction can be finely balanced *in vitro*. *In vivo*, this balance might be a determinant of the yield of hepatic antigen from AQ, and consequently a factor in the idiosyncratic hepatotoxicity associated with AQ.

Dealkylation was the major pathway of AQ metabolism in both human and rat liver microsomes but the formation of the putative N-oxide was unique to the latter.

The use of isozyme-selective inhibitors (Halpert *et al.* 1994) indicated that dealkylation is catalysed to P4503A4 in human liver microsomes. Inhibition of de-ethylation did not increase protein conjugation. The presumed N-oxidation pathway of rat liver microsomes, unlike dealkylation, was not inhibited by ketoconazole, and therefore might have been catalysed largely by a flavine-containing monoxygenase (FMO) rather than P450; FMO metabolizes a variety of alkyl- and arylamines (Hines *et al.* 1994). Interspecies differences in the rates of N-oxidation of N-substituted anilines have been reported (Gorrod and Gooderham 1977).

Substitution of a fluorine for the hydroxyl group in AQ, as noted previously with unlabelled DFAQ (O'Neill *et al.* 1994), appeared to block metabolic activation completely in rat: negligible amounts of [^{3}H]DFAQ became irreversibly bound to liver protein and the sole biliary metabolite was resistant to γ-glutamyl transpeptidase. In comparison, 1% of intraportally administered [^{14}C]AQ underwent irreversible binding and 9% was eliminated in bile as a glutathione adduct. Although certain fluoroaromatics substituted *para* to an amine function undergo oxidative defluorination (Rietjens and Vervoort 1991, Boersma *et al.* 1993), the very large increase in oxidation potential association with deshydroxyfluorination (O'Neill *et al.* 1994) effectively precluded this biotransformation. In the absence of compensatory conjugation reactions, the biliary excretion of [^{3}H]DFAQ was only 29% of that of [^{14}C]AQ and N-dealkylation supplanted oxidation–glutathione conjugation as the principal metabolic pathway. Biosteric substitution of the 4-hydroxyl group with fluorine was clearly the optimal modification for the prevention of quinoneimine formation. Furthermore, such modification does not affect antimalarial potency (O'Neill *et al.* 1994). However, further modification(s) were necessary to enable clearance via other pathways, e.g. glucuronidation. Unfortunately, complex structure–activity relationships make it difficult to predict both the extent and site of conjugation, particularly glucuronidation (Tephly *et al.* 1988, Ebner and Burchell 1993).

Insertion of an N-hydroxyethyl moiety was chosen because a number of drugs bearing primary alkanol functions undergo glucuronidation *in vivo* (Zak *et al.* 1963, Ruijten *et al.* 1984, Wallis *et al.* 1988). Specifically, in rat, the N-hydroxyethyl derivative of chloroquine, hydroxychloroquine (McChesney 1983), is metabolized to an *O*-glucuronide, which undergoes biliary clearance (Harrison *et al.* 1993). The corresponding derivative of AQ, consistent with AQ's resistance to conjugation, yielded only an alkylic ether glucuronide. The extent to which the regioselectivity of HAQ's glucuronidation is dictated by hindrance of the phenolic hydroxyl is emphasized by the exclusive metabolism of certain alcohol–phenol substrates to phenolic ether glucuronides (Jackson *et al.* 1991). Notwithstanding its direct conjugation, and reflecting the ready oxidation of the *p*-hydroxyanilino moiety (O'Neill *et al.* 1994), HAQ still yielded twice as much biliary glutathione adduct as glucuronide; however, a novel route of metabolism, *viz.* glucuronidation of the hydroxyethyl side chain, has been introduced into the AQ molecule.

Unlike HAQ, HDFAQ was eliminated in bile to a greater extent than its parent compound (DFAQ). However, this was unambiguously attributable to the formation of HDFAQ glucuronide in only three of the nine male Wistar rats. The majority of the rats, though their biliary elimination of radiolabel exceeded that of the rats given DFAQ, excreted a much more complex mixture of metabolites, amongst which the glucuronide of HDFAQ was not pre-eminent. The other

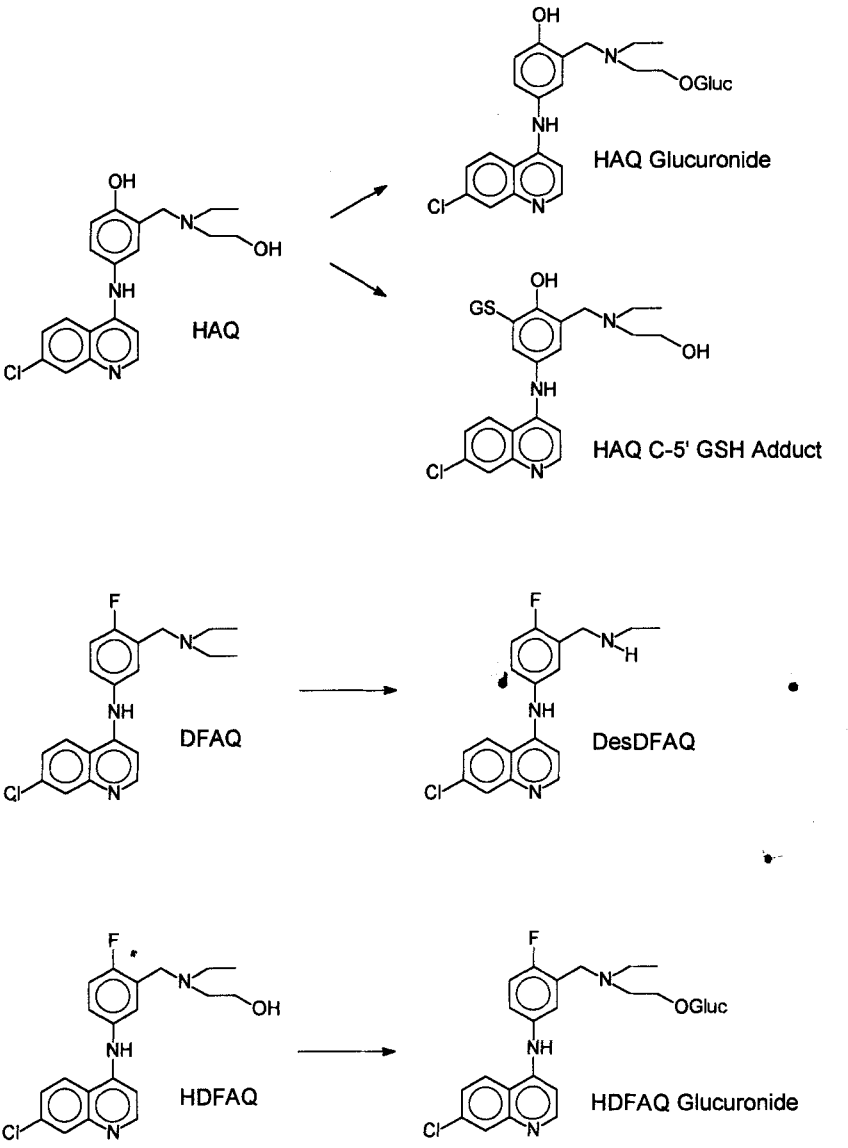


Figure 9. Biliary metabolites of the analogues of AQ in rat.

metabolites were not identified but may be products of oxidative biotransformations. Although HDFAQ glucuronide was apparently the principal determinant of the extent of biliary elimination of HDFAQ, a metabolic basis for the dimorphism of this elimination remains to be defined. A discontinuous variation in glucuronyltransferase activity toward 3α-hydroxysteroids in Wistar rat has been described (Tephly *et al.* 1988)—50% of individuals possess very low hepatic activity—but the enzyme appears highly specific for endogenous steroid substrates. Alternatively, differential removal of HDFAQ via oxidative pathways might be primarily responsible for the observed variation in the excretion of glucuronide. This appears to be the case with

the female Wistar rat in which the lower biliary elimination in two out of nine animals is associated with greater N-deshydroxyethylation (unpublished data).

In conclusion, this study has shown that the hepatic metabolism of AQ in the rat involves N-dealkylation, N-oxidation and oxidation to a quinoneimine that either conjugates to thiols or undergoes reduction to amodiaquine. There was little renal clearance of the drug or direct conjugation by either sulphotransferases or glucuronyltransferases. An attempt to replace oxidation–glutathione conjugation with direct conjugation by chemical modification of the diethylamine group achieved only partial success because of competing oxidation of the hydroxyaniline group. However, selective inhibition of oxidative bioactivation of the hydroxyaniline group was achieved by the introduction of fluorine into the 4-position (figure 9). Such analogues can now be used to investigate the role of bioactivation in the hepatoxicity of AQ.

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